Transcriptional Analysis of the tet(P) Operon from Clostridium perfringens

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The Clostridium perfringens tetracycline resistance determinant from the 47-kb conjugative R-plasmid pCW3 is unique in that it consists of two overlapping genes, tetA(P) and tetB(P), which mediate resistance by different mechanisms. Detailed transcriptional analysis has shown that the inducible tetA(P) and tetB(P) genes comprise an operon that is transcribed from a single promoter, P3, located 529 bp upstream of the tetA(P) start codon. Deletion of P3 or alteration of the spacing between the −35 and −10 regions significantly reduced the level of transcription in a reporter construct. Induction was shown to be mediated at the level of transcription. Unexpectedly, a factor-independent terminator, T1, was detected downstream of P3 but before the start of the tetA(P) gene. Deletion or mutation of this terminator led to increased read-through transcription in the reporter construct. It is postulated that the T1 terminator is an intrinsic control element of the tet(P) operon and that it acts to prevent the overexpression of the TetA(P) transmembrane protein, even in the presence of tetracycline.

The Tet P determinant from the 47-kb conjugative R-plasmid pCW3 from Clostridium perfringens is unique among tetracycline resistance determinants in that it consists of two overlapping genes, tetA(P) and tetB(P), which mediate resistance by different mechanisms. The tetA(P) gene is 1,260 bp in length and encodes a 46-kDa protein, TetA(P), which is responsible for the active efflux of tetracycline from the cell (44). The TetA(P) protein is predicted to contain 12 transmembrane domains but is atypical because it does not have the typical structure or conserved motifs that are common to the other classes of tetracycline efflux proteins (7, 21). The tetB(P) gene, which overlaps the tetA(P) gene by 17 nucleotides (nt), is 1,956 bp in length and encodes a putative 72.6-kDa protein. The TetB(P) protein has significant amino acid sequence identity (37 to 39%) to Tet(M)-like cytoplasmic ribosomal protection proteins (44).

The tet(P) genes are the most widely distributed tetracycline resistance genes in C. perfringens, being found in both conjugative and nonconjugative tetracycline-resistant strains from diverse geographical locations and environmental sources (1, 24). Conjugative transfer of tetracycline resistance is invariably associated with plasmids that are either identical to or closely related to pCW3 (2, 3, 41). In these conjugative isolates, resistance is inducible. Inducible resistance is also observed when pCW3 is introduced into derivatives of strains CW234 and CW362, whereas in a strain 13 background tetracycline resistance is constitutively expressed, suggesting that induction requires an as-yet-unidentified host-encoded factor (19, 38). Resistance is also constitutively expressed in nonconjugative isolates.

Analysis of the approximately 1 kb of sequence data that are available upstream of tetA(P) (44) has revealed that this region is AT rich, having an overall G+C content of 22%, which is similar to the normal 24 to 27% G+C content of C. perfringens DNA (9, 20). There is a highly AT-rich region between bp 377 and 575, which has a G+C content of only 14% (44). Although several sequences with similarity to the consensus C. perfringens σ70-like promoter sequence (39) can be identified, the AT-rich nature of the upstream region has prevented the precise identification of the tet(P) promoter. No recognizable promoter appears to be present between the start codons of the tetA(P) and tetB(P) genes, and a potential factor-independent terminator (ΔG = −21.3 kcal mol⁻¹) is present at the end of the tetB(P) gene, suggesting that these genes comprise an operon (44).

The objective of this study was to carry out detailed transcriptional analysis of the pCW3-encoded tet(P) genes. The results have shown that the tetA(P) and tetB(P) genes comprise an operon that is transcribed from a single promoter, P3, located 529 bp upstream of the tetA(P) start codon. Induction was shown to be at the level of transcription. A potential factor-independent terminator, T1, which is located some 390 bp downstream from the transcriptional start point but before the start of the tetA(P) gene, was also identified.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial plasmids used in this study are described in Table 1. Escherichia coli strains were derivatives of DH5α (Life Technologies) and were routinely grown at 37°C in 2% yeast extract-triptone (YT) supplemented with ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), erythromycin (150 μg/ml), or tetracycline (10 μg/ml). C. perfringens strains were derivatives of the chlorate- and streptomycin-resistant strain JIR33 (19) and were cultured under anaerobic conditions at 37°C in fluid thioglycolate medium (Difco), nutrient broth (38), or Trypticase peptone glucose broth (40). Solid media were prepared by the addition of 1.5% (wt/vol) bacteriological agar (Oxoid) prior to sterilization. C. perfringens strains grown on agar medium were incubated in an atmosphere of 80% N₂-10% H₂-10% CO₂ in an anaerobic jar (Oxoid) or in an anaerobic chamber (COY Laboratory Products Inc.). Antibiotics were added where appropriate, unless otherwise stated, to the indicated concentrations: chloramphenicol (5 μg/ml), erythromycin (50 μg/ml), tetracycline (0.5 or 5 μg/ml), or streptomycin (1 mg/ml).
Biosystems) and an ABI 373A automated PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied
test the DNA chain termination method using a T7 Sequencing kit (Pharmacia) or the ABI
described previously (26).

DNA isolation and molecular techniques. Plasmid DNA was routinely isolated
from E. coli strains using the Magic Minipreps DNA Purification System
(Promega), the High Pure Plasmid Isolation kit (Roche Molecular Biochemi-
ty was carried out with

Plasmid DNA was prepared from

(32). Plasmid DNA was prepared from

(C. perfringens)

(44) (GenBank accession no. L20800).

Nucleotide sequence analysis was performed either by the dideoxynucleotide
method using Taq DNA polymerase (Roche Molecular Biochemi-
)(chlorate-resistant) strains were cultured on the appropriate solid medium con-
taining 1% (vol/vol) saturated potassium chlorate solution.

DNA isolation and molecular techniques. Plasmid DNA was routinely isolated
from E. coli strains using the Magic Minipreps DNA Purification System
(Promega), the High Pure Plasmid Isolation kit (Roche Molecular Biochemi-
cals), or an alkaline lysis method (32). Plasmid DNA was prepared from

(pPSV Em r

(pJIR1645 pPSV

(pJIR1716 Random mutant of pJIR71, shows increased tetracycline resistance, contains G-to-A change at bp 941b)

at bp 941b and A-to-G change at bp 2218b(T386A)

plasmid pPSV (29) to construct pJIR1438.

Construction of the deletion derivatives pJIR1617, pJIR1618, and pJIR1494. These deletion derivatives were constructed from a pJIR1438 template by splice overlap extension (SOE)-PCR (15, 16). Initially, for each deletion two separate

TABLE 1. Relevant characteristics of plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristicsa</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCW3</td>
<td>47 kb; Tc' Traa</td>
<td>2</td>
</tr>
<tr>
<td>pJR71</td>
<td>pUC1801(PsIc); pJR39, 2.9 kb, contains tetA(P) and part of tetB(P); Tc'</td>
<td>1</td>
</tr>
<tr>
<td>pPSV</td>
<td>Em' lacZ; catP; pL404 replication origin (C. perfringens); pUC18 replication origin (E. coli); pBR322 origin (E. coli)</td>
<td>29</td>
</tr>
<tr>
<td>pJR418</td>
<td>CM' Em' lacZ'; pL404 replication origin (C. perfringens); pUC18 replication origin (E. coli)</td>
<td>45</td>
</tr>
<tr>
<td>pJR4138</td>
<td>pPSV HindIII/SphI[pHindIII/SphI]; pJR71, 839 bp, contains upstream region tetA(P)</td>
<td>This study</td>
</tr>
<tr>
<td>pJR1494</td>
<td>pPSV HindIII/SphI[AP3 SOE-PCR product; HindIII/SphI, 810 bp]</td>
<td>This study</td>
</tr>
<tr>
<td>pJR1617</td>
<td>pPSV HindIII/SphI[AT1 SOE-PCR product; HindIII/SphI, 808 bp]</td>
<td>This study</td>
</tr>
<tr>
<td>pJR1618</td>
<td>pPSV HindIII/SphI[EPE2 SOE-PCR product; HindIII/SphI, 810 bp]</td>
<td>This study</td>
</tr>
<tr>
<td>pJR1644</td>
<td>pPSV HindIII/SphI[pHindIII/SphI]; pJR1694, 838 bp, has a ΔΔ at bp 525b)</td>
<td>This study</td>
</tr>
<tr>
<td>pJR1645</td>
<td>pPSV HindIII/SphI[pHindIII/SphI]; pJR1716, 839 bp, contains G-to-A change at bp 941b</td>
<td>This study</td>
</tr>
<tr>
<td>pJR1716</td>
<td>Random mutant of pJR71, shows increased tetracycline resistance, contains G-to-A change at bp 941b</td>
<td>This study</td>
</tr>
<tr>
<td>pJR1722</td>
<td>pJR1634 Asp718/T4 polymerase (EcOR/ HindIII/T4 polymerase; pJR1465, 0.25 kb, contains RK2 oriT)</td>
<td>This study</td>
</tr>
</tbody>
</table>

a CM', chloramphenicol resistance; Em', erythromycin resistance; Tc', tetracycline resistance; Tra', transfer proficient.

b The coordinates relate to the published nucleotide sequence (44).

c Confirmed by restriction digestion and by sequencing.

d Confirmed by the dideoxynucleotide chain termination method using a T7 Sequencing kit (Pharmacia) or the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and an ABI 373A automated fluorescent sequencing apparatus in accordance with the manufacturer's instructions. Sequence analysis was carried out with Sequencher 3.0 software (Gene Codes Corporation).

Nucleotide sequence analysis was performed either by the dideoxynucleotide chain termination method using a T7 Sequencing kit (Pharmacia) or the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and an ABI 373A automated fluorescent sequencing apparatus in accordance with the manufacturer's instructions. Sequence analysis was carried out with Sequencher 3.0 software (Gene Codes Corporation).

TABLE 2. Oligonucleotide primers

<table>
<thead>
<tr>
<th>Primer no.</th>
<th>Sequence (5'-3')</th>
<th>Position (template)</th>
<th>Usea</th>
</tr>
</thead>
<tbody>
<tr>
<td>212</td>
<td>TCCGGGACATTACTA</td>
<td>2449–2464c</td>
<td>LP</td>
</tr>
<tr>
<td>274</td>
<td>AACCTTGTTTAACTG</td>
<td>3683–3667c</td>
<td>LP</td>
</tr>
<tr>
<td>1366</td>
<td>CACAGATGTTAGGGATTAG</td>
<td>1364–1385ca</td>
<td>PCR, LP</td>
</tr>
<tr>
<td>1367</td>
<td>CTTTATAGAAACGACAGT</td>
<td>2128–2107c</td>
<td>PCR, LP</td>
</tr>
<tr>
<td>1369</td>
<td>CGTCACTGTCGCCCTAATAGT</td>
<td>2107–2128c</td>
<td>PCR, RT-PCR, S</td>
</tr>
<tr>
<td>1370</td>
<td>ATGTGCAATAATATCTTGT</td>
<td>2657–2636c</td>
<td>PCR, RT</td>
</tr>
<tr>
<td>1947</td>
<td>TTATACACCTTAAATCTACCC</td>
<td>1073–1052c</td>
<td>PCR, PE, S</td>
</tr>
<tr>
<td>2980</td>
<td>AAATTAAACAGGATACGCTT</td>
<td>1657–1679c</td>
<td>LP</td>
</tr>
<tr>
<td>2981</td>
<td>GCTCCTTGAGAGTTCAGTAGT</td>
<td>2344–2322c</td>
<td>LP</td>
</tr>
<tr>
<td>3333</td>
<td>GACCTTCAAAGCTTATATGAG</td>
<td>2412–2395c</td>
<td>PE, S</td>
</tr>
<tr>
<td>3334</td>
<td>GCCCTGATACATCAATACCAA</td>
<td>933–912c</td>
<td>PE, S</td>
</tr>
<tr>
<td>3534</td>
<td>AGAAGTGACAGGATATGTC</td>
<td>296–276c</td>
<td>SOE, S</td>
</tr>
<tr>
<td>3644</td>
<td>CAAGATTTGTTCATCATC</td>
<td>659–642c</td>
<td>PE, S, LP</td>
</tr>
<tr>
<td>4392</td>
<td>ATACGGGTAGTAAATATGATGCAATCATATT</td>
<td>773–750c</td>
<td>SOE</td>
</tr>
<tr>
<td>4393</td>
<td>AACGCCTGCaATATAACCCCTAAATATATACAA</td>
<td>923–901c</td>
<td>SOE</td>
</tr>
<tr>
<td>4400</td>
<td>GGTATATGATCGCCTTTTTGTTTATAGGG</td>
<td>955–977c</td>
<td>SOE</td>
</tr>
<tr>
<td>4401</td>
<td>ACACTCTACGACGATTATTTTTATCTTGTATC</td>
<td>802–824c</td>
<td>LP</td>
</tr>
<tr>
<td>4402</td>
<td>ATATATATATATATGTCCTGCTAAATATTACAC</td>
<td>534–555c</td>
<td>SOE, LP</td>
</tr>
<tr>
<td>4403</td>
<td>CATATATATATATATATATATATTATATATTATAC</td>
<td>504–482c</td>
<td>SOE</td>
</tr>
<tr>
<td>8034</td>
<td>CACGGTATTTTTTATGTCAATTTT</td>
<td>802–824c</td>
<td>LP</td>
</tr>
<tr>
<td>8133</td>
<td>AACATATAATATATAGCTTAA</td>
<td>906–889c</td>
<td>LP</td>
</tr>
</tbody>
</table>

a S, sequencing; LP, oligonucleotides used for preparing labeled probes; PE, primer extension.
b Refers to relevant position within the Tn4451 sequence (6), GenBank accession no. U15027; see also the work of Lyras et al. (26).
c Refers to relevant position within the Tet P determinant (44), GenBank accession no. L20800.
d Refers to the work of Lyra and Rood (25).
e Refers to relevant position within the erm B determinant of C. perfringens (8), GenBank accession no. U19831.
f Oligonucleotide contains 9 bp at the 5' end which are complementary to the corresponding primer, which was used in deletion of the putative promoter regions.
g Refers to relevant position within the catP sequence (48), GenBank accession no. M74769.1.
stream of each of these endpoints. The resultant PCR products contained com-
plementary sequences and were purified from a 1.0% low-melting-point agarose (FMC Bioproducts) gel with the Magic PCR Prep DNA Purification system (Promega). The two products specific for each deletion derivative were then mixed, and a third PCR was performed using primers UP and 3534. The PCR consisted of 30 cycles of 1 min of denaturation at 91°C, 1 min of annealing at 37°C, and 3 min of extension at 72°C. The resultant SOE-PCR products were excised and extracted as before. These products were digested with HindIII and Spolig and ligated to HindIII/Spolig-digested PSV DNA. The resultant recombi-
nant plasmids pJR1617, pJR1618, and pJR1494 carried deletions in the Tl, PEZ, and P3 regions, respectively. The insert in each of the recombinant plasmids was sequenced to confirm that the precise deletion had occurred and that no other changes were present.

Determination of chloramphenicol MICs. For each of the deletion mutants, chloramphenicol MICs were determined in both E. coli and C. perfringens at 37°C as described previously (25). Briefly, for E. coli strains, overnight broth cultures were diluted 1:25 into fresh 2× YT broth containing erythromycin and grown until the turbidity at 550 nm was 0.7 to 0.8. Cultures were then diluted 1:100 in fresh broth. Duplicate 10-μl aliquots were then placed onto 2× YT containing chloramphenicol at concentrations ranging from 0 to 200 μg/ml. The cultures were incubated for 18 to 20 h at 37°C, and the MIC was determined as the lowest concentration of chloramphenicol that caused inhibited growth. Assays were repeated three times. For C. perfringens, an essentially identical procedure was followed with the exceptions that brain heart infusion medium was substituted for 2× YT medium and chloramphenicol concentrations in the range of 0 to 80 μg/ml were used.

Preparation of C. perfringens RNA. Total RNA was extracted from 20 or 100 ml of C. perfringens broth cultures using Trizol reagent (Gibco-BRL) as de-
scribed previously (25) with the additional step of treatment with RNase-free DNase (Promega). For strain JIR33(pCW3), RNA was extracted from cultures described previously (25) with the additional step of treatment with RNase-free DNase (Promega). For strain JIR33(pCW3), RNA was extracted from cultures that harbored pCW3. Unfortunately, a hybridiz-
ation system (Promega) in accordance with the manufacturer's instructions. RNA was transferred overnight at 4°C to a Hybond-N+ nylon membrane by capillary transfer using 10× SSC. The nylon membrane was air dried and cross-linked under UV light (312 nm) for 3 to 5 min. DNA probes specific for the tet(P) upstream region and the cat(P) and erm(B) genes were derived by PCR as described for the dot blots. The RNA probe specific for the tet(P) gene was derived in an analogous manner, with primers 1366 and 1367 (Table 2). Prehybridization, hybridization, and detection were carried out as described for dot blot hybridization analysis.

RESULTS

Transcriptional organization of the tet(P) genes. To determine if the tet(A) and tet(B) genes were arranged in an operon, Northern blots were initially performed with separate gene-specific probes on RNA that was isolated from C. perfringens cells that harbored pCW3. Unfortunately, a hybridiz-
ing smear was consistently observed for both the tet(A) and tet(B) probes (data not shown), suggesting that the RNA transcript was unstable. Therefore, the alternative method of RT-PCR analysis was performed using primer 1370, which was specific for the putative tet(A)-tet(B) transcript and bound 323 nt downstream of the tet(B) start codon (Fig. 1), and RNA from JIR33(pCW3), which exhibits an inducible tetracy-
cline resistance phenotype (19). The resultant cDNA mole-
ules were then amplified using PCR with primers 1369 and 1370, which produce a product of 550 bp. The oligonucleotide 1369 binds within tet(A), 179 bp upstream of the tet(B) start codon. An RT-dependent product was observed, indicating that the tet(A) and tet(B) genes were transcriptionally coupled (Fig. 1). To determine whether a promoter was present upstream of the tet(B) gene, primer extension analysis was carried out on RNA from JIR33(pCW3) using primer 3333, which was complementary to a region 133 nt downstream of the initiation codon of tet(B). No cDNA products were de-
tected (data not shown). Based on these results, it was con-
cluded that the tet(A) and tet(B) genes formed an operon.
Primer extension analysis of the tet(P) operon. To identify promoters upstream of tetA(P), primer extension analysis was performed on RNA extracted from JIR33(pCW3) cells that had been grown in the presence and absence of tetracycline (5 μg/ml), from starter cultures grown in the presence and absence of subinhibitory but inducing levels of tetracycline (0.5 μg/ml). Total RNA extracted from JIR33 was used as the negative control. Primer extension reactions were carried out with primer 1947, which was complementary to the beginning of the tetA(P) coding region. Three major cDNA products were consistently obtained from RNA extracted from JIR33(pCW3) cells that had been exposed to tetracycline, whereas either smaller amounts of product or no products were observed for cells grown in the absence of tetracycline (Fig. 2A).

Primer extension endpoints may represent transcriptional start points and thereby identify promoters. However, they may also result from mRNA processing or from the presence of secondary mRNA structures that lead to RT stalling. The smallest product observed in these experiments was located approximately 102 nt upstream of the tetA(P) translational initiation codon and appeared to be a result of the latter process (Fig. 3). It was subsequently shown to represent the site of a transcriptional terminator, designated T1. The second product, designated PE2, was mapped to a position 256 nt upstream of the tetA(P) initiation codon (Fig. 2B), and the third was mapped to a point 522 nt upstream of this ATG codon (Fig. 2C). The latter was subsequently shown to be the transcript originating from the tet promoter, P3.

Mutagenesis and analysis of T1, PE2, and P3. To examine the roles of T1, PE2, and P3 in gene expression, SOE-PCR (15, 16) was used to construct separate plasmids that each contained deletions in the regions upstream of the primer extension endpoints. Because of difficulties experienced with the genetic manipulation of shuttle vector constructs containing the tet(P) operon, it was necessary to use a reporter construct containing an alternative antibiotic resistance gene. An 839-bp HindIII/SphI pJJ71-derived fragment, which carried the relevant upstream region and the start of the tetA(P) gene (44), was cloned into the C. perfringens promoter probe shuttle vector pPSV (29) to construct pJIR1438 (Fig. 4). SOE-PCR was then used to delete each of the T1, PE2, and P3 regions, and each of the resultant PCR fusion products was cloned into pPSV to construct pJIR1617, pJIR1618, and pJIR1494, respectively (Fig. 4). pPSV contains a promoterless chloramphenicol acetyltransferase gene, catP, which confers chloramphenicol resistance (29).

In addition to these constructs, two other plasmids that contained mutations in the tet(P) upstream region were analyzed. These plasmids were derived from pJIR1694 and pJIR1716, which had been detected during previous studies that involved random mutagenesis of the tetA(P) gene (7). The plasmid pJIR1694 (7) had a deletion of an A residue at position 525, between the −35 and −10 regions of the putative promoter P3 (Fig. 3). This deletion resulted in a reduction of the tetracycline MIC in E. coli from 30 to 15 μg/ml (7). The plasmid pJIR1716 had a mutation of a G residue to an A residue at bp 941, which was within the T1 region (Fig. 3). This plasmid also had a change from an A to a G residue at bp 2218 [TetA(P)-T386A]. The result of these two mutations was a hyper-tetracycline-resistance phenotype in E. coli, with a tetracycline MIC of 50 μg/ml (T. Bannam and J. Rood, unpublished data). The HindIII/SphI fragments, containing the upstream regions and the start of the tetA(P) genes from pJIR1694 and pJIR1716, were cloned into pPSV to construct pJIR1644 and pJIR1645, respectively. Note that the TetA(P)-T386A mutation encoded on pJIR1716 was not present in the upstream region used to construct pJIR1645. Therefore, any phenotypic changes observed with this plasmid must be solely the result of the G941A mutation in the T1 region.

All of the pPSV derivatives, as well as the recombinant plasmid pJIR418 (45), which carries the wild-type catP gene and its native promoter, were introduced into C. perfringens strain JIR33 for further analysis. The effect of each of the deletions and mutations on promoter activity was assayed in vivo by determining the MICs of chloramphenicol. The results (Table 3) showed that deleting (pJIR1617) or mutating (pJIR1645) the T1 region led to a sixfold increase in MIC. Similarly, deleting the PE2 region (pJIR1618) resulted in a greater-than-twofold increase in MIC. By contrast, deleting the putative promoter region P3 (pJIR1494), or mutating it by changing the spacing between the −35 and −10 sequences from 17 to 16 bp, resulted in a decrease in MIC, essentially to that observed for the vector control, pPSV. These results suggested that P3 was the promoter responsible for transcription of the tet(P) operon.
Transcriptional analysis of the tetA(P)-catP fusions. To determine the transcriptional status of the upstream regions, primer extension analysis was performed on each of the reporter constructs of the deletion derivatives and the random mutants. Analysis of RNA extracted from cells carrying either pJIR1617 (ΔT1) or pJIR1645 (T1-G941A) revealed cDNA products corresponding to those previously mapped downstream of T1, PE2, and P3 (data not shown). Analysis of pJIR1618 (ΔPE2) revealed cDNA products corresponding to those detected downstream of T1 and P3 but barely detectable levels of product 2. Finally, for pJIR1494 (ΔP3), no cDNA products corresponding to those products mapped downstream of PE2 and P3 were detected and only extremely low levels of product 1 were observed (data not shown). Only low levels of all three major cDNA products were observed for pJIR1644 (P3-525 ΔA). Taken together, these results provided further evidence that P3 was the tet(P) promoter.

Quantitative RNA dot blot analysis of the transcriptional fusions. Dot blot hybridization analysis was performed on RNA isolated from JIR33 derivatives harboring the shuttle vector constructs. The RNA was hybridized with DNA probes that were specific for the regions between P3 and PE2 (P3-PE2 probe), PE2 and T1 (PE2-T1 probe), and for the catP gene (catP probe) (Fig. 4E). As a quantitative control for RNA
concentration, the RNA preparations were also probed using an *erm*(B)-specific probe. Since the *erm*(B) gene located on pPSV is constitutively expressed, the level of transcription of this gene should be equivalent for each of the derivatives studied (Fig. 4D).

When the wild-type upstream region was present (pJIR1438), the level of *catP* mRNA was slightly higher than that of background (Fig. 4C). Hybridization analysis with the other probes showed that in cells containing pJIR1438 there was more upstream transcript present than *catP* transcript (Fig. 4A and B). Changes in the T1 region, whether by deletion (pJIR1617) or point mutation (pJIR1645), resulted in an increase in the level of *catP* transcript. This increase was greater in the deletant than in the random mutant (Fig. 4C). In the upstream region, lower levels of transcript than that seen for the *catP* probe were observed. There was also a decreased amount of transcript within the P3-to-PE2 region compared to that observed for the wild type. Again, this effect was more distinct in the deletant (Fig. 4A).

Deletion of the PE2 region (pJIR1618) resulted in a slight increase in the amount of *catP* transcript, to a level less than twofold higher than that observed for the wild type (Fig. 4C). The effect of this deletion on the upstream transcript(s) was more dramatic (Fig. 4A and B), with an increase of just under threefold and slightly more than eightfold compared to the wild type for the P3-PE2 and P2-T1 probes, respectively (Fig. 4A). By contrast, a wild-type level of hybridization was observed for both the T1 deletant and the T1-G941A mutant with the PE2-T1 probe (Fig. 4B).

Deletion of the P3 promoter (pJIR1494) resulted in a decrease in the level of all transcripts to that of background (pPSV) (Fig. 4A to C). Slightly higher levels of hybridization compared to background were observed for the P3-521/214A mutant, pJIR1644. These results were in agreement with the primer extension analysis and MIC determinations. They confirmed that P3 was the *tet*(P) promoter. When this promoter was deleted or even changed slightly by altering the spacing between the 35 and 10 regions, reporter gene expression was reduced to background levels. This result implied that there were no significant promoters downstream of P3. The results also implied that sequences downstream of the P3 transcriptional start point were involved in modulating *catP* expression resulting from the P3 promoter.

**Putative RNA secondary structures in the *tet*(P) upstream region.** Examination of the region upstream of the *tet*(P) gene for RNA folding predictions using the programs FoldRNA (52) and mfold (28, 53) revealed that the T1 and PE2 regions were associated with potential RNA stem-loop secondary
structures with $\Delta G$ values of $-17.1$ and $-13.3$ kcal/mol, respectively. Modeling of the upstream regions of the $\Delta$PE2 and $\Delta$T1 transcripts revealed no significant changes from that observed for the wild type, apart from the deletion of the appropriate structures. The T1 structure consisted of a stem containing four G-C pairs followed by a string of T residues and resembled the characteristic structure of a factor-independent terminator (10, 36, 37). Analysis of this potential structure by use of an algorithm (10) designed to predict Rho-independent terminators suggested that it was a good terminator candidate. This algorithm calculates a value, $d$, which correlates with a predicted termination efficiency, expressed as a percentage. In general, positive $d$ values are regarded as the necessary requirement to describe the structure of $E. coli$ Rho-independent terminators (10). The T1 region was calculated as having a $d$ value of $35.51$, which correlated with an in vitro termination efficiency of $\sim 86\%$. The deletion of this region would inhibit the formation of this structure, and changes in this stem-loop structure caused by the G-to-A transition at position 941, which prevents the formation of a GC base pair, reduce the $\Delta G$ to $-11.1$ kcal/mol. This potentially creates a less efficient terminator with a calculated $d$ value of $17.95$, which correlates with a termination efficiency of $\sim 60\%$. Based on these data and in conjunction with the MIC and dot blot hybridization results, it was concluded that the structure located in the T1 region represented a transcriptional terminator.

Examination of the PE2 structure revealed that it was also followed by a string of T residues, with an A residue separating the T residues from the stem-loop structure. This structure also bears some resemblance to factor-independent terminators, but analysis similar to that performed for T1 revealed that the PE2 region did not meet the minimum conditions that are used to define a terminator. This result does not rule out the possibility that this region of RNA secondary structure is acting as a terminator in $C. perfringens$. Even in $E. coli$, a small proportion of terminators do not conform to the constraints of this algorithm and have negative $d$ values (10).

Evidence for small RNA transcripts derived from the $tet(P)$ upstream region. If the predicted secondary structures were formed in vivo, then short 5'-proximal RNA transcripts repre-
senting terminated mRNA molecules would be present. To detect these molecules, Northern hybridization analysis was performed on purified RNA preparations extracted from the JIR33 derivatives used in the previous experiments, using radioactively labeled P3-PE2 and \( \text{catP} \)-specific probes (Fig. 4E).

Using the P3-PE2 probe, two small hybridizing bands, which were estimated to be approximately 250 and 410 nt in size, were observed for the wild-type construct, pJIR1438 (Fig. 5A, lane 2). If the 5' ends of these bands mapped to the transcriptional start point downstream of P3, their 3' ends would map near the PE2 and T1 structures, respectively. Further evidence that the 410-nt transcript represented a product terminating at T1 came from examination of the profile of the A71 and G941A mutants. When probed with the P3-PE2 probe, only the 250-nt RNA band was observed for the T1 deletant (Fig. 5A, lane 3), whereas both the 250-nt band and a faintly hybridizing 410-nt band were observed for the T1-G941A mutant (Fig. 5A, lane 7). Two larger bands estimated as approximately 1.43 and 0.77 kb in size were also observed for both mutants (Fig. 5A). These bands also hybridized to the \( \text{catP} \) probe (Fig. 5B, lanes 3 and 7). Weakly hybridizing bands of approximately 1.43 and 0.77 kb in size were also observed for wild-type and \( \Delta \text{PE2} \) RNA when probed with \( \text{catP} \) (Fig. 5B, lane 4).

Promoter fusion constructs initiating at P3 and terminating at the \( \text{catP} \) transcriptional terminator would yield an \( \sim 1,460 \)-nt transcript. As a result, it was concluded that the 1.43-kb band (Fig. 5) represented a full-length mRNA transcript initiating at the transcriptional start point downstream of P3. The 0.77-kb and 1.0- to 1.1-kb hybridizing bands observed in the T1 deletant and T1 mutant most probably represent transcripts resulting from RNA processing or degradation.

Analysis of the PE2 deletant with the P3-PE2 probe revealed that the two smaller hybridizing bands were present but at greater intensity than that observed for the wild type (Fig. 5A, lane 4). The first of these bands was smaller, correlating with the 29-bp deletion of the PE2 region. The presence of the second band, slightly less than 250 nt, in this deletant indicated that the 3' end of the smaller RNA transcript did not map to the PE2 stem-loop structure, as this sequence was not present in the deletant. The 3' end of this transcript may map further downstream, or alternatively, it may represent a breakdown product obtained from RNA processing. In agreement with the results already obtained, no hybridization to any of the probes was observed with RNA from the P3 deletant (Fig. 5, lane 5), and only a very faintly hybridizing 410-nt P3-specific band was observed for RNA from the P3-525\( \Delta \text{A} \) mutant (Fig. 5, lane 6).

The observation of the smaller RNA transcripts in the reporter system prompted two questions. Firstly, could the small transcripts be observed from the upstream region of the \( \text{tet}(P) \) operon on the native plasmid, pCW3? Secondly, did the regulation of the \( \text{tet}(P) \) genes involve a transcriptional attenuation mechanism similar to that proposed for the \( \text{tet}(M) \) gene of \( \text{Te}n916 \) (49)? If such a mechanism existed, then in the absence of tetracycline, \( \text{tet}(P) \) transcription would terminate in the upstream region, whereas when tetracycline was added, there would be read-through of transcription from the upstream region into the \( \text{tet}(P) \) coding region.

To answer these questions, Northern hybridization analysis was performed using total RNA extracted from JIR33(pCW3) cultures grown in the presence or absence of tetracycline and using probes specific for the P3-PE2 and \( \text{tet}(P) \) regions. Northern analysis with the P3-PE2 probe showed that both of the small RNA bands observed with the reporter constructs were present in the pCW3-derived RNA preparations (Fig. 6). In addition, hybridizing smears that presumably represented a highly unstable full-length transcript were also observed from cells exposed to tetracycline. Interestingly, the intensity of hybridization of both the small RNA bands and the smeared RNA increased upon tetracycline induction. Although the signal intensity observed with the \( \text{tet}(P) \)-specific probe was relatively low, a broadly smeared band, which commenced at the 23S rRNA band and increased in intensity upon exposure to tetracycline, was observed (Fig. 6). Similar profiles were observed when these experiments were repeated with different RNA preparations. Despite numerous attempts, it was not possible to obtain better resolution of the full-length \( \text{tet}(P) \)

### TABLE 3. MICs for the deletion mutants of \( E. \) \( \text{coli} \) and \( C. \) \( \text{perfringens} \)

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mutation</th>
<th>( E. ) ( \text{coli} ) (DH5( \alpha ))</th>
<th>( C. ) ( \text{perfringens} ) (JIR33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>ND( ^{a} )</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>pJIR418</td>
<td>Positive control</td>
<td>&gt;200</td>
<td>60</td>
</tr>
<tr>
<td>pPSV</td>
<td>Vector control</td>
<td>15</td>
<td>1.5</td>
</tr>
<tr>
<td>pJIR1438</td>
<td>Wild type</td>
<td>52.5</td>
<td>4</td>
</tr>
<tr>
<td>pJIR1617</td>
<td>( \Delta \text{T1} )</td>
<td>&gt;200</td>
<td>25</td>
</tr>
<tr>
<td>pJIR1618</td>
<td>( \Delta \text{PE2} )</td>
<td>67.5</td>
<td>10</td>
</tr>
<tr>
<td>pJIR1649</td>
<td>( \Delta \text{P3} )</td>
<td>22.5</td>
<td>1.5</td>
</tr>
<tr>
<td>pJIR1644</td>
<td>P3-525( \Delta \text{A} )</td>
<td>ND</td>
<td>2.5</td>
</tr>
<tr>
<td>pJIR1665</td>
<td>T1-G941A</td>
<td>ND</td>
<td>25</td>
</tr>
</tbody>
</table>

\( ^{a} \) ND, not done.

\( \text{FIG. 5.} \) Northern hybridization analyses of RNA from each of the promoter mutants. Total RNA was isolated from cells of JIR33 derivatives grown in the presence of erythromycin (50 \( \mu \text{g/ml} \)). RNA (20 \( \mu \text{g} \)) was subjected to electrophoresis through 1.5% formaldehyde gels and transferred to nylon membranes, which were subsequently probed with the \( \gamma^{32} \text{P} \)-labeled P3-PE2 and \( \text{catP} \)-specific probes, respectively (Fig. 4). The arrow represents full-length \( \text{tet}(P) \)-\( \text{catP} \) transcript. The asterisks mark the faintly hybridizing 1.0- to 1.1-kb transcripts observed in the blot probed with \( \text{catP} \). The size of RNA markers is indicated at the left in bases. Lanes: 1, pPSV; 2, pJIR1438; 3, pJIR1617; 4, pJIR1618; 5, pJIR1494; 6, pJIR1644; 7, pJIR1645.
of the P3 promoter. Analysis of this promoter revealed that there were no functional promoters located downstream of the P3 promoter to the consensus sequence (39). Although several potential promoter sequences were present between P3 and the start of the tetA(P) gene, analysis of the ΔP3 and P3-525ΔA mutants provided strong evidence that there were no functional promoters located downstream of the P3 promoter. Analysis of this promoter revealed that it was identical to the consensus Clostridium perfringens σ70 promoter (39) and contained only one mismatch to the E. coli σ70 consensus sequence (14). The spacing between the −35 and −10 regions, at 17 nt, is the optimal spacing observed for Clostridium perfringens (39) and E. coli (13) promoter sequences. This spacing appears to be a stringent constraint on promoter function because it is required for recognition by the RNA polymerase holoenzyme (5, 14, 30, 33, 47, 51). Deviations from this spacing, as observed for the P3ΔA mutant with the reporter construct in this study, have a severe effect on expression. The close similarity of the promoter to the consensus σ70 sequence suggests that this promoter, if not subjected to any regulatory constraints, would act as a strong promoter in vivo (14, 35).

Transcription of the tet(P) operon appears to be under tight regulatory control, which is not surprising given that the TetA(P) protein is a transmembrane protein that is involved in the active efflux of tetracycline from the cell (44). In E. coli, the constitutive expression of tetracycline efflux proteins has been shown elsewhere to reduce the competitive fitness of the resultant strain (23, 34). High-level expression of the tetracycline resistance proteins TetA(B) and TetA(K) has also been shown previously to be highly toxic to the host cell (11, 12).

In gram-positive bacteria, tetracycline resistance genes may be regulated by transcriptional or translational attenuation (17, 22, 31, 49), translational coupling (46), or a tetracycline-responsive repressor protein (50). The results from this study are consistent with the hypothesis that in C. perfringens the induction of tetracycline resistance is at the level of initiation of transcription from the tet(P) promoter, P3. This postulate suggests the involvement of a regulatory protein that modulates P3 promoter activity. We have been unable to identify such a protein, although we have previously shown that inducible expression of the tet(P) operon requires an as-yet-unidentified host-encoded factor (19).

Once induced, transcription of the tet(P) operon is dependent upon read-through of the factor-independent transcriptional terminator, T1, which is located 390 bp downstream of the transcriptional start point. Northern blots using probes specific for the upstream region identified two small RNA transcripts, approximately 410 and 250 nt in size. The size of the larger fragment is consistent with a transcript originating from the P3 promoter and terminating at T1. Mutations that affected the formation or stability of this structure (ΔT1 and T1-G941A) resulted in high-level expression of the downstream gene. The presence of such a structure suggested the possibility that regulation was mediated by a transcriptional attenuation mechanism. However, the experimental data did not support this hypothesis (Fig. 6). Instead, it is postulated that T1 is an intrinsic control element that acts to prevent the overexpression of the TetA(P) protein, even in the presence of tetracycline. The role of the PE2 region is more difficult to understand. Deletion of PE2 in the reporter construct leads to significantly increased levels of the mRNA transcripts detected by the P3-PE2 and PE2-T1 probes and slightly increased chloramphenicol resistance. However, the nature of these transcripts appears unaltered compared to that of the wild type (Fig. 5A), apart from the expected small reduction in size specifically from the deletion. It is possible that, like T1, PE2 is also a terminator sequence but is less efficient and that its effects are modulated by the presence of the downstream T1 terminator. Alternatively, deletion of PE2 may act to stabilize the transcripts originating from the P3 promoter.

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