Tetracycline resistance is the most common antibiotic resistance phenotype found in the anaerobic pathogen *Clostridium perfringens* (18, 20). This organism harbors both conjugative and nonconjugative tetracycline resistance determinants (3, 4, 18). The conjugative determinants are carried on a group of transmissible plasmids which are all either identical to or closely related to pCW3, the prototype tetracycline resistance plasmid from *C. perfringens*, and they all probably carry the same tetracycline resistance determinant (2–4). This determinant, designated Tet P, encodes two functional genes, *tetA* (P) and *tetB* (P), which overlap by 17 bp (25). The *tetA* (P) gene encodes a putative 46-kDa transmembrane protein which mediates active efflux of tetracycline from the cell, while *tetB* (P) encodes a putative 72.6-kDa protein which has significant similarity to Tet M-like tetracycline resistance proteins (J. Sloan, L. M. McMurtry, D. Lyras, S. B. Levy, and J. I. Rood, Mol. Microbiol. 11:403–415, 1994). In the present study, hybridization and PCR analysis of 81 tetracycline-resistant isolates of *C. perfringens* showed that they all carried the *tetA* (P) gene. Most of these isolates (93%) carried a second tetracycline resistance gene, with 53% carrying *tetB* (P) and 40% carrying a *tet* (M)-like gene. Despite the wide distribution of the *tetB* (P) and *tet* (M) genes, no isolate which carried both of these determinants was detected. In isolates that carried both *tetA* (P) and *tetB* (P) these genes overlapped, as in pCW3. Isolates carrying this combination of genes originated from diverse geographical locations and environmental sources. The single *Clostridium paraputreficum* isolate examined carried *tetA* (P), indicating that this gene is not confined to *C. perfringens*. However, neither *tetA* (P) nor *tetB* (P) was detected in the nine *Clostridium difficile* isolates tested. Nucleotide sequence analysis of isolates lacking *tetB* (P) revealed that they contained the *tetA*408 (P) gene, which lacked the codons for the 12 carboxy-terminal amino acids of the TetA (P) protein.

**MATERIALS AND METHODS**

*Bacterial strains, plasmids, and growth media.* All *Escherichia coli* strains were derivatives of DH5α (Bethesda Research Laboratories). The *C. difficile* isolates included the Australian isolate AM1180 (1), two isolates from Japan (15), one isolate from England (11), and five isolates, including strain 630, from Germany (30). The *C. perfringens* isolates included 49 porcine and human isolates from the United States (18a, 21), 16 porcine isolates from Australia (19), 6 isolates from Belgium (1, 4) (obtained from D. E. Mahony), and 1 isolate from Germany (29). The single *C. paraputreficum* isolate, CW409, was a porcine isolate from the United States (21).

The plasmids used in the study were the Trn16-containing plasmid pAM120 (10), the *tet* (M) plasmid pJ13 (8), and the *tetA* (P)- and *tetBP*-specific probe plasmids pIR666 and pIR667, respectively (see Fig. 1A). *E. coli* strains were grown on 2YT agar medium (12) supplemented with ampicillin (100 μg/ml) or tetracycline (10 μg/ml). *C. perfringens* and *C. paraputreficum* strains were cultured at 37°C in Trypticase-peptone-glucone broth (21), brain heart infusion (Oxoid), fluid thioglycolate medium (Difco), or nutrient agar (18) supplemented with minocycline (5 μg/ml), nalidixic acid (20 μg/ml), rifampin (20 μg/ml), or tetracycline (5 μg/ml). The *C. difficile* strains were grown in BHIS medium (26) supplemented with tetracycline (5 μg/ml). Clostridial agar cultures were grown in an atmosphere of 10% H2:10% CO2:80% N2. All strains were grown at 37°C.

**DNA techniques.** Plasmid DNA from *E. coli* was isolated by an alkaline lysis procedure (14). PCR products for nucleotide sequencing were purified by isolation from a low-melting-temperature agarose gel (Scoplaque; FMC BioProducts) with the Magic PCR Prep DNA Purification System (Promega) according to the manufacturer’s protocol. Total genomic DNA from the clostridial isolates was prepared by a method developed for *C. perfringens* (3). Transformation of *E. coli* (23) and *C. perfringens* (24) cells was as described before. All enzymes involved in the manipulation of DNA were used according to the manufacturer’s specifications (Boehringer Mannheim). Primers used for PCR or nucleotide sequencing were synthesized on an Applied Biosystems 392 DNA/RNA Synthesizer and are listed in Table 1.

**DNA sequencing.** For nucleotide sequence analysis we used the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI 373 A automated fluorescent sequencing apparatus (Applied Biosystems). Double-stranded PCR products, together with the appropriate oligonucleotide primers, were used as the templates in the sequencing reactions (Table
TABLE 1. Synthetic oligonucleotides used in the study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence</th>
<th>Specificity</th>
<th>Coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1366</td>
<td>5'-CACAGATGTATGAGGGATTAGG-3'</td>
<td>tetA(P)</td>
<td>1364–1385</td>
</tr>
<tr>
<td>1367</td>
<td>5'-CATTATAGAAAGCACTAGACC-3'</td>
<td>tetA(P)</td>
<td>2128–2107</td>
</tr>
<tr>
<td>1369</td>
<td>5'-GCTACTGGTTTCTATATAAGT-3'</td>
<td>tetA(P)</td>
<td>2107–2128</td>
</tr>
<tr>
<td>1370</td>
<td>5'-ATGCTGAAAATATACATTCTG-3'</td>
<td>tetB(P)</td>
<td>2657–2636</td>
</tr>
<tr>
<td>1371</td>
<td>5'-ACAAGAATATTTGGACATC-3'</td>
<td>tetB(P)</td>
<td>2636–2657</td>
</tr>
<tr>
<td>1374</td>
<td>5'-ATCTCCCTGATTTCTCACTGG-3'</td>
<td>tetA(P)</td>
<td>4097–4076</td>
</tr>
<tr>
<td>1567</td>
<td>5'-ATTCTTGTAACACGGGACGG-3'</td>
<td>tet(M)</td>
<td>13887–13866</td>
</tr>
<tr>
<td>1568</td>
<td>5'-ACCTGATATATCTGCTAGAC-3'</td>
<td>tet(M)</td>
<td>12447–12468</td>
</tr>
<tr>
<td>1569</td>
<td>5'-GTGCATGAAATATATACGAGT-3'</td>
<td>tet(M)</td>
<td>12468–12447</td>
</tr>
<tr>
<td>2114</td>
<td>5'-GACCGATTTAGGATCCATGC-3'</td>
<td>tet(M)</td>
<td>2162–2183</td>
</tr>
<tr>
<td>2477</td>
<td>5'-GATAGGGATTTGATAGAATCG-3'</td>
<td>tet(M)</td>
<td>2195–2216</td>
</tr>
<tr>
<td>2491</td>
<td>5'-CCACTACTAATATAGGAAGCC-3'</td>
<td>tetP(M)</td>
<td>1562–1541</td>
</tr>
<tr>
<td>2541</td>
<td>5'-TCTACTCCCTGAACTTGGAGC-3'</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2581</td>
<td>5'-CTTCCCTCTTCTGAGAGAG-3'</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2745</td>
<td>5'-CCCAGGTCTGATTATCCCTATG-3'</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

a Primers 1367, 1370, and 1568 were complementary to primers 1369, 1373, and 1569, respectively.

b The tetA(P)- and tetB(P)-specific primers were derived from previously published sequences (25), as were the tet(M)-specific primers (9).

c The coordinates relate to the published nucleotide sequences.

d ---, see text for details.

1. The sequences were compiled by using Sequencher software (Gene Codes Corporation). The nucleotide sequences were compared with database entries by using the BLAST program (5).

2. Inverse PCR. A total of 3 to 5 μg of total genomic DNA from the various isolates was digested with HindIII, extracted with phenol-chloroform (23), and ligated for 16 h at 14°C in a final volume of 500 μl with 1 U of T4 DNA ligase. The ligated DNA was precipitated with ethanol and resuspended in 20 μl of distilled H2O. The DNAs from the various isolates were then used as templates in separate PCRs with oligonucleotide primers that were derived from tetA(P)-specific sequences (Table 1).

3. Dot blot and Southern hybridization analysis. Genomic DNA was placed directly onto nylon membranes (Amersham) or was digested with the appropriate restriction endonucleases, subjected to agarose gel electrophoresis, and then transferred to nylon membranes (23). Positive and negative controls were included in every experiment. All blots were analyzed by using the DIG DNA Labelling and Detection Kit (Boehringer Mannheim) according to the manufacturer's instructions. Hybridization was carried out at 65°C in 5X SSC (0.75 M NaCl plus 0.075 M sodium citrate [pH 7.0]), and subsequent washes were done twice, for 15 min each time, at either 65 or 55°C in 0.1X SSC-0.1% (wt/vol) sodium dodecyl sulfate for high- or medium-stringency washes, respectively.

RESULTS AND DISCUSSION

Hybridization analysis of C. perfringens isolates. To determine which tetracycline resistance determinants were carried by the various C. perfringens isolates, segments of three genes were used as probes in dot blot hybridization experiments. Two of the probes, pJIR666 and pJIR667, carried fragments internal to the tetA(P) and tetB(P) genes, respectively (Fig. 1A). The 1.8-kb HindIII-Asp718 fragment from pJIR68 (8), which was internal to the tet(M) gene of Tn916 (7, 27), was used as the third probe.

Analysis of the 81 tetracycline-resistant C. perfringens isolates indicated that they all carried tetA(P). These isolates included both conjugative and nonconjugative derivatives from diverse sources (Table 2). Of these isolates, 75 (93%) carried a second tetracycline resistance gene, with 43 (53%) carrying tetB(P) and, unexpectedly, 32 (40%) carrying a tet(M)-like gene which hybridized to the tet(M) gene from Tn916. Despite the wide distributions of both the tetB(P) and tet(M)-like genes, no isolate which carried both of these determinants was detected. The results obtained with the tetA(P)-specific probe were in agreement with previous results (1).

All of the C. perfringens isolates which hybridized with the tetB(P)- or tetM(M)-specific probes were minocycline resistant (5 μg/ml). This result was expected since previous studies have shown that tetB(P) (25) and tetM (28) encode minocycline resistance, whereas tetA(P) does not (25). Of the six isolates hybridizing only with tetA(P), three were minocycline resistant, indicating the presence of another determinant encoding this phenotype. Since 96% of the isolates tested carried tetA(P)
together with another determinant which encodes minocycline resistance, it was concluded that tetracycline-resistant *C. perfringens* isolates generally carry two tetracycline resistance determinants.

Isolates which hybridized to both tetA(P) and tetB(P) originated from diverse geographical locations which included the United States, Australia, France, Belgium, Japan, Canada, and Germany. The six isolates which hybridized only to tetA(P) originated from the United States and Australia. By contrast, the isolates which hybridized to both tetA(P) and tet(M) were exclusively from North America. The locations of the tetA(P) and tetB(P) genes on conjugative plasmids such as pCW3 and pIP401 (3, 25) may account for the broad geographic distribution of these determinants. Furthermore, the nonconjugative tetA(P)+ tetB(P)+ isolates probably also carry these determinants on plasmids, since preliminary transformation experiments with total genomic DNA resulted in transformants which were tetracycline and minocycline resistant (data not shown).

In contrast, the determinants from tetA(P)+ tetB(P)+ and tetA(P)+ tet(M)+ isolates are probably chromosomally located since their markers could not be transformed (data not shown). These results are in agreement with previous observations which showed that 10 tetracycline resistance plasmids from *C. perfringens* strains from a variety of sources hybridized to tetA(P) but not to tet(M) (22).

**Hybridization analysis of other tetracycline-resistant clostridia.** Hybridization analysis of other clostridial strains revealed that a *C. paraputrefaciens* isolate (CW498) hybridized to the tetA(P) probe, which indicates that tetA(P) is not confined to *C. perfringens*. This isolate also hybridized to the tet(M) probe. The nine *C. difficile* isolates hybridized to tet(M) but not tetA(P) or tetB(P). The results obtained with the tetA(P)-specific probe for the *C. paraputrefaciens* and *C. difficile* isolates were in agreement with previous results (1).

Previous workers (17) probed a large number of *C. difficile* isolates using the 0.8-kb EcoRI-PstI fragment from pJIR39 (2). This 858-bp fragment contains regions specific for parts of both the tetA(P) gene (188 bp) and the tetB(P) gene (686 bp), as well as the 17-bp region which overlaps both genes. Therefore, hybridization experiments carried out by using this fragment as a probe will not discriminate between these genes. Fifteen *C. difficile* isolates which hybridized with the pJIR39-derived probe were detected (17). Two of the Tet-P-hybridizing strains reported in that study were ATCC 9689, the *C. difficile* type strain, and ATCC 17857. We have obtained both strains directly from the American Type Culture Collection and have found that both were susceptible to tetracycline (5 μg/ml).

Neither isolate hybridized to the tetA(P)-, tetB(P)-, or tet(M)-specific probes. Furthermore, a culture of ATCC 17857 (obtained from M. Roberts, University of Washington), although resistant to tetracycline, did not hybridize to any of the probes. In view of these results and the fact that the genes encoded by the strains examined in the previous study (17) have not been further characterized, the previous observation (17) that the Tet P determinant is present in *C. difficile* must remain unconfirmed.

**Use of PCR to determine the genetic organization of tet genes.** Since all of the tetracycline-resistant *C. perfringens* isolates carried tetA(P) and either tetB(P) or tet(M), we decided to determine if the tetB(P) gene was always arranged with tetA(P) in an operon-like structure. In addition, we decided to see if, in the tet(M)-containing strains, the tet(M) gene had directly replaced the tetB(P) gene and was now associated with tetA(P).

Five sets of PCR primers were synthesized. Three of these primer pairs were specific for the tetA(P), tetB(P), and tet(M) genes, respectively. One primer pair amplified the overlapping tetA(P)-tetB(P) gene region, whereas the final set of primers would detect overlapping tetA(P)-tet(M) genes (Fig. 1B).

In all of the isolates that carried both tetA(P) and tetB(P), PCR analysis showed that these genes overlapped, as they did in pCW3; that is, the 550-bp product was detected in both conjugative and nonconjugative isolates. Nucleotide sequence analysis of the 550-bp PCR products from two conjugative and two nonconjugative isolates confirmed that this region was identical to the pCW3 sequence. Since no products were observed when primers 1369 and 1569 (Fig. 1B) were used in the PCRs, the results indicated that there was no overlap between the tetA(P) and tet(M) genes in isolates carrying these determinants. This result was confirmed by subsequent nucleotide sequence analysis. Note that when the tet(M)-specific primers 1567 and 1568 were used in the PCRs an amplified product was observed in the appropriate isolates.

**Nucleotide sequence analysis of the distal (3’) end of tetA(P) in isolates lacking tetB(P).** Since a significant number of isolates carried tetA(P) but not tetB(P), the nucleotide sequences of the 3’ end of tetA(P) and its flanking DNA were determined in representative isolates lacking tetB(P). The objective of these experiments was to find the divergence point between the various sublines.

Southern hybridization analysis was performed on HindIII-digested DNA from four tetA(P)- tetM+ isolates, two tetA(P)+ isolates, and the single tetA(P)+ tetM+ *C. paraputrefaciens* isolate (data not shown). A fragment of approximately 4 kb hybridized to the tetA(P)-specific probe in all of the *C. perfringens* isolates, indicating that the tetA(P) genes were in the same genomic location and that these gene regions may therefore have a common origin. A hybridizing fragment of about 2.3 kb was observed from the *C. paraputrefaciens* isolate (data not shown).

Inverse PCR methods and, subsequently, direct PCR methods were used to generate the required fragments for sequencing. Inverse PCR was performed on HindIII-digested and religated DNA preparations by using the tetA(P)-specific primers 2477 and 2491 (Table 1) for the six *C. perfringens* isolates and primers 1367 and 2114 for the *C. paraputrefaciens* isolate, resulting in 3.5- and 2.2-kb PCR products, respectively, which hybridized to the tetA(P)-specific probe. Sequence analysis of these products allowed new primers to be synthesized. These new primers were subsequently used to amplify the desired DNA region directly from genomic DNA.

The 3’ 270 nucleotides of the tetA(P) gene were sequenced to determine the point of divergence of tetA(P) from the previously published sequence. The nucleotide sequences ob-
genes which diverged from ing, it is possible that the differences between the nucleotide sequences of tetA(P) and tetA408(P) do not result in phenotypic or functional differences.

The point of divergence between the nucleotide sequences of tetA(P) and tetA408(P) was at nucleotide 2280 (Fig. 2). Therefore, in the tetA408(P) region, no tetB(P)-associated ribosome-binding sites or coding sequences were present (Fig. 2). On the basis of these observations, it is postulated that the PCW3-derived determinant evolved by replacement of the 3' end of a progenitor tetA(P) gene with the tetB(P) gene region. This event would have introduced a new stop codon for tetA(P) within tetB(P), thus creating the unusual overlapping gene arrangement observed previously (25). If such an event did occur, it is possible that tetA408(P) was the progenitor of tetA(P).

The results indicated that the six C. perfringens isolates lacking tetB(P) which were analyzed all carried identical tetA408(P) genes which diverged from tetA(P) at the same site. These results imply that these diverse tetA408(P) gene regions all have a common evolutionary origin. It seems unusual that the C. paraputrificum isolate was found to carry tetA408(P) rather than tetA(P), since acquisition of tetA408(P) by another species would be considered to be a more likely event given its location on a conjugative plasmid.

Several additional PCRs were performed with the same six C. perfringens isolates and the C. paraputrificum isolate to broadly map the extent of common sequences flanking tetA408(P). Three primer pairs were used, each involving primer 1369 and primer 2541, 2581, or 2745 (Table 1); approximately 0.2 kb of each of the resultant PCR products encoded tetA408(P) sequences. Primers 2541 and 2581 were generated by using sequences external to tetA408(P) from C. perfringens, and primer 2745 was generated by using C. paraputrificum sequences. PCR with primer pair 1369-2581 resulted in a 0.4-kb product from all isolates. A 3.0-kb product with primer pair 1369-2541 was generated only with the C. perfringens isolate. It is therefore concluded that at least 0.2 kb of a highly similar sequence flanking tetA408(P) is common to both C. perfringens and C. paraputrificum but that these sequences diverge 1.6 kb from tetA408(P).

On the basis of these results, a model which describes the genetic organization of the C. perfringens tet genes was derived (Fig. 3). In this model, the three combinations of tet genes found in C. perfringens are shown, these being tetA(P) with tetB(P) (Fig. 3A), tetA408(P) with tet(M) (Fig. 3B), and tetA408(P) alone (Fig. 3C). In addition, the C. difficile strains examined...
carried only tet(M) (Fig. 3D). Further studies involving comparative analysis of these gene regions from \textit{C. perfringens} and other clostridia may yield additional insights into the acquisition and evolution of tetracycline resistance determinants by the clostridia.

**ACKNOWLEDGMENTS**


This research was supported by a grant from the Australian National Health and Medical Research Council.

**REFERENCES**