ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1996, p. 2500–2504 Vol. 40, No. 11
0066-4804/96/$04.00+0

Genetic Organization and Distribution of Tetracycline Resistance Determinants in *Clostridium perfringens*

DENA LYRAS* AND JULIAN I. ROOD

Department of Microbiology, Monash University, Clayton, Victoria 3168, Australia

Received 17 April 1996/Returned for modification 19 June 1996/Accepted 15 August 1996

The Tet P determinant from the conjugative *Clostridium perfringens* R plasmid pCW3 encodes two functional overlapping tetracycline resistance genes, *tetA*(P) and *tetB*(P). 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. McMurry, 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 paraputrificum* 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 *tetA408*(P) gene, which lacked the codons for the 12 carboxy-terminal amino acids of the TetA(P) protein.

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. The *tetB*(P) gene encodes a putative 72.6-kDa protein which has significant similarity to Tet M-like tetracycline resistance proteins. These genes appear to be linked in an operon, which represents a novel genetic arrangement for tetracycline resistance determinants (25).

Hybridization studies showed that Tet P was present in eight nonconjugative *C. perfringens* isolates and in a *Clostridium paraputrificum* isolate but was not present in five tetracycline-resistant *Clostridium difficile* isolates and a *Clostridium sporogenes* isolate (1). Since those data were obtained, the nucleotide sequence of Tet P has been determined (25), and it is now evident that the 0.8-kb *Sph*I-*Eco*RI fragment used as a probe in the earlier studies was *tetA*(P) specific. Therefore, the published results apply only to the distribution of the *tetA*(P) gene.

The aims of the study described here were to determine both the genetic organization and the distribution of the *tetA*(P) and *tetB*(P) genes in a wide range of clostridial isolates. The results indicated that conjugative and nonconjugative tetracycline-resistant isolates of *C. perfringens* from diverse sources all carried the *tetA*(P) gene. Most of these isolates also carried a second tetracycline resistance gene, either *tetB*(P) or a *tet*(M)-like gene. In all of the isolates that carried both *tetA*(P) and *tetB*(P), these genes had the overlapping gene arrangement found in pCW3.

* Corresponding author. Phone: (61) (3) 9905 4821. Fax: (61) (3) 9905 4811. Electronic mail address: dena.lyras@med.monash.edu.au.

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 G. N. Dutta), strains CP590 and CP600 from France (6), 5 isolates from Japan (13), 2 isolates from Canada (obtained from D. E. Mahony), and 1 isolate from Germany (29). The single *C. paraputrificum* isolate, CW498, was a porcine isolate from the United States (21).

The plasmids used in the study were the Tn*916*-containing plasmid pAM120 (10), the *tet*(M) plasmid pJI3 (8), and the *tetA*(P)- and *tetB*(P)-specific probe plasmids pJIR666 and pJIR667, 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. paraputrificum* strains were cultured at 37°C in Trypticase-peptone-glucose 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% H_2–10% CO_2–80% N_2. 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 (Seaplaque; FMC BioProducts) with the Magic PCR Preps 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

Primer	Nucleotide sequence[a]	Specificity[b]	Coordinates[c]
1366	5′-CACAGATTGTATGGGGATTAGG-3′	*tetA*(P)	1364–1385
1367	5′-CATTTATAGAAAGCACAGTAGC-3′	*tetA*(P)	2128–2107
1369	5′-GCTACTGTGCTTTCTATAAATG-3′	*tetA*(P)	2107–2128
1370	5′-ATGTGTCAAATAATATTCTTGT-3′	*tetB*(P)	2657–2636
1373	5′-ACAAGAATATTATTTGACACAT-3′	*tetB*(P)	2636–2657
1374	5′-ATTCTTTTGAATTTTCTACTGG-3′	*tetB*(P)	4097–4076
1567	5′-ATTCTTGAATACACCGAGCAGG-3′	*tet*(M)	13887–13866
1568	5′-ACTCGTATATTATTTCATGCAC-3′	*tet*(M)	12447–12468
1569	5′-GTGCATGAAATAATATACGAGT-3′	*tet*(M)	12468–12447
2114	5′-GACCGATTATAGGAATCATAGC-3′	*tetA*(P)	2162–2183
2477	5′-CAGTAAGTATTGGTATAGTATG-3′	*tetA*(P)	2195–2216
2491	5′-CCACTAACTATAATAGGAAGCC-3′	*tetA*(P)	1562–1541
2541	5′-TCTACTCCTGAACTTGGAGCCT-3′	—[d]	—
2581	5′-TTCTCCCTTCTGAGAGAG-3′	—	—
2745	5′-CCCAGGTCTGATTATCCCTATG-3′	—	—

[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).

Inverse PCR. A total of 3 to 5 μg of total genomic DNA from the various isolates was digested with *Hin*dIII, 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 H_2O. 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).

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 5× 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 0.1× 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 *Hin*dIII-*Asp*718 fragment from pJI3 (8), which was internal to the *tet*(M) gene of Tn*916* (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 Tn*916*. 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 *tet*(M)-specific probes were minocycline resistant (5 μg/ml). This result was expected since previous studies have shown that *tetB*(P) (25) and *tet*(M) (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)

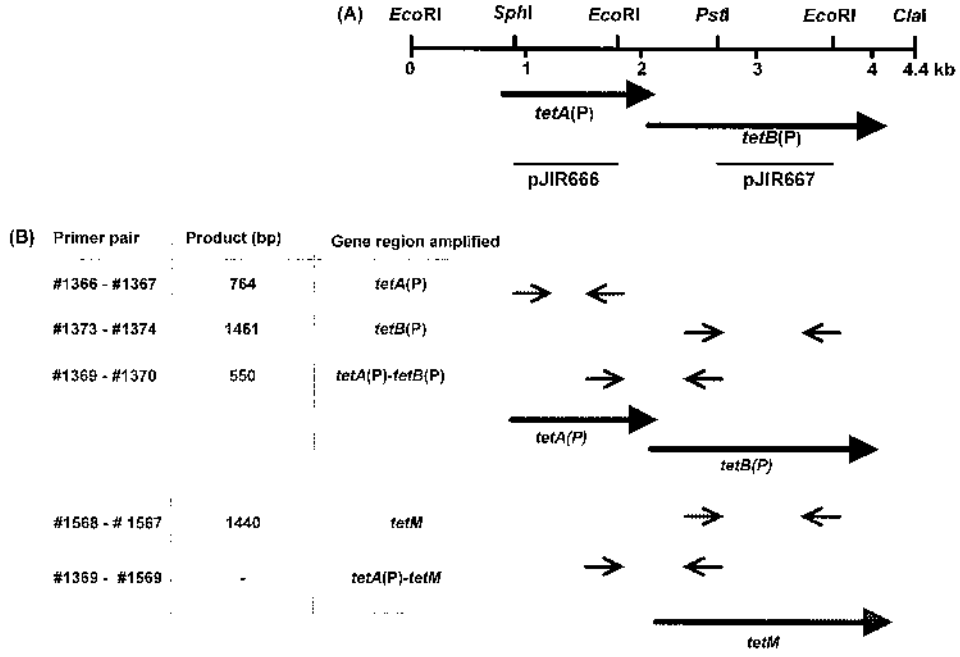

FIG. 1. (A) Restriction and genetic map of the Tet P determinant. Plasmid pJIR666 was constructed by cloning a 0.9-kb *Sph*I-*Eco*RI fragment internal to *tetA*(P) into the equivalent sites of the vector pPR328 (16). Plasmid pJIR667 was constructed by cloning a 1.1-kb *Pst*I-*Eco*RI fragment internal to *tetB*(P) into the equivalent sites of pPR328. (B) Determination of genetic organization of tetracycline resistance determinants by PCR. The product sizes are based on those expected from the nucleotide sequence of the Tet P determinant (25) and of *tet*(M) from Tn*916* (9).

TABLE 2. Hybridization analysis of tetracycline-resistant *C. perfringens* isolates

Group[a]	No. (%) of isolates	Hybridization with *tet* gene fragments from:		
		pJIR666 [*tetA*(P)]	pJIR667 [*tetB*(P)]	pJI3 [*tet*(M)]
Conjugative, *tetA*(P)$^+$ *tetB*(P)$^+$	13 (16)	+	+	−
Nonconjugative				
tetA(P)$^+$ *tetB*(P)$^+$	30 (37)	+	+	−
tetA(P)$^+$ *tet*(M)$^+$	32 (40)	+	−	+
tetA(P)$^+$	6 (7)	+	−	−

[a] The isolates were grouped on the basis of their ability to transfer their tetracycline resistance by conjugation (4, 13, 18, 18a, 19). The nonconjugative isolates were further subdivided on the basis of hybridization results.

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 distributions 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)$^+$ *tet*(M)$^+$ and *tetA*(P)$^+$ 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. paraputrificum* 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. paraputrificum* 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 *Eco*RI-*Pst*I 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 *Hin*dIII-digested DNA from four *tetA*(P)$^+$ *tet*(M)$^+$ isolates, two *tetA*(P)$^+$ isolates, and the single *tetA*(P)$^+$ *tet*(M)$^+$ *C. paraputrificum* 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. paraputrificum* 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 *Hin*dIII-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. paraputrificum* 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-

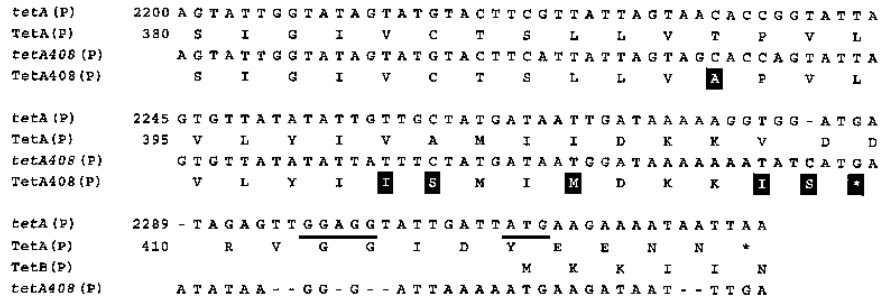

FIG. 2. Comparison of the nucleotide sequences of *tetA*(P) and *tetA408*(P) and of the amino acid sequences encoded by these genes. The coordinates cited are from the published nucleotide sequence of the Tet P determinant and from the amino acid sequence of the TetA(P) protein (25). The light shading highlights nucleotide differences between *tetA*(P) and *tetA408*(P), and the dark shading highlights amino acid differences between the encoded proteins. Stop codons are indicated by asterisks. Dashes indicate spaces which have been introduced to align the two sequences. The underlined sequences in *tetA*(P) represent the *tetB*(P) ribosome-binding site and start codon. The portion of the amino acid sequence of TetB(P) which is encoded within *tetA*(P) is also shown.

tained from the six *C. perfringens* isolates and the *C. paraputrificum* isolate were identical in both the *tetA*(P) gene region and the flanking DNA, although they were somewhat different from that from pCW3. For ease of discussion, the gene from isolates lacking *tetB*(P) was designated *tetA408*(P), for reasons which will become apparent. Note that the nucleotide sequence of the DNA flanking the *tetA408*(P) gene from these isolates had no significant similarity to sequences in the databases.

When the *tetA*(P) and *tetA408*(P) sequences were compared, several differences were observed (Fig. 2). First, *tetA408*(P) was 37 nucleotides shorter than *tetA*(P), resulting in a protein consisting of 408 amino acids, which would be 12 amino acids smaller than that encoded by *tetA*(P). There were 11 nucleotide differences within the sequenced coding regions, 2 of which did not alter the encoded amino acid. The other changes resulted in six amino acid substitutions (Fig. 2).

We have previously proposed a transmembrane model for the structure of TetA(P) (25). In this model, the last transmembrane domain at the carboxy-terminal end of the protein is from amino acids 385 to 403; the remaining amino acids are proposed to reside in the cytoplasm. The amino acid changes in TetA408(P) which were within this putative transmembrane domain (i.e., amino acids 391, 399, 400, and 403) were conservative amino acid changes which would not result in significant charge alterations and which would therefore probably not significantly alter the arrangement of this efflux protein in the membrane. Furthermore, truncation of the protein in the putative cytoplasmic carboxy terminus, following amino acid 403, would be unlikely to affect the transmembrane domains required for the efflux of tetracycline. Therefore, it is presumed that the differences between 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 *tetA*(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* isolates. In contrast, a 1.8-kb product obtained with primer pair 1369-2745 only resulted from the *C. paraputrificum* 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

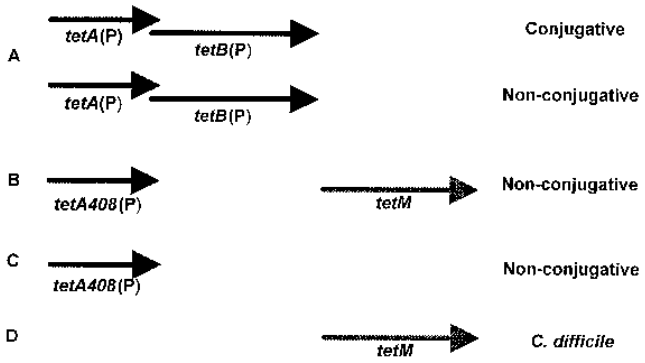

FIG. 3. Tetracycline resistance genes carried by *C. perfringens* (A to C) and *C. difficile* (D).

carried only *tet*(M) (Fig. 3D). Further studies involving comparative analysis of these gene regions from *C. perfringens* and other clostridia may yield additional insights into the acquisition and evolution of tetracycline resistance determinants by the clostridia.

ACKNOWLEDGMENTS

We thank Stephen Billington for helpful discussions and Pauline Howarth, Joan Sloan, Tracy Warner, and Ishara Gunesekere for technical assistance. We thank the following for generously providing strains: V. Burdett, D. B. Clewell, J. W. Dale, G. N. Dutta, D. E. Mahony, Y. Miyoshi, S. Nakamura, M. C. Roberts, W. H. Traub, R. G. Wilkinson, and J. Wüst.

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

REFERENCES

1. **Abraham, L. J., D. I. Berryman, and J. I. Rood.** 1988. Hybridization analysis of the class P tetracycline resistance determinant from the *Clostridium perfringens* R-plasmid, pCW3. Plasmid **19:**113–120.
2. **Abraham, L. J., and J. I. Rood.** 1985. Cloning and analysis of the *Clostridium perfringens* tetracycline resistance plasmid, pCW3. Plasmid **13:**155–162.
3. **Abraham, L. J., and J. I. Rood.** 1985. Molecular analysis of transferable tetracycline resistance plasmids from *Clostridium perfringens*. J. Bacteriol. **161:**636–640.
4. **Abraham, L. J., A. J. Wales, and J. I. Rood.** 1985. Worldwide distribution of the conjugative *Clostridium perfringens* tetracycline resistance plasmid, pCW3. Plasmid **14:**37–46.
5. **Altschul, J. D., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
6. **Brefort, G., M. Magot, H. Ionesco, and M. Sebald.** 1977. Characterization and transferability of *Clostridium perfringens* plasmids. Plasmid **1:**52–66.
7. **Burdett, V.** 1990. Nucleotide sequence of the *tet*(M) gene of Tn*916*. Nucleic Acids Res. **18:**6137.
8. **Burdett, V., J. Inamine, and S. Rajagopalan.** 1982. Heterogeneity of tetracycline resistance determinants in *Streptococcus*. J. Bacteriol. **149:**995–1004.
9. **Flannagan, S. E., L. A. Zitzow, Y. A. Su, and D. B. Clewell.** 1994. Nucleotide sequence of the 18-kb conjugative transposon Tn*916* from *Enterococcus faecalis*. Plasmid **32:**350–354.
10. **Gawron-Burke, C., and D. B. Clewell.** 1984. Regeneration of insertionally inactivated streptococcal DNA fragments after excision of transposon Tn*916* in *Escherichia coli*: strategy for targeting and cloning of genes from gram-positive bacteria. J. Bacteriol. **159:**214–221.
11. **Hayter, P. M., and J. W. Dale.** 1984. Detection of plasmids in clinical isolates of *Clostridium difficile*. Microbios Lett. **27:**151–156.
12. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Miyoshi, Y., and A. Higa.** 1984. Interrelationship between drug resistance and bacteriocinogeny of *Clostridium perfringens*. Microbiol. Immunol. **28:** 281–289.
14. **Morelle, G.** 1989. A plasmid extraction procedure on a miniprep scale. Focus **11:**7–8.
15. **Nakamura, S., K. Yamakawa, S. Nakashio, S. Kamiya, and S. Nishida.** 1987. Correlation between susceptibility to chloramphenicol, tetracycline and clindamycin, and serogroups of *Clostridium difficile*. Med. Microbiol. Immunol. **176:**79–82.
16. **Quigley, N. B., and P. R. Reeves.** 1987. Chloramphenicol resistance cloning vector based on pUC9. Plasmid **17:**54–57.
17. **Roberts, M. C., L. V. McFarland, P. Mullany, and M. E. Mulligan.** 1994. Characterization of the genetic basis of antibiotic resistance in *Clostridium difficile*. J. Antimicrob. Chemother. **33:**419–429.
18. **Rood, J. I.** 1983. Transferable tetracycline resistance in *Clostridium perfringens* strains of porcine origin. Can. J. Microbiol. **29:**1241–1246.

18a. **Rood, J. I.** Unpublished data.

19. **Rood, J. I., J. R. Buddle, A. J. Wales, and R. Sidhu.** 1985. The occurrence of antibiotic resistance in *Clostridium perfringens* from pigs. Aust. Vet. J. **62:** 276–278.
20. **Rood, J. I., and S. T. Cole.** 1991. Molecular genetics and pathogenesis of *Clostridium perfringens*. Microbiol. Rev. **55:**621–648.
21. **Rood, J. I., E. A. Maher, E. B. Somers, E. Campos, and C. L. Duncan.** 1978. Isolation and characterization of multiply antibiotic-resistant *Clostridium perfringens* strains from porcine feces. Antimicrob. Agents Chemother. **13:** 871–880.
22. **Saksena, N. K., and N. Truffaut.** 1992. Cloning of tetracycline-resistance genes from various strains of *Clostridium perfringens* and expression in *Escherichia coli*. Can. J. Microbiol. **38:**215–221.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Scott, P. T., and J. I. Rood.** 1989. Electroporation-mediated transformation of lysostaphin-treated *Clostridium perfringens*. Gene **82:**327–333.
25. **Sloan, J., L. M. McMurry, D. Lyras, S. B. Levy, and J. I. Rood.** 1994. The *Clostridium perfringens* Tet P determinant comprises two overlapping genes: *tetA*(P), which mediates active tetracycline efflux, and *tetB*(P), which is related to the ribosomal protection family of tetracycline-resistance determinants. Mol. Microbiol. **11:**403–415.
26. **Smith, C. J., S. M. Markowitz, and F. L. Macrina.** 1981. Transferable tetracycline resistance in *Clostridium difficile*. Antimicrob. Agents Chemother. **19:**997–1003.
27. **Su, Y. A., P. He, and D. B. Clewell.** 1992. Characterization of the *tet*(M) determinant of Tn*916*: evidence for regulation by transcription attenuation. Antimicrob. Agents Chemother. **36:**769–778.
28. **Taylor, D. E., and A. Chau.** 1996. Tetracycline resistance mediated by ribosomal protection. Antimicrob. Agents Chemother. **40:**1–5.
29. **Traub, W. H.** 1990. Comparative *in vitro* bactericidal activity of 24 antimicrobial drugs against *Clostridium perfringens*. Chemotherapy (Basel) **36:**127–135.
30. **Wüst, J., and U. Hardegger.** 1983. Transferable resistance to clindamycin, erythromycin, and tetracycline in *Clostridium difficile*. Antimicrob. Agents Chemother. **23:**784–786.