Comparison of Tn5397 from *Clostridium difficile*, Tn916 from *Enterococcus faecalis* and the CW459tet(M) element from *Clostridium perfringens* shows that they have similar conjugation regions but different insertion and excision modules

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Comparative analysis of the conjugative transposons Tn5397 from *Clostridium difficile* and Tn916 from *Enterococcus faecalis*, and the CW459tet(M) element from *Clostridium perfringens*, has revealed that these tetracycline-resistance elements are closely related. All three elements contain the tet(M) resistance gene and have sequence similarity throughout their central region. However, they have very different integration/excision modules. Instead of the *int* and *xis* genes that are found in Tn916, Tn5397 has a large resolvase gene, *tndX*. The *C. perfringens* element encodes the putative Int459 protein, which is a member of the integrase family of site-specific recombinases but is not closely related to Int from Tn916. Based on these studies it is concluded that the clostridial elements have a modular genetic organization and were derived independently from distinct mobile genetic elements.

Keywords: conjugative transposons, gene transfer, antibiotic resistance, mobile elements

INTRODUCTION

Conjugative transposons are mobile DNA elements that encode all the necessary functions for intracellular transposition and intercellular conjugation. They are found in a wide variety of both Gram-positive and Gram-negative bacteria and are important in the dissemination of antibiotic-resistance genes (Scott & Churchward, 1995). The first conjugative transposon identified, and the most extensively studied, is the 18 kb element Tn916 from *Enterococcus faecalis* strain DS16 (Franke & Clewell, 1981). Tn916 and the closely related element Tn1545 from *Streptococcus pneumoniae* (Caillaud *et al.*, 1987; Courvalin & Carlier, 1987) form the basis of a family of conjugative transposons that have an extremely broad host range (Rice, 1998). All of

the members of this family carry the tetracycline–minocycline-resistance gene, *tet*(M) (Burdett, 1991); many also carry genes encoding resistance to other antimicrobial agents.

Conjugative transposition of members of the Tn916 family involves excision from the donor genome, circularization of the element, and then transfer to a new host and subsequent insertion into a new target site. Transposition requires the products of the transposon-encoded *int* and *xis* genes, which encode Int, a site-specific recombinase of the integrase family, and Xis, respectively (Poyart-Salmeron *et al.*, 1990). Both Int and Xis are essential for excision but only Int is required for integration (Senghas *et al.*, 1988; Poyart-Salmeron *et al.*, 1990; Lu & Churchward, 1995; Rudy *et al.*, 1997; Marra & Scott, 1999). Int-mediated excision of Tn916 is similar to the excision of λ prophage DNA as it utilizes a mechanism that involves staggered cuts at the ends of the element, circularization, and subsequent transfer to a new host. However, the recombination sites of λ are

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homologous, unlike those of Tn916 (Caparon & Scott, 1989).

Members of the Tn916 family also encode their own conjugative transfer. Analysis of the complete DNA sequence of Tn916 reveals open reading frames (ORFs) that encode putative polypeptides with sequence similarity to proteins known to be involved in conjugation (Flannagan *et al.*, 1994). For example, Orf18 has similarity to the anti-restriction protein Ard of plasmid Collb-P9 and Orf23 is related to the MbeA mobilization protein of plasmid ColE1. A functional *oriT* site is also present between *orf21* and *orf20* (Jaworski & Clewell, 1995).

Work in our laboratories has concentrated on determining the molecular basis for tetracycline resistance in the anaerobic pathogens *Clostridium difficile* and *Clostridium perfringens*. In *C. difficile*, resistance is encoded by the conjugative transposon Tn5397, which mediates transfer between *C. difficile* strains and to and from *Bacillus subtilis* (Mullany *et al.*, 1990). DNA hybridization analysis and partial DNA sequence analysis have shown that Tn5397 contains the *tet(M)* gene and is closely related to Tn916. However, Tn5397 differs from Tn916 in that it contains a Group II intron inserted into *orf14* (Mullany *et al.*, 1996) and has different sequences at its ends (Wang *et al.*, 2000a).

In *C. perfringens*, tetracycline resistance is the most common antimicrobial resistance phenotype (Lyras & Rood, 1996). In most strains, this resistance is non-transferable; however, conjugative transfer of tetracycline resistance is not uncommon (Rood, 1983; Abraham *et al.*, 1985). Transfer is always associated with large conjugative plasmids that are either identical to, or closely related to, the prototype R-plasmid, pCW3 (Abraham *et al.*, 1985; Abraham & Rood, 1985). pCW3 carries the well-characterized tetracycline-resistance determinant Tet(P), which comprises two tetracycline-resistance genes, *tetA(P)* and *tetB(P)* (Sloan *et al.*, 1992). The *tetA(P)* gene encodes a 46 kDa protein that mediates active efflux of tetracycline from the cell. The *tetB(P)* gene encodes a putative 72.6 kDa protein that has significant similarity to Tet(M)-like tetracycline-resistance proteins.

Hybridization and PCR analysis of a large number of conjugative and non-conjugative tetracycline-resistant *C. perfringens* isolates has shown that they all carry the *tetA(P)* gene. Most (93%) of these isolates carry a second tetracycline-resistance gene, with 53% carrying a *tetB(P)* gene and 40% carrying a *tet(M)*-like gene (Lyras & Rood, 1996). No isolates have been detected that carry both the *tetB(P)* and the *tet(M)*-like genes. Conjugative transfer of the latter gene has not been demonstrated in *C. perfringens*.

In this paper, we compare the complete nucleotide sequences of Tn5397, Tn916 and the partial sequence of a *tet(M)*-like element from *C. perfringens* strain CW459. The results show that all three elements are closely related but have different excision modules. The comparison of these three elements provides valuable

insights into the evolution and dissemination of conjugative transposons in Gram-positive bacteria.

METHODS

Bacterial strains and culture conditions. All *Escherichia coli* strains were derivatives of DH5 α (Life Technologies) or DH5 α MCR' (Gibco-BRL) and were grown at 37 °C in 2 $\times$ YT (Sambrook *et al.*, 1989) or LB medium (Sambrook *et al.*, 1989) in the presence of 100 μ g ampicillin ml⁻¹, 10 μ g tetracycline ml⁻¹ or 10 μ g minocycline ml⁻¹. *C. difficile* strain 630 was grown in Brain Heart Infusion (BHI) broth or agar (Oxoid) at 37 °C in an anaerobic chamber (Don Witley Scientific) in an atmosphere of 80% N₂, 10% H₂ and 10% CO₂ in the presence of 10 μ g tetracycline ml⁻¹ and 10 μ g erythromycin ml⁻¹. *C. perfringens* strain CW459 was grown at 37 °C in fluid thioglycollate medium (Difco) or trypticase peptone glucose broth (Rood *et al.*, 1978b) or on nutrient (Rood, 1983) or BHI agar in an atmosphere of 80% N₂, 10% H₂ and 10% CO₂ in an anaerobic jar (Oxoid). Tetracycline (5 μ g ml⁻¹) was added when appropriate.

Molecular techniques. Plasmid DNA was isolated from *E. coli* using the Qiagen Miniprep kit or the High Pure Plasmid Isolation Kit (Boehringer Mannheim) in accordance with the manufacturers' instructions or using a modified small-scale mini alkaline/lysis/PEG precipitation procedure (Applied Biosystems). Genomic DNA was prepared from *C. difficile* by using the Puregene Gram-positive genomic DNA isolation kit (Flowgen) and from *C. perfringens* using dye-buoyant gradient ultracentrifugation at 260 000 g for 20 h at 20 °C (Abraham & Rood, 1985).

A genomic library of *C. perfringens* strain CW459 (Rood *et al.*, 1978a) was constructed by digesting purified chromosomal DNA with *Pst*I and ligating it to *Pst*I-digested pSU39 (Bartolome *et al.*, 1991). After overnight ligation at 14 °C, the DNA was introduced into rubidium chloride competent *E. coli* DH5 α cells (Sambrook *et al.*, 1989). The library was screened on media containing tetracycline (10 μ g ml⁻¹). Recombinant clones that were tetracycline resistant were screened for minocycline resistance (10 μ g ml⁻¹). The recombinant plasmid pJIR1470 was found to confer resistance to both tetracycline and minocycline.

DNA sequencing and analysis. DNA sequencing was carried out using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an Applied Biosystems Perkin Elmer 310 Genetic Analyser or an Applied Biosystems 373 DNA Sequencer. DNA sequence analysis was carried out using the DNAsIS (Hitachi), NCBI tools (<http://www.ncbi.nlm.nih.gov/>), European Bioinformatics Institute tools (<http://www.ebi.ac.uk/>), Seqed (Applied Biosystems), Sequencer 3.0 Software (Gene Codes Corporation) and GeneJockey (Biosoft) programs.

Dot blot hybridization analysis. Plasmid DNA from *E. coli* and chromosomal DNA from *C. perfringens* and *C. difficile* were applied to Hybond-N+ nylon membranes (Amersham) without prior treatment (Sambrook *et al.*, 1989) using a dot blot apparatus (Minifold SRC 96; Schleicher & Schuell). Analysis of dot blots was performed using the DIG DNA Labelling and Detection Kit (Boehringer Mannheim) in accordance with the manufacturer's instructions. Hybridization was carried out at 65 °C in 5 $\times$ SSC (0.75 M NaCl, 0.075 M sodium citrate pH 7.0) with subsequent washes of 2 $\times$ 15 min at 65 °C in 0.1 $\times$ SSC, 0.1% (w/v) SDS. Digoxigenin-11-dUTP-labelled probes were amplified by PCR from Tn916 template DNA and spanned the length of the Tn916 transposon (Fig. 1).

RESULTS AND DISCUSSION

The Tet(M) determinant from *C. perfringens* strain CW459 is located on a Tn916-like element

Previous studies have identified *C. perfringens* strains that carry *tet(M)* genes (Lyras & Rood, 1996). The *tet(M)* determinant from one such strain, CW459, was cloned into the plasmid vector pSU39 to generate pJIR1470. Restriction endonuclease digestion showed that this plasmid contained a 28.1 kb *Pst*I insert and DNA hybridization confirmed that it contained the *tet(M)* gene (data not shown). To see if pJIR1470 carried homologues of other Tn916-encoded genes, the plasmid was hybridized with a series of separate PCR-derived DIG-labelled probes that together encompassed most of Tn916 (Fig. 1). *C. difficile* strain 630, which carries Tn5397, *C. perfringens* strain JIR4229 (Awad & Rood, 1997), which carries a chromosomal copy of Tn916, and pAM120, an *E. coli* plasmid carrying Tn916, were included as positive controls. The results (Fig. 1) showed that pJIR1470 hybridized to each of the Tn916-derived probes, suggesting that a complete Tn916-like element was present in this plasmid and consequently in strain CW459. However, since this strain is non-conjugative (Rood *et al.*, 1978a), and no evidence for the transposition of its *tet(M)* gene has been obtained, this region was designated the CW459*tet(M)* element but not given a formal transposon designation. As expected, *C. difficile* strain 630 also hybridized to these probes (Hächler *et al.*, 1987).

Comparison of the overall genetic organization of Tn5397, Tn916 and CW459*tet(M)*

Previous studies involved the sequence analysis of the first 328 bp of the left end and the last 2442 bp of the right end of Tn5397 (Wang *et al.*, 2000a). The remaining sequence of Tn5397 was determined on both strands by primer walking on the plasmids pPPM5.3 and pPPM1.6 (Mullany *et al.*, 1996). Gaps in the sequence were filled by designing PCR primers based on the Tn916 sequence (GenBank accession no. U09422) and the unpublished *C. difficile* sequence (http://www.sanger.ac.uk/Projects/C_difficile/). To avoid PCR-induced errors, each PCR product was amplified and sequenced at least three times. The results showed that Tn5397 was 20658 bp in length (GenBank accession no. AF333235) and had 21 potential ORFs that had a suitably spaced ribosome-binding site upstream of the putative start codon. The ORF within the Group II intron (Mullany *et al.*, 1996) and *tndX* (Wang *et al.*, 2000a; Wang & Mullany, 2000) have been described previously. Seventeen of the ORFs were very similar to corresponding ORFs from Tn916 (Flannagan *et al.*, 1994) and were labelled accordingly (Fig. 2).

The CW459*tet(M)* element was sequenced on both strands by primer walking on a pJIR1470 template. An 8691 bp region including the *tet(M)* gene and the right end of the element was sequenced (GenBank accession no. AF329848). The G+C content of this region was

35.9 mol% compared to 38.3 mol% for Tn5397 and 38.8 mol% for Tn916. Since CW459*tet(M)* is not a functional mobile element, the left end of the element was not completely sequenced. The overall structure of both clostridial elements is very similar to that of Tn916 (Fig. 2) although there are numerous insertions, deletions and rearrangements, both within ORFs and in the intergenic regions. This finding also applies to the incomplete single-stranded sequence that was obtained from the left end of the CW459*tet(M)* element (data not shown). Nine of the ten sequenced CW459*tet(M)* ORFs were closely related to those of Tn916 and were named accordingly (Fig. 2). A comparison of each of these putative protein products is shown in Table 1.

Tn5397, CW459*tet(M)* and Tn916 have different excision/insertion modules

The most notable differences between the three elements occurred at the extreme right end, where both clostridial elements diverged from Tn916 and from each other (Fig. 2). In these elements, divergence from Tn916 occurs within *orf5*, suggesting that this region is a recombinational hotspot. Tn5397 only has the 3' end of *orf5* but is still capable of conjugative transfer, providing evidence that *orf5* is not essential for the transfer of this element.

In Tn916, the *int* and *xis* genes, the products of which are essential for excision/insertion, are located to the right of *orf5* (Fig. 2). In the clostridial elements, these genes have been replaced with other genes that encode different site-specific recombinases. Database searches with the putative protein encoded at the right end of the CW459*tet(M)* element revealed 21–28% identity to several bacteriophage or transposon-encoded integrases from Gram-positive bacteria (GenBank accession nos AAA85500, AAC48867, A69774 and C55205) and 17.5% identity to Int from Tn916. Therefore, the CW459-derived ORF was designated *int459* (integrase CW459). A comparison of Int459 with other members of the integrase family showed that the conserved integrase motif (Abremski & Hoess, 1992) was present in the putative Int459 protein. This protein may have played a role in the transposition and introduction of the CW459*tet(M)* element into CW459. However, since conjugative transfer of the CW459*tet(M)* element is not observed, it appears that either this protein is not produced, is not functional, or is not sufficient on its own for transposition. As observed for the transposition of Tn916 (Jaworski *et al.*, 1996; Marra & Scott, 1999), the excision, and therefore transposition, of the CW459*tet(M)* element may require additional proteins, such as an excisionase, which are not present in this element.

An additional 170 bp incomplete ORF was identified at the right of the sequenced CW459*tet(M)* region and was designated *gmp* because the deduced protein product had 70% identity over 47 amino acids to a GMP synthetase protein from *B. subtilis* (Mantsala & Zalkin, 1992). Since this enzyme is a housekeeping protein we

Fig. 1. Dot blot analysis of regions of Tn916 against strains containing Tn5397, CW459tet(M) or derivatives of these elements. (a) Map of Tn916 with the size and orientation of genes represented by the arrowed boxes. The names of the ORFs are as in Flannagan *et al.* (1994). The next line shows the scale in kb; the next line shows the binding site and direction of priming of oligonucleotides used in PCR to generate probes used in the dot blot analysis. The extent of the various probes is indicated under the oligonucleotides. (b) Dot blot analysis. Plasmid or genomic DNA was spotted onto the membrane and probed with fragments of Tn916.

Fig. 2. Genetic organization of Tn5397 and CW459tet(M) compared to Tn916. The top line for all three elements indicates the size in kb. Underneath this is the genetic structure of the elements. Arrowed boxes represent the ORFs, with the proposed direction of transcription shown by the arrow. The ORFs are labelled underneath. In Tn5397, the Group II intron is shaded. In Tn916, a black arrowhead depicts the *oriT*. Only the sequenced area of CW459tet(M) is shown.

concluded that it was outside the CW459tet(M) element. Therefore, the right terminus of the element appeared to be located within the 67 bp region between *int459* and *gmp* (Fig. 2). Although this region contained several repeat sequences, it did not have any similarity to the ends of Tn916, Tn5397 or any other known conjugative transposons.

Downstream of *orf8* in Tn5397 (Fig. 2) is the *tndX* gene, which encodes a 61.5 kDa protein, TndX (Wang *et al.*, 2000a), that has 37% identity to TnpX, a large resolvase from the *C. perfringens* transposon Tn4451 (Bannam *et al.*, 1995) and the *C. difficile* transposon Tn4453a (Lyras

& Rood, 2000). Functional analysis has shown that TndX is involved in the excision and integration of Tn5397 in *C. difficile*. The *tndX* gene ends 10 bp before the right terminus of Tn5397 (Wang *et al.*, 2000a; Wang & Mullany, 2000).

The Tn5397 and CW459tet(M) elements do not have the tet(M) leader peptide *orf12*

Just upstream of the *tet(M)* gene in Tn916 there is a small ORF, *orf12*, which encodes a *tet(M)* leader peptide that is involved in the regulation of *tet(M)* expression by

Table 1. Comparative analysis of the Orf14–Orf5 region of the CW459*tet*(M) element, Tn916 and Tn5397

Percentage identity is shown.

	Tn916	CW459
Orf14		
Tn916	100.0	
CW459	71.5	100.0
Tn5397	87.4	66.3
Orf13		
Tn916	100.0	
CW459	52.1	100.0
Tn5397	87.4	52.7
Tet(M)		
Tn916	100.0	
CW459	99.7	100.0
Tn5397	90.5	90.5
Orf6		
Tn916	100.0	
CW459	100.0	100.0
Tn5397	87.1	87.1
Orf9		
Tn916	100.0	
CW459	99.1	100.0
Tn5397	83.2	84.0
Orf10*		
Tn916	100.0	
CW459	43.4	100.0
Tn5397	47.8	39.1
Orf7		
Tn916	100.0	
CW459	68.6	100.0
Tn5397	85.8	63.8
Orf8		
Tn916	100.0	
CW459	78.9	100.0
Tn5397	85.5	69.7
ΔOrf5†		
Tn916	100.0	
CW459	53.2	100.0
Tn5397	70.9	59.6

* Over 23 amino acids

† Over 62 amino acids.

a transcriptional attenuation mechanism (Su *et al.*, 1992; Celli & Trieu-Cuot, 1998). In both Tn5397 and the CW459*tet*(M) element there has been a deletion of all or part of *orf12*. In Tn5397, the 88 bp deletion is associated with a CCCAGT direct repeat (Fig. 3). In the CW459*tet*(M) element, there is a deletion of 155 bp, which is associated with a CCTTTT direct repeat (Fig. 3). Therefore, both deletions appear to have arisen by homologous recombination. CW459 expresses tetracycline resistance constitutively (J. Rood, unpublished

results), but also carries a second tetracycline-resistance gene, *tetA*(P). Strain 630 is inducible but the mechanism of induction of tetracycline resistance is not known (A. Roberts & P. Mullany, unpublished results). However, although *orf12* is partially deleted, this region still contains an inverted repeat ($\Delta G = -61.7 \text{ kJ mol}^{-1}$) that has similarity to the repeats which in Tn916 form the upstream transcriptional terminator.

The Tet(M) proteins from Tn916 and the CW459*tet*(M) element are more closely related to each other than to Tet(M) from Tn5397

The deduced amino acid sequence of the Tet(M) protein from the CW459*tet*(M) element was almost identical to the Tet(M) protein from Tn916 (Su *et al.*, 1992; Flannagan *et al.*, 1994) but was less closely related to the Tet(M) protein from Tn5397 (Table 1). Comparative analysis was also performed with other Tet(M) proteins from a variety of bacterial genera. A high degree of sequence identity (91–99%) was observed (Fig. 4). Tet(M) from Tn5397 had slightly higher identity (93%) to the Tet(M) protein from Tn1545 (Martin *et al.*, 1986) than to the Tet(M) protein from CW459 or Tn916 (Table 1). These results suggest that the acquisition of the CW459 resistance determinant from a precursor carrying a Tn916-like *tet*(M) gene was a relatively recent event and that the *tet*(M) genes in CW459 and Tn5397 were not derived from each other but originated from different donors at different times.

The regions between *tet*(M) and the recombinase genes are similar but not identical to the equivalent region of Tn916

The regions between *tet*(M) and the recombinase genes are closely related in all three elements (Table 1). However, the CW459*tet*(M) ORFs are more closely related to those of Tn916 than to Tn5397 (Table 1). Celli & Trieu-Cuot (1998) showed that *orfs* 7, 8 and 9 encoded putative regulatory proteins. Homologues of these ORFs are present in similar size and arrangement in the clostridial elements (Table 1). The proposed regulatory region in Tn916 includes the palindromes *pal*_{orf10a} and *pal*_{orf10b}, which are involved in the termination of readthrough transcription from the upstream *tet*(M) promoter. Similar but not identical palindromes are found in both clostridial elements.

In both Tn5397 and the CW459*tet*(M) element there was an intergenic region after *orf8*, followed by the remnants of *orf5*. In both elements, the N-terminal encoding region of *orf5* is missing. In Tn916, the *orf8*–*orf5* intergenic region contains the promoter region for the *xis* and *int* genes (Celli & Trieu-Cuot, 1998). The intergenic region in the CW459*tet*(M) element was similar in size and nucleotide sequence identity (79%) to the equivalent region of Tn916. The *xis*–*int* promoter region was present but there were two single base changes. Two regions of dyad symmetry, which have some similarity to the imperfect inverted repeats *pal*_{orf8a}

Fig. 3. Comparative DNA sequence analysis of the region just upstream of *tet(M)* in Tn5397, Tn916 and CW459tet(M). The deduced amino acid sequence of the Orf12 leader and Tet(M) of Tn916 is shown in the single letter code underneath the nucleotide sequence. Ribosome-binding sites are underlined and shown in bold. Inverted repeats within *orf12* are underlined or overlined with half arrows. Direct repeats (CCTTTT) associated with the 155 bp deletion region upstream of the CW459tet(M) *tet* gene are overlined by half arrows. Direct repeats (CCCAGT) associated with the 88 bp deletion in Tn5397 are overlined by half arrows.

Fig. 4. Phylogenetic relationship between ribosomal protection proteins. The relationship of ribosomal protection proteins was estimated using the neighbour-joining distance procedure (Thompson *et al.*, 1994). The alignment was constructed using CLUSTAL W (Thompson *et al.*, 1994). The reliability of the branching was assessed using bootstrapping analysis (1000 replications). Statistical significant support (<70%) for a branch is indicated at the node as a percentage. The branch lengths are scaled in proportion to the extent of the change per position as indicated by the scale bar. No correction for multiple substitution was made. The tree showing the evolutionary relationships between these proteins was constructed using TreeView (Rhoderic D. M. Page). The phylogenetic tree was constructed from amino acid sequences from the following proteins: Tet(S) from *Lactococcus lactis* (accession no. CAA63528), Tet(O) from *Campylobacter jejuni* (accession no. AAA23033), Tet(W) from *Butyrivibrio fibrisolvens* (accession no. CAA10975), Tet(T) from *Streptococcus pyogenes* (accession no. AAF01499), TetB(P) from *C. perfringens* (accession no. AAA20117) and OtrA from *Streptomyces rimosus* (accession no. S18572). TetB(P) and OtrA were used as outgroups as they are the most distantly related ribosomal protection proteins.

and *pal*_{orf8b} from Tn916, were present. These repeats are believed to be involved in transcriptional termination from the upstream *orf7* promoter (Celli & Trieu-Cuot, 1998). In contrast, the smaller *orf8-orf5* intergenic

region of Tn5397 has sequence identity (74%) to only part of the equivalent Tn916 region. In Tn5397, a major deletion has occurred at the beginning of this region. This deletion effectively removes the promoter area of

the missing *xis* and *int* genes and the *pal*_{orf8} repeats. To the right of the remainder of *orf5* the sequence of the clostridial elements diverges dramatically as already discussed.

Tn5397 contains homologues of the Tn916 conjugation genes

As previously described, the 180 bp at the left end of Tn5397 are not related to Tn916 (Wang *et al.*, 2000a). The first seven nucleotides of *orf24* are also missing. Following this region, the two transposons are very closely related (Fig. 2) although several insertions and deletions are present in Tn5397. The deduced amino acid sequences of Orfs 23–16 have 90–100% identity to their Tn916 homologues; Orf15 has 80% identity. Following *orf21* is a 392 bp intergenic region which contains a sequence that is homologous to the functional *oriT* region of Tn916 (Jaworski & Clewell, 1995). Within the *oriT* sites of Tn916 and the F plasmid there is a conserved nick site, 5'-TGGTGTGG-3'. This site is identical in Tn5397.

Preliminary sequence analysis of the CW459*tet*(M) element indicated that sequences with similarity to much of the region encompassing the conjugation genes of Tn916 appear to be present. However, its exact genetic organization and precise relationship to the equivalent region in Tn916 remain to be determined. The conserved *oriT* nick site is present.

The first ORF of the double-stranded sequenced region of the CW459*tet*(M) element, *orf15*, was only partially sequenced. However, the amino acid sequence of the deduced protein had only 21% identity to a corresponding 288-amino-acid region of Orf15 from Tn916 (Flannagan *et al.*, 1994) and 23.9% identity to a corresponding region of Orf15 from Tn5397. By comparison with Tn916, Orf15 from both Tn5397 and the CW459*tet*(M) element contained C-terminal truncations of 25 and 57 amino acids, respectively. In Tn5397, the next ORF, *orf14*, is disrupted by a Group II intron (Mullany *et al.*, 1996). Neither the CW459*tet*(M) element nor Tn916 contains a Group II intron in *orf14* (Fig. 2). Comparative analysis of the deduced amino acid sequences showed that Orf14 (minus the Group II intron) from Tn5397 is most closely related to Orf14 from Tn916.

Evolution of Tn5397 and the CW459*tet*(M) element

Both Tn5397 and the CW459*tet*(M) element appear to consist of four distinct modules that may have originated from separate mobile genetic elements. The first module is common although not identical and consists of the Tn916-like conjugation region (*orf23–orf13*), which is present in Tn5397 and most probably in the CW459*tet*(M) element. The resistance determinant comprises the second module, with the *tet*(M) genes of Tn916 and CW459 being very closely related and distinct from that of Tn5397, which is more closely related to the *tet*(M) gene from Tn1545 (Fig. 4). The third module

comprises the putative regulatory region (*orf12–orf8*). The regulatory and resistance modules may overlap, as *orf12* is involved in sensing tetracycline and consequently regulating *tet*(M) expression. The fourth major module is responsible for the excision and insertion of the transposons or their precursors and consists of *xis* and *int* in Tn916, *int459* in CW459*tet*(M) and *tndX* in Tn5397. Therefore, the three elements have different excision and integration systems.

Tn916-like sequences have also been found in other mobile genetic elements. The *tet*(M) genes of the large conjugative plasmids of *Neisseria gonorrhoeae* and *Neisseria meningitidis* are associated with Tn916-like sequences that encompass regions upstream and downstream of the resistance determinant (Swartley *et al.*, 1993). Other studies involving the non-conjugative multiple antibiotic resistance plasmid pK214 from *L. lactis*, which carries resistance determinants for streptomycin, tetracycline and chloramphenicol, have revealed that the tetracycline-resistance gene, *tet*(S), is linked to sequences with similarity to *orf7*, *orf9* and *orf6*, which have a similar genetic organization to that observed in Tn916. This Tn916-like region is linked to distinct gene regions from three other bacterial species as well as to numerous insertion sequences (Perreten *et al.*, 1997; Teuber *et al.*, 1999). Finally, the integrase from a lysogenic *Mycoplasma arthritidis* bacteriophage has greater similarity to Int from Tn916 than to any other integrase (Voelker & Dybvig, 1998), suggesting a common evolutionary origin.

There are several hypotheses that may explain the evolution of the non-functional CW459*tet*(M) element. Firstly, it is possible that a modular conjugative transposon may have been initially formed by recombination events between different mobile genetic elements in another bacterium and then subsequently transferred to *C. perfringens*. Upon insertion into the chromosome, deletion or rearrangement events may have occurred which resulted in the loss of mobility of the transposon. Similar events, resulting in deletions of sections of Tn916, have been observed in *Neisseria* (Swartley *et al.*, 1993). Altered Tn916-like elements, which are no longer capable of conjugative transfer, have also been detected in the non-conjugative plasmid pKQ1, from *Enterococcus faecium* (Fletcher & Daneo-Moore, 1992). Secondly, a composite element may have been formed in *C. perfringens* strain CW459 by a recombination event between a previously acquired, ancestral Tn916 or Tn916-like transposon and a second mobile genetic element, which carried an Int459-based excision/integration system. Alternatively, the recombination event may have involved a resident element containing *int459* and an incoming Tn916-like transposon. Note that the conjugative transposition of Tn916 into the chromosome of *C. perfringens* from both *E. coli* and *Enterococcus faecalis* has been observed (Kaufmann *et al.*, 1996). Tn916 has also been shown to transfer into *C. difficile* from *B. subtilis* (Mullany *et al.*, 1991) and natural isolates of *C. difficile* that carry Tn916 have recently been identified (Wang *et al.*, 2000b).

Although direct transfer of genetic information between *C. perfringens* and *C. difficile* has not been demonstrated, closely related transposons have been detected in both species, indicating that genetic exchange whether directly, or indirectly through another bacterial host, can occur (Lyras *et al.*, 1998). The TndX protein, which is involved in the excision and integration of Tn5397, is more closely related to the TnpX resolvase that is responsible for the movement of the *C. perfringens* transposon Tn4451 and the closely related *C. difficile* Tn4453 transposons than to any other resolvase (Wang *et al.*, 2000a; Lyras & Rood, 2000). It has been proposed that one of these elements may have been involved in recombination events with an ancestral Tn916-like element that resulted in the formation of Tn5397 (Wang *et al.*, 2000a).

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