Genetic analysis of a tetracycline resistance element from *Clostridium difficile* and its conjugal transfer to and from *Bacillus subtilis*

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A tetracycline resistance (Tc^r) determinant from *Clostridium difficile* strain 630 was cloned into the *Escherichia coli* plasmid vector pUC13. The resulting plasmid pPPM20, containing an insert of 3.4 kbp, was mapped and a 1.1 kbp *SacI*-*HindIII* fragment wholly within the Tc^r gene was identified. Dot-blot hybridization studies with the 1.1 kbp fragment showed that the Tc^r gene belonged to hybridization class M. Tc^r could be transferred between *C. difficile* strains and to *Bacillus subtilis* at a frequency of 10^{-7} per donor cell. The element could be returned from *B. subtilis* to *C. difficile* at a frequency of 10^{-8} per donor cell. This is the first demonstration of *C. difficile* acting as a recipient in intergeneric crosses. DNA from *C. difficile* transconjugants digested with *EcoRV* always has two hybridizing fragments of 9.5 and 11.0 kbp when probed with pPPM20. DNA from *B. subtilis* transconjugants digested with *EcoRV* produced one hybridizing band of variable size when probed with pPPM20. The behaviour of the element was reminiscent of the conjugative transposons. Therefore we compared the element to the conjugative transposon Tn916. The *HincII* restriction maps of the two elements differed and no hybridization was detected to oligonucleotides directed to the ends of Tn916. However, the elements do have some sequence homology, detected by hybridization analysis.

Introduction

Clostridium difficile has been shown to be the most important cause of antibiotic-associated pseudomembranous colitis and antibiotic-associated diarrhoea (Larson & Price, 1977; George *et al.*, 1978; Bartlett *et al.*, 1978). Resistance to antibiotics may also play a significant role in the development of the disease, as multiply resistant strains are often responsible for nosocomial outbreaks of *C. difficile*-associated disease (Pierce *et al.*, 1982; Wust *et al.*, 1982).

Resistance to tetracycline and macrolide antibiotics is known to be transferable between strains of *C. difficile*; and the transfer of tetracycline resistance (Tc^r) has been found to be independent of the transfer of other antibiotic resistances (Wust & Hardegger, 1983). Hybridization studies indicated that the transferable Tc^r element contains sequences homologous to 95% of the conjugative transposon Tn916, which carries a Tc^r gene of hybridization class M (Hachler *et al.*, 1987a). Conjugative transposons are widespread in Gram-positive bacteria and are important in the dissemination of antibiotic resistance genes in many Gram-positive

genera (for a review see Clewell & Gawron-Burke, 1986).

This paper describes the cloning and partial genetic and physical characterization of the *C. difficile* Tc^r element.

Methods

Bacterial strains and plasmids. All bacterial strains and plasmids used in this study are shown in Table 1.

Growth media. Clostridial strains were grown in pre-reduced Nakamura broth (Nakamura *et al.*, 1981) or on Wilkins-Chalgren agar (Oxoid) and incubated at 37 °C for 18 h under anaerobic conditions. All other strains were grown in Luria-Bertani broth or on Luria-Bertani agar (Maniatis *et al.*, 1982).

DNA preparation. *C. difficile* and *B. subtilis* genomic DNA was prepared by a method developed in this laboratory (Wren & Tabaqchali, 1987). Plasmid DNA from *E. coli* was prepared by the method of Birnboim & Doly (1979).

Construction and screening of a *C. difficile* genomic library. *C. difficile* genomic DNA was partially cleaved with *Sau3AI* and fragments between 2 and 10 kbp were pooled. The fragments were ligated into the *lacZ* gene of *Bam*HI-cleaved pUC13 plasmid DNA and transformed into competent *E. coli* JM109 cells by the method of Hanahan (1985).

Table 1. *Bacterial strains and plasmids*

Bacterial strains	Genotype and/or phenotype*	Source or reference
<i>C. difficile</i>		
C	Tc ^s Rif ^s ; PAGE type strain	Tabaqchali <i>et al.</i> (1984)
D	Tc ^s Rif ^s ; PAGE type strain	Tabaqchali <i>et al.</i> (1984)
630	Tc ^r Rif ^s ; Tc ^r donor	Hachler <i>et al.</i> (1987a)
CD37	Tc ^s Rif ^r ; recipient strain	Hachler <i>et al.</i> (1987a)
SB1	Tc ^r Rif ^r ; 630 × CD37 transconjugant	This study
SB2	Tc ^r Rif ^r ; 630 × CD37 transconjugant	This study
SB3	Tc ^r Rif ^r ; 630 × CD37 transconjugant	This study
FM29	Tc ^r Rif ^r ; BS1 × CD37 transconjugant	This study
FM30	Tc ^r Rif ^r ; BS2 × CD37 transconjugant	This study
FM75	Tc ^r Rif ^r ; CD37 :: Tn916	P. Mullany <i>et al.</i> , unpublished
<i>E. coli</i>		
D20-15	<i>tetA</i>	PHLS, Colindale, UK
D20-16	<i>tetB</i>	PHLS, Colindale, UK
D20-6	<i>tetC</i>	PHLS, Colindale, UK
D22-2	<i>tetD</i>	PHLS, Colindale, UK
D22-14	<i>tetE</i>	PHLS, Colindale, UK
JIR741	<i>tetP</i>	Abraham <i>et al.</i> (1988)
CGC120	pAM120 (::Tn916)	Gawron-Burke <i>et al.</i> (1984)
2B1	Contains pAM120 from which Tn916 has spontaneously excised (pAM120LT)	This study
2A1	JM109 containing pPPM20	This study
2A87	HB101 containing pCDC1	This study
JM109	<i>recA1 endA1 gyrA96 thi hsdR17</i> (r _K m _K) <i>supE44 relA1</i> λ ⁻ Δ(<i>lac-proAB</i>) (F' <i>traD36 proAB lacI^hΔM15</i>)	Yanisch-Perron <i>et al.</i> (1985)
HB101	F ⁻ <i>hsd20</i> (r _K m _K) <i>supE44 ara-14</i> λ ⁻ <i>galK12 lacY1 proA2 rpsL20 xyl-5 mtl-1 recA13</i>	Maniatis <i>et al.</i> (1982)
<i>B. subtilis</i>		
CU2189	Tc ^s ; recipient strain	Christie <i>et al.</i> (1987)
BS1	Tc ^r ; 630 × CU2189 transconjugant	This study
BS2	Tc ^r ; SB2 × CU2189 transconjugant	This study
BS3	Tc ^r ; SB3 × CU2189 transconjugant	This study
Plasmids	Description/derivation	Origin or reference
pUC13	Cloning vector	Yanisch-Perron <i>et al.</i> (1985)
pPPM20	pUC13 containing a 3.4 kbp insert of <i>C. difficile</i> DNA encoding Tc ^r	This study
Homer III	5.3 kbp cosmid	Amersham
pCDC1	Homer III containing a 22.4 kbp insert of <i>C. difficile</i> DNA encoding Tc ^r	This study
pAM120	Tn916 :: pGL101	Gawron-Burke & Clewell (1984)
pAM120LT	pAM120ΔTn916	This study

* Rif^r, rifampicin resistant; Rif^s, rifampicin sensitive; Tc^r, tetracycline resistant; Tc^s, tetracycline sensitive.

Transformants were selected on plates containing ampicillin (50 µg ml⁻¹) and the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal, Sigma). The colourless, Lac⁻ colonies, which contained inserts of *C. difficile* DNA, were replica-plated onto agar containing 24 µg tetracycline ml⁻¹.

Restriction enzyme analysis and transposon mutagenesis. The clone with the smallest DNA insert (pPPM20) was mapped with a variety of restriction enzymes (*AccI*, *AvaI*, *BamHI*, *BclI*, *DdeI*, *EcoRI*, *EcoRV*, *HincII*, *HindIII*, *MboI*, *PstI*, *SmaI*, *SstI*, *TaqI*, *XbaI* and *XhoI*) under conditions specified by the suppliers (NBL). To localize the Tc^r gene further, transposon mutagenesis using Tn1000 (γδ region of the F factor

of *E. coli* K12) was performed to generate insertion mutations of pPPM20 which were no longer resistant to tetracycline. These insertions were localized by digesting independently with *HindIII* and *EcoRI* essentially as described by Taylor *et al.* (1987).

Preparation of radioactive DNA probes and hybridization procedures. DNA fragments were radiolabelled *in vitro* with [³²P]dCTP (Amersham) by the random hexamer primer method of Feinberg & Vogelstein (1983). Colony hybridization was performed on nitrocellulose filters (NEN Research Products) by the method of Grunstein & Hogness (1975). Further analysis of digested genomic DNA from *C. difficile* and *B. subtilis* strains was performed by Southern blots

(Southern, 1975) using Gene-screen hybridization membranes (NEN Research Products). Hybridizations were carried out under conditions recommended by NEN Research Products. All blots were dried, and exposed to Fuji RX X-ray film at -70°C for up to 14 d.

Filter mating. Cultures were grown to mid-exponential phase ($\text{OD}_{650} = 0.45$) and mixed in a donor : recipient ratio of 1 : 1 on 0.45 μm cellulose nitrate filters (Whatman). The filters were placed on blood agar plates (Oxoid) and incubated anaerobically for 24 h at 37°C . Transconjugants were selected on Wilkins-Chalgren agar supplemented with 7% (v/v) defibrinated horse blood, rifampicin ($20\text{ }\mu\text{g ml}^{-1}$, to inhibit the growth of the donor) and tetracycline ($20\text{ }\mu\text{g ml}^{-1}$). When *C. difficile* was the recipient the plates were incubated anaerobically for 48 h. In matings where *B. subtilis* was the recipient, transconjugants were selected on Wilkins-Chalgren agar plates supplemented with tetracycline ($20\text{ }\mu\text{g ml}^{-1}$) and were incubated aerobically (to inhibit the growth of the *C. difficile* donor) at 37°C for 24 h.

Stability of the Tc^r element. A single colony of the transconjugant FM29 was inoculated into 10 ml Wilkins-Chalgren broth and incubated anaerobically at 37°C . The mixture was subcultured daily for 14 d.

Cosmid cloning. A library of genomic DNA from *C. difficile* strain 630 was constructed by ligating DNA fragments generated by partial cleavage with *Hind*III into the unique *Hind*III site of the 5.34 kbp cosmid Homer III. The procedures of Maniatis *et al.* (1982) were followed. The chimeric cosmids were packaged *in vitro* using the Gigapack Plus packaging extract (Stratagene) and transduced into *E. coli* HB101. Transduced cells were plated directly onto solid medium containing tetracycline ($30\text{ }\mu\text{g ml}^{-1}$) and 10 resistant colonies were selected for further study.

Preparation of oligonucleotides. Oligonucleotides homologous to the first 18 nucleotides of the left ($5' \text{ TAAACAAAGTATAAATTT } 3'$) and first 19 nucleotides of the right ($5' \text{ GATACTAGATTTT-TATGC } 3'$) ends of Tn916 were prepared from the data of Clewell *et al.* (1988).

Results and Discussion

Cloning and hybridization analysis of the *C. difficile* Tc^r determinant

The Tc^r gene of *C. difficile* strain 630 (see Table 1) was cloned into plasmid pUC13. Transformants were plated on tetracycline-containing agar, which yielded 13 tetracycline-resistant colonies out of 1200 tested. The inserts ranged in size between 3.4 and 6.0 kbp. Plasmid pPPM20 (Fig. 1) contained the smallest insert (3.4 kbp) and was selected for further study; pPPM20 conferred Tc^r on *E. coli* with an MIC of $64\text{ }\mu\text{g ml}^{-1}$.

The restriction map of the DNA cloned in pPPM20 was determined (Fig. 1), and mapping of a limited number of Tn1000 insertions showed that a 1.1 kbp *Sac*I–*Hind*III fragment is wholly within the Tc^r gene. Dot-blot hybridization of the pPPM20 *Sac*I–*Hind*III fragment with DNA from *E. coli* strains carrying Tc^r genes *tetA*–*E* and *tetM* (Mendez *et al.*, 1980; Gawron-Burke & Clewell, 1984; Marshall *et al.*, 1986) and the *tetP* gene of *Clostridium perfringens* (Abraham *et al.*, 1988)

Fig. 1. Map of restriction enzyme recognition sites and Tn1000 insertions into plasmid pPPM20. —, Cloned *C. difficile* DNA; ---, vector DNA. Triangles indicate Tn1000 insertions; all abolish Tc^r .

resulted in strong hybridization to the *tetM* gene and weak non-specific hybridization to other strains, suggesting that the *C. difficile* Tc^r gene is of class M (data not shown). Analysis of pPPM20-directed polypeptides synthesized in an *in vitro* transcription-translation system indicated that the plasmid encoded a protein of 68 kDa (results not shown), which is similar to the size of the prototype TetM protein (Martin *et al.*, 1986). Deletion of the *Sac*I–*Sac*I or *Hind*III–*Hind*III fragments of pPPM20 abolished the production of the 68 kDa protein, further confirming that the *Sac*I–*Hind*III fragment used in hybridization analysis is indeed wholly within the Tc^r gene. Furthermore, the *tetM* determinant has a 1.1 kbp *Sac*I–*Hind*III fragment. However, the Tc^r gene of *C. difficile* also contains a *Hinc*II site which is not found in the *tetM* determinant of Tn916.

Transfer of tetracycline resistance to *C. difficile* strains and to *B. subtilis*

Filter mating using *C. difficile* strains 630 and CD37 as donor and recipient, respectively, produced Tc^r transconjugants at a frequency of 10^{-7} per donor cell; three independent transconjugants, designated SB1–SB3 were selected for further study. Tc^r was also transferred from *C. difficile* 630 to *B. subtilis* CU2189 at a frequency of 10^{-7} per donor cell; transconjugant BS1 was selected for further study. Tc^r previously transferred between *C. difficile* 630 and CD37 was further transferred from SB2 and SB3 into *B. subtilis*, at the same frequency, and BS2 and BS3 were selected from these matings. Tc^r was reintroduced into *C. difficile* from *B. subtilis* BS1 and BS2 at a frequency of 10^{-8} per donor cell. Transconjugants FM29 and FM30 respectively were selected from these matings. *B. subtilis* CU2189 also became spontaneously Tc^r at a frequency of 10^{-8} per surviving cell. The spontaneously resistant cells had an MIC of $32\text{ }\mu\text{g ml}^{-1}$, the same as the transconjugants.

The transfer of Tc^r from *B. subtilis* BS1 to Tc^s *C. difficile* RADIO-PAGE type strains C and D (Tabaqchali *et al.*, 1984) was attempted but no transconjugants were obtained in any of several experiments. Similarly, there was no transfer of Tn916-borne Tc^r from *B. subtilis*

Fig. 2. Southern blot analysis of *C. difficile* genomic DNA digested with *Hind*III (lanes 1–5) or *Eco*RV (lanes 6 and 7) and probed with the 32 P-labelled 3.4 kbp insert of pPPM20. Lanes: 1, 630; 2, SB1 (630 $\times$ CD37); 3, SB2 (630 $\times$ CD37); 4, SB3 (630 $\times$ CD37); 5, CD37; 6, SB1; 7, 630.

CU2189::Tn916 to these strains, although *C. difficile* CD37 was a competent recipient in these experiments (P. Mullany and others, unpublished results).

In all matings Tc^r was transferred independently of the transferable erythromycin resistance determinant, as previously observed by Wust & Hardegger (1983).

Hybridization analysis of transconjugants

DNA from the parent and transconjugant strains of the mating *C. difficile* 630 $\times$ *C. difficile* CD37 was restricted with *Hind*III or *Eco*RV and probed with the 3.4 kbp insert of plasmid pPPM20. In the *Hind*III-digested DNA (Fig. 2), no hybridization occurred with recipient CD37 (lane 5); DNA from the donor strain 630 (lane 1) and from transconjugants SB1, SB2 and SB3 (lanes 2–4) yielded two bands, of 3.6 and 5.5 kbp. With *Eco*RV-digested DNA (Fig. 2, lanes 6 and 7), the donor strain 630 produced a single 9.5 kbp band (lane 7) while in transconjugant SB1 (lane 6) there were two bands, of 9.5 and 11.0 kbp. The latter bands were also detected in each of 20 further transconjugants tested (data not shown). The 11 kbp fragment was always observed in *C. difficile* transconjugants. The results of *Eco*RV digests probed with the 3.4 kbp fragment indicate that the transconjugants contain a second copy of the Tc^r element (there are no *Eco*RV sites in the probe). The fact that only two hybridizing fragments were observed when the 3.4 kbp probe was used against *Hind*III-digested genomic DNA of the transconjugants implies that the 5.5 and 3.6 kbp fragments are adjacent and internal to the element and that the Tc^r gene spans these fragments.

Fig. 3. Southern blot analysis of genomic DNA isolated from *C. difficile* and *B. subtilis* digested with *Eco*RV and probed with the 32 P-labelled 3.4 kbp insert of plasmid pPPM20. Lanes: 1, BS1 (630 $\times$ CU2189); 2, BS2 (SB2 $\times$ CU2189); 3, CU2189; 4, BS3 (SB3 $\times$ CU2189); 5, 630; 6, 630; 7, FM30 (BS2 $\times$ CD37); 8, CD37.

B. subtilis genomic DNA from strains BS1, BS2 and BS3 was digested with *Eco*RV and probed with the 3.4 kbp insert of pPPM20 (Fig. 3). For each transconjugant a hybridizing band of different size was produced (lanes 1, 2 and 4). When DNA isolated from the spontaneously Tc^r *B. subtilis* cells was probed with the 3.4 kbp insert no hybridization was observed (data not shown). Ives & Bott (1989) have shown that *B. subtilis* can possess a cryptic Tc^r gene that only expresses on amplification or after a promoter up mutation. This gene does not hybridize with *tetM*. It is likely that we are observing the expression of such a gene.

Transfer of Tc^r back into *C. difficile* CD37 from the *B. subtilis* transconjugants BS1 and BS2 restored the hybridization pattern, with two bands of 9.5 and 11.0 kbp (Fig. 3, lane 7), which had been observed in *Eco*RV-digested DNA from matings in which both donor and recipient were *C. difficile* (Fig. 2, lane 6).

A chromosomal location for the Tc^r element is implied by the fact that when the element is transferred to *B. subtilis*, fragments of variable size are observed. If the element were located on a plasmid, fragments of the same size would be expected. The data do not rule out the possibility that the element acts as a plasmid in *C. difficile* and integrates into the chromosome of *B. subtilis*. An alternative explanation of the data is that in *C. difficile* the element has a specific attachment site(s) in the chromosome, and in *B. subtilis* the element integrates at multiple sites. Transduction mapping using the transducing phage PBS1 will allow the position of the Tc^r determinant in the chromosome of *B. subtilis* to be determined unambiguously.

The possibility also remains that the *C. difficile* Tc^r element is on a plasmid that is often integrated into the chromosome. Certain strains of *Streptomyces ambofaciens* contain a 11 kbp conjugative plasmid, pSAM2,

that can be present in an integrated or a free state. The integration-excision system of this plasmid has been found to be related to that of the lambdoid phages and a requirement for two enzymes that display local homology with Xis and Int has been shown (Bocard *et al.*, 1989a). The conjugative transposon Tn1545 has also been shown to contain an integration-excision system related to the lambdoid phages; furthermore, Tn1545 is closely related to Tn916, and the region required for Tn916 excision correlates with the gene required for Tn1545 excision (Poyart-Salmeron *et al.*, 1989). As the *C. difficile* element is homologous with 95% of Tn916 it is also likely to contain a related integration-excision system. pSAM2 integration involves a site-specific recombination event within a 58 bp *att* sequence present on both plasmid and chromosome. The *att* site is found in three distantly related *Streptomyces* strains and a wide host range within the genus *Streptomyces* is implied (Bocard *et al.*, 1989b). The conjugative transposons Tn916 and Tn1545 do not appear to require long *att* sequences in most hosts. This probably reflects their ability to recognize degenerate *att* sites in most species. However, in *C. difficile* both the Tc^r element and Tn916 (P. Mullany and others, unpublished results) enter the genome at specific sites. The inability to transfer the *C. difficile* element and Tn916 into some strains of *C. difficile* may be due to the lack of a suitable attachment site which is required by and common to both elements.

Work by Volk *et al.* (1988) has shown that bi-directional transfer of Tn916 between *Clostridium tetani* and *Streptococcus faecalis* can occur *in vitro*. Hachler *et al.* (1987b) have shown that *C. difficile* is able to transfer erythromycin resistance to *Staphylococcus aureus* *in vitro* via a conjugation-like mechanism, possibly on a conjugative transposon. Our results extend the above observations to include bi-directional transfer of Tc^r between *C. difficile* and *B. subtilis*; this observation represents the first demonstration that *C. difficile* can act as a recipient in intergeneric crosses, thus paving the way for a more extensive genetic analysis of this species.

Tc^r is widespread in bacteria and the *tetM* gene has been found in many different genera (recently reviewed by Salyers *et al.*, 1990). The conjugative transposons are often responsible for mediating this resistance type. This communication demonstrates a further extension of the host range of these promiscuous elements.

Stability of the Tc^r element

After daily subculture for 14 d all of 100 colonies tested from the final subculture were resistant to tetracycline.

Fig. 4

Fig. 4. Southern blot analysis of plasmid DNA digested with *Hinc*II and probed with ³²P-labelled genomic DNA isolated from FM30 (lanes 1 and 2) and CD37 (lanes 3 and 4). Lanes: 1, pCDC1; 2, pAM120; 3, pCDC1; 4, pAM120.

Fig. 5

Fig. 5. Southern blot analysis of *C. difficile* genomic DNA digested with *Hind*III and probed with an oligonucleotide recognizing the right end of Tn916 (lanes 1 and 2) and an oligonucleotide recognizing the left end of Tn916 (lanes 3 and 4). Lanes: 1 and 4, SB1; 2 and 3, FM75.

Relationship of Tn916 to the *C. difficile* Tc^r element

DNA from *C. difficile* 630 was partially digested with *Hind*III and cloned into the unique *Hind*III site of the 5-34 kbp cosmid Homer III. The Tc^r clones had inserts of approximately 22.4 kbp, and all produced identical patterns on digestion with *Hind*III (data not shown). One cosmid, pCDC1 (Table 1), was selected for further study.

Gawron-Burke & Clewell (1984) have shown that pAM120 when digested with *Hinc*II generates five Tn916 internal and two junction fragments. Some properties of Tn916 have been mapped to these fragments (Senghas *et al.*, 1988). To determine whether Tn916 and the *C. difficile* element had any *Hinc*II sites in common, genomic DNA (from the transconjugant FM30 and the recipient strain CD37) was radiolabelled and used to probe *Hinc*II-restricted pAM120 and pCDC1 (Fig. 4). *Hinc*II treatment of pCDC1 generates fragments of 6.0, 4.4, 2.8, 2.2, 1.6, 1.4, 1.1, 0.7 and 0.6 kbp. Of these, all except the 6.0 and 2.1 kbp fragments show

some homology with FM30 (Fig. 4, lane 1); the fragments of 4.4 and 1.3 kbp show the strongest hybridization to the probe, possibly indicating double bands at these points. There is no homology to the vector sequences (results not shown).

Digestion of pAM120 with *HincII* generates fragments of 5.4, 4.8, 3.7, 2.8, 2.7, 1.8, 1.2 and 0.4 kbp (Senghas *et al.*, 1988). All the fragments show some homology to the probe (Fig. 4, lane 2) but the 2.7 kbp fragment shows only very poor homology. This fragment is likely to be from pAM120LT, a derivative of pAM120 lacking Tn916 due to spontaneous deletion (see Table 1), which is always present in preparations of pAM120 (Gawron-Burke & Clewell, 1984). The fifth Tn916 internal fragment, at 0.4 kbp, was too small to be detected in these experiments. The only hybridizing band that pAM120 and pCDC1 have in common is the 2.8 kbp fragment. When CD37 was used as the probe (Fig. 4, lanes 3 and 4) hybridization was observed to the 4.4 kbp *HincII* fragment of pCDC1 (lane 3) and to the 5.4 kbp fragment of pAM120 (lane 4).

At least 10.4 kbp of pCDC1 appears to be part of the Tc^r element as this hybridizes specifically to DNA from FM30; 4.1 kbp hybridizes to DNA from CD37. CD37 differs from FM30 only by the absence of the Tc^r element. Hybridization of genomic DNA of the Tc^s *C. difficile* recipient CD37 to a fragment of Tn916 has also been demonstrated by Hachler *et al.* (1987a). The reason for this homology cannot be explained at present. However, it is possible that CD37 once contained a functional Tn916-like element which could have undergone an aberrant transposition event, leaving a truncated element in the genome. This could act as a substrate for homologous recombination, explaining the site-specificity of the element as observed in *C. difficile*.

In order to characterize further the relationship between Tn916 and the *C. difficile* element, *HindIII* digests of genomic DNA from CD37 containing Tn916 (FM75) and CD37 containing the *C. difficile* element (SB1) were probed with oligonucleotides homologous to the left and right ends of Tn916. Fig. 5 shows FM75 DNA probed with an oligonucleotide which recognizes the right end of Tn916 (lane 2) and an oligonucleotide which recognizes the left end of Tn916 (lane 3). Hybridization is seen to two fragments in each lane, indicating that there are two copies of Tn916 in FM75. Lanes 1 and 4 in Fig. 5 contain DNA from SB1 digested with *HindIII* and probed with the right (lane 1) and left (lane 4) ends of Tn916. No hybridization was observed to this DNA. The fact that the *C. difficile* element shows no hybridization to oligonucleotides homologous to both ends of Tn916 shows further differences between the two elements. The nucleotide sequence of the extremities of Tn916 and the only other conjugative transposon (from a

Gram-positive host) for which data are available, Tn1545, is almost identical for at least 250 bp (Caillaud & Courvalin, 1987; Clewell *et al.*, 1988). Our results suggest that the *C. difficile* element is likely to be a conjugative transposon related to but distinct from Tn916 and Tn1545. Further work is required to establish exactly how close the relationship is.

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