Typing of *Clostridium difficile* strains by PCR-amplification of variable length 16S–23S rDNA spacer regions

VOLKER GÜRTLER*

Department of Microbiology, Heidelberg Repatriation Hospital, West Heidelberg, Australia

(*Received 6 July 1993; accepted 14 July 1993*)

To develop a rapid and accurate method of typing large numbers of clinical isolates of *Clostridium difficile*, four regions of the rRNA operon [A, 15–1407 and B, 907–1407 (16S–16S); C, 1392–507 and D, 907–507 (16S–23S)] were enzymically amplified from 24 strains. When region A was hybridized to *Hin*dIII-digested genomic DNA isolated from *C. difficile* strains, all of the variable length restriction fragments hybridized. When region B was hybridized to *Hin*dIII-digested genomic DNA isolated from *C. difficile* strains, a set of variable length restriction fragments (Group II) hybridized predominantly. When region C was separated by agarose gel electrophoresis, a series of products ranging in size from approximately 800–1300 bp was obtained. When regions C and D were digested with *Hin*dIII, a constant region of 430 bp was found in both products and in all strains. From the above experiments it was concluded that the variable length Group II restriction fragments and the variable length region C amplification products were due to variable length 16S–23S spacer regions between alleles of the one strain. When region C amplification products were separated by denaturing PAGE, 16 variable length rRNA alleles (*rrn*A–P) were demonstrated from 24 *C. difficile* strains ranging in size from 852–1210 bp. After analysis with maximum parsimony, the 24 strains were divided into 14 ribotypes. The product C ribotypes and band sizes were stable after 14 single colony passages on horse blood agar plates and stable *in vivo*, since ribotype G was isolated twice from one patient and ribotype E was isolated three times from another patient (all on separate occasions). The ribotyping method described here has clear advantages over existing *C. difficile* typing methods; it has universal applicability, it is objective and is moderately rapid.

Introduction

Clostridium difficile is closely associated with pseudomembranous colitis and antibiotic-associated diarrhoea in man (Bartlett *et al.*, 1978; George *et al.*, 1978). Nosocomial colonization and disease caused by *C. difficile* has been demonstrated (Johnson *et al.*, 1990; McFarland *et al.*, 1989). A method which is sufficiently stable and analyses large numbers of isolates quickly and accurately would be invaluable for clinical epidemiological studies of *C. difficile*.

The methods which have been used to type *C. difficile* include toxin detection (Wust *et al.*, 1982), isolation of plasmids (Clabots *et al.*, 1988*a*, *b*; Mulligan *et al.*, 1988; Wust *et al.*, 1982), a bacteriophage/bacteriocin typing system (Sell *et al.*, 1983), antibiotic susceptibility testing (Delmee & Avesani, 1988), SDS-PAGE (Tabaqchali *et al.*, 1984), immunoblotting (Heard *et al.*, 1986), restriction endonuclease analysis (Wren & Tabaqchali, 1987) and Southern hybridization of the ribosomal RNA operon (Bowman *et al.*, 1991). Molecular genetic methods are more discriminating than the above methods.

The rRNA operon is present in varying copy number in all bacteria with some regions highly conserved and others highly variable (Neefs *et al.*, 1990). Consequently, when genomic DNA digested with a restriction enzyme is hybridized to rRNA operons, several bands are detected (Garnier *et al.*, 1991). The Southern hybridization of rRNA operons (ribotyping) to detect restriction fragment length polymorphisms (RFLPs) between strains has been reported in many bacterial species including *Salmonella typhi* strains (Altwegg *et al.*, 1989), *E. coli* strains (LiPuma *et al.*, 1989), *Xanthomonas maltophilia* (Bingen *et al.*, 1991), *Legionella pneumophila* strains (Harrison *et al.*, 1992), and *Staphylococcus* spp. and subspp. (DeBuyser *et al.*, 1992). However, Southern hybridization is slow and labour intensive.

The rRNA operon has a very high genetic stability and the length of the 16S rRNA gene is constant in all eubacteria (Neefs *et al.*, 1990). The numerous bands

*Author for correspondence. Tel. +61 3 4962743; fax +61 3 4962178.

Table 1. *Source and properties of C. difficile strains*

Abbreviations: T, toxin production; RT, PCR-ribotype of *C. difficile* strains (Table 3). The plasmid types are designated: −, none detected; +, extrachromosomal bands detected (A, B and C are the different sized band patterns obtained); ND, not determined. The *C. bifermentans* strains have been studied previously (Gürtler *et al.*, 1991).

Strain	T	RT	Plasmid	Year of isolation	Source
C. difficile					
H17, H18	+	G	−	1990	Heidelberg Repatriation Hospital, Melbourne, Australia
H23	+	F	−	1990	
H24	+	J	ND	1990	
H25	+	D	ND	1990	
H26	−	K	ND	1991	
H27	+	G	ND	1990	
H28	+	L	ND	1990	
H29	+	H	ND	1991	
H30	+	M	ND	1991	
H31	+	D	ND	1991	
H32	+	N	ND	1991	
H33	+	E	ND	1991	
H14, H16, H20	+	E	+ (A)	1990	
H13	−	O	−	1990	
H19	−	P	+ (B)	1990	
H15	+	D	−	1990	
6390	+	H	ND	1993	
6048	+	G	ND	1993	
AM690	ND	Q	ND	1982	St Vincent's Hospital, Melbourne, Australia
ATCC 9689	+	R	−		American Type Culture Collection
630	+	F	+ (C)	1987	H. Hächler, Switzerland
C. bifermentans					
ATCC 638					American Type Culture Collection
AM312					Dr R. Wilkinson, University of Melbourne, Australia
AM360					

Table 2. *Regions of the rRNA operon amplified and their corresponding primers*

The products amplified cover the regions shown where S = 16S rRNA gene and L = 23S rRNA gene. The sense of the primers used is shown by R = reverse, F = forward and * = identical region with R1391 being the complement of R1391F. The positions of all the primers are in regions which are highly conserved in eubacteria (Neefs *et al.*, 1990; Guttell & Fox, 1988). The nucleotide numbering system is that of the *E. coli* operon (Brosius *et al.*, 1978). The positions of each product are schematically represented in Fig. 1.

PCR product	Gene	Region amplified	16S–23S spacer	Primer code	Primer position and direction	Sequence
A	16S	15(S)–1408(S)	−	R015	15–27(F)	GATCCTGGCTCAG
				R1391*	1408–1391(R)	GACGGGCGGTGTGTACAA
B	16S	907(S)–1408(S)	−	R907	907–926(F)	AAACTCAAATGAATTGACGG
				R1391*	1408–1391(R)	GACGGGCGGTGTGTACAA
C	16S and 23S	1392(S)–507(L)	+	R1391F*	1391–1408(F)	TTGTACACACCGCCCGTC
				LR488	488–507(R)	CCTTTCCCTCACGGTACTG
D	16S and 23S	907(S)–507(L)	+	R907	907–926(F)	AAACTCAAATGAATTGACGG
				LR488	488–507(R)	CCTTTCCCTCACGGTACTG

detected with ribotyping of *C. difficile* correspond to different rDNA alleles with variation in the regions flanking the 16S rRNA gene (Gürtler *et al.*, 1991). The PCR-analysis of the 16S rRNA gene has been used to demonstrate species-specific differences (Gürtler *et al.*, 1991) and strain differences (Vaneechoutte *et al.*, 1992) in various bacterial species. Allelic species-specific differences within the 16S rRNA gene have been demonstrated in clostridia (Gürtler *et al.*, 1991). The rRNA alleles of *E. coli* (Brosius *et al.*, 1981) and *B. subtilis* (Loughney *et al.*, 1982) have been shown to have variable length 16S–23S rRNA spacer regions. In this report evidence is provided to show that allelic differences seen in ribotyping of strains of *C. difficile* is explained by

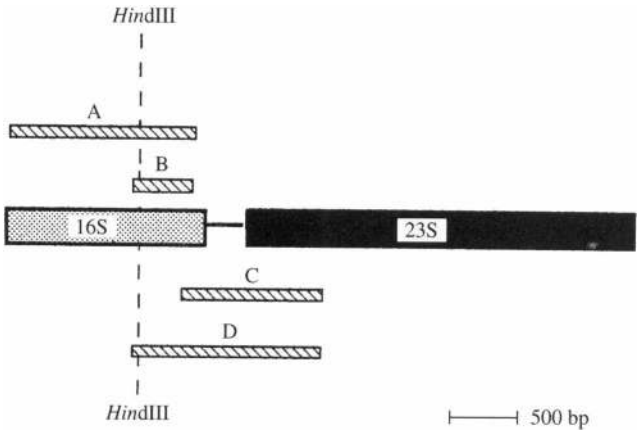

Fig. 1. Approaches used for the detection of rRNA alleles in *C. difficile* by Southern hybridization and PCR. The hatched bars (A, B, C and D) show positions of the respective PCR products (Table 2), the shaded bar denotes the 16S rRNA gene , the solid bar denotes the 23S rRNA gene, and the line joining the 16S and 23S genes depicts the spacer regions. The *Hin*dIII site is at position 975 of the 16S rRNA gene (Gürtler *et al.*, 1991).

variable length 16S–23S rRNA spacer regions. A method is described which uses PCR to amplify the 16S–23S spacer region and demonstrate differences between *C. difficile* strains. The potential of this method in clinical epidemiology of *C. difficile* is discussed.

Methods

Bacterial strains. The bacterial strains used are listed in Table 1.

Cultivation. The identity of all strains was determined by biochemical tests (Cato *et al.*, 1986) and confirmed by GLC (Sutter *et al.*, 1985). Purified stocks were stored in cooked-meat broth at room temperature or in glycerol broth at −20 °C. All strains were grown in brain heart infusion broth (BHI, Gibco). The stability of ribotype patterns was tested by passaging single colonies from horse blood agar plates every 2–3 d over a 5 week period. Toxin B production by *C. difficile* strains was detected by the method of Boondeekhun *et al.* (1993).

DNA isolation. Genomic and plasmid DNA was isolated from all clostridia by the protocol of Gürtler *et al.* (1991).

DNA amplification. For DNA amplifications, the protocol of Gürtler *et al.* (1991) was followed with some modifications. The reaction volume and the amount of DNA were halved and 1·25 units *Taq* polymerase (Boehringer) was used. The regions that were amplified and their corresponding primers are shown in Table 2.

Restriction enzyme analysis. Purified PCR products R907–LR507 and R1391–LR507 were digested singly or doubly with 10–15 units *Hin*dIII and *Cfo*I, as instructed by the manufacturer (Boehringer). Genomic DNA was digested with 30 units *Hin*dIII. The digested and undigested PCR products were resolved on 2% (w/v) low-gelling-temperature plus 2% (w/v) 'AR' agarose gels. The *Hin*dIII-digested genomic DNA was resolved on 1% (w/v) 'AR' agarose gels.

Southern hybridization. For hybridizations, the protocol of Gürtler *et al.* (1991) was followed with one exception. The PCR products A and B (Table 2) were amplified from *C. difficile* 630 and labelled with digoxigenin.

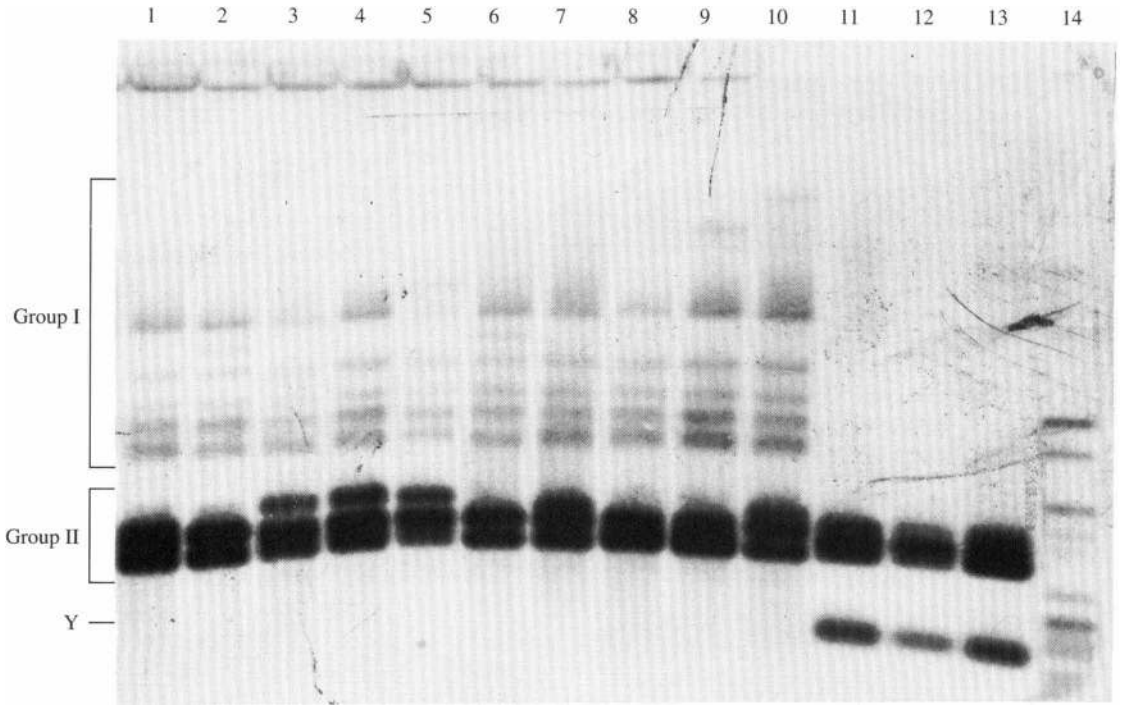

Fig. 2. Hybridization of PCR product B to Group II bands in genomic DNA isolated from *C. difficile* and *C. bifermentans* strains. Lanes: 1–10, *C. difficile* strains H13, H15, H16, H17, H18, H19, H20, H23, 9689 and 9689, respectively; 11–13, *C. bifermentans* strains AM312, AM360, and AM818, respectively; 14, pBR328 DNA digested with *Bgl*I and *Hin*fI, labelled with photodigoxigenin. Y indicates the position of an extra band visible in *C. bifermentans* products.

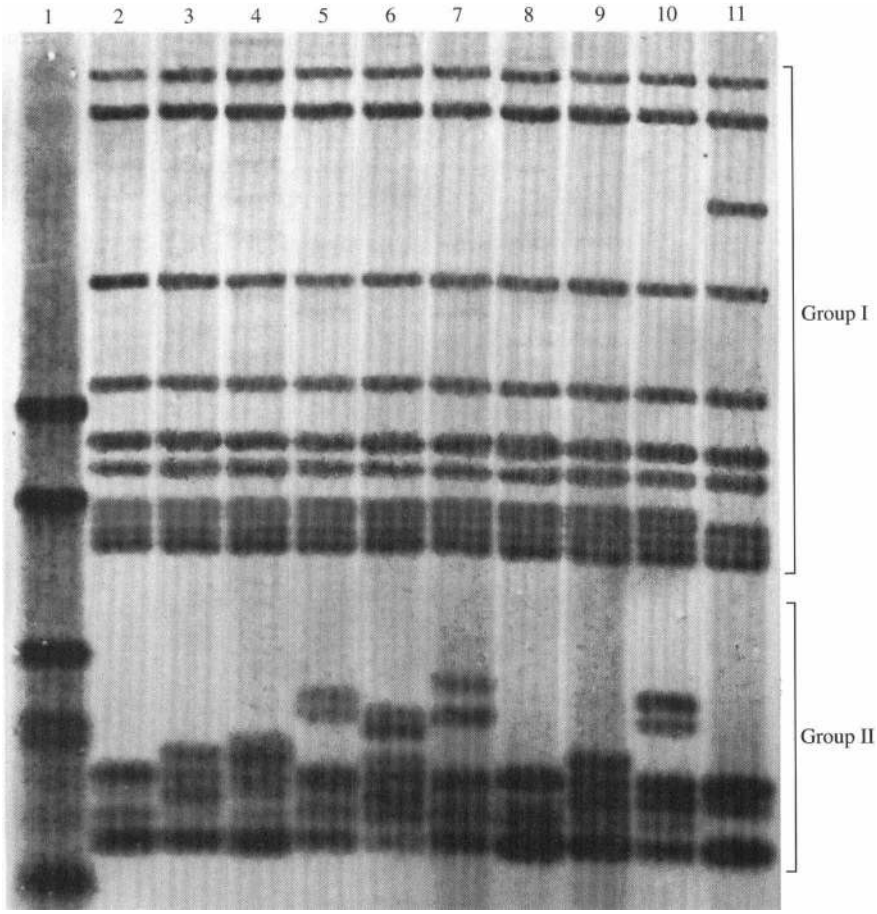

Fig. 3. Hybridization of PCR product A to Group I and II bands in genomic DNA isolated from *C. difficile* strains. Lanes: 1, pBR328 DNA digested with *Bgl*I and *Hin*fI, labelled with photodigoxigenin; 2–11, H24, H25, H26, H27, H28, H29, H30, H31, H32 and H33, respectively.

Denaturing PAGE. The above amplification protocol was followed with some modifications. The reaction volume was decreased by a factor of two and 2 μCi [α-^{32}P]dATP (DuPont or Amersham) was added. The reduction of the cold dNTP's by a factor of four increased the yield of labelled product. Radiolabelled DNA fragments were separated on a 0·4 mm thick, 38 cm wide and 50 cm high (Bio-Rad), 3·5% (w/v) polyacrylamide gel containing 7 M-urea (Sambrook *et al.*, 1989). Gels were dried in a vacuum slab gel drier (Bio-Rad) for 2 h at 80 °C. Autoradiography was for 18–96 h.

Data analysis. The presence or absence of PCR product C bands was analysed by the following methods. (1) The presence or absence of bands (corresponding to region R1391–LR507) on autoradiograms was analysed by using the program BioImage (Millipore). The average sizes of the 16 alleles (*rrn*A–P) were calculated from five separate gels ranging from 1–51 determinations for the respective alleles. Using these sizes as internal standards, molecular masses were assigned to respective bands from strains 630, 9689, H14, H15 or H23. Twenty-four strains from four gels were then compared at once. Presence or absence of bands was scored by a 1 or 0, respectively. (2) The resulting data matrix prepared from four gels was analysed by maximum parsimony (Swofford, 1985).

Results

The DNA typing approaches used are shown in Fig. 1 and Table 2. Products A and B were hybridized to *Hin*dIII-digested genomic DNA isolated from *C. difficile* and *C. bifermentans* strains. Differences in *Hin*dIII sites on both flanking sides of the 16S rRNA gene were sought within and between strains. Products C and D were amplified from *C. difficile* strains in an attempt to find differences in the length of the 16S–23S spacer region within and between strains.

The bands detected by Southern hybridization (ribotyping) have been divided into Groups I and II, showing numerous Group II differences between strains and fewer Group I differences. Ribotyping of 21 isolates of *C. difficile* from the Heidelberg Repatriation Hospital and one from St Vincent's Hospital, Melbourne, Australia, produced 14 restriction fragment length polymorphism (RFLP) types, 10 of which are shown in Fig. 3. There were 10 group I bands (Fig. 3) demonstrating 10 rRNA alleles in *C. difficile*.

Products A and B are comprised only of parts of the 16S rRNA gene (Table 2). It has been shown previously that the 16S rRNA gene is of constant length between alleles and strains of *C. difficile* (Gürtler *et al.*, 1991). When PCR product B was hybridized to *C. difficile* genomic DNA, Group II bands hybridized predominantly (Fig. 2); the Group I bands hybridized faintly because product B included 62 bp 5′ of the *Hin*dIII site

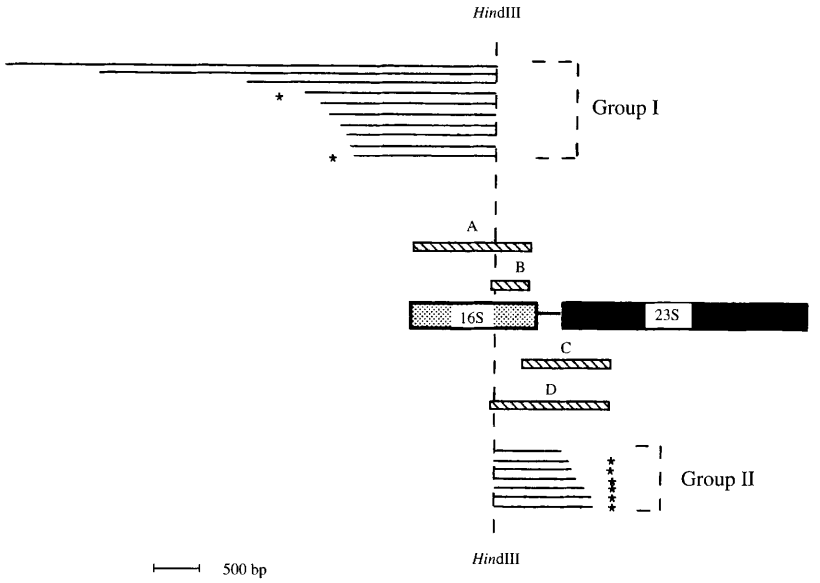

Fig. 4. Detection of rRNA alleles in *C. difficile* strains by Southern hybridization. The symbols, box shadings and the position of the *Hin*dIII site are described in the legend to Fig. 1. Bands depicted as Group I (Figs 3 and 4) correspond to fragments 5′ of the *Hin*dIII site and Group II bands (Figs 3 and 4) correspond to fragments 3′ of the *Hin*dIII site. ★ refers to bands which are not present in all strains.

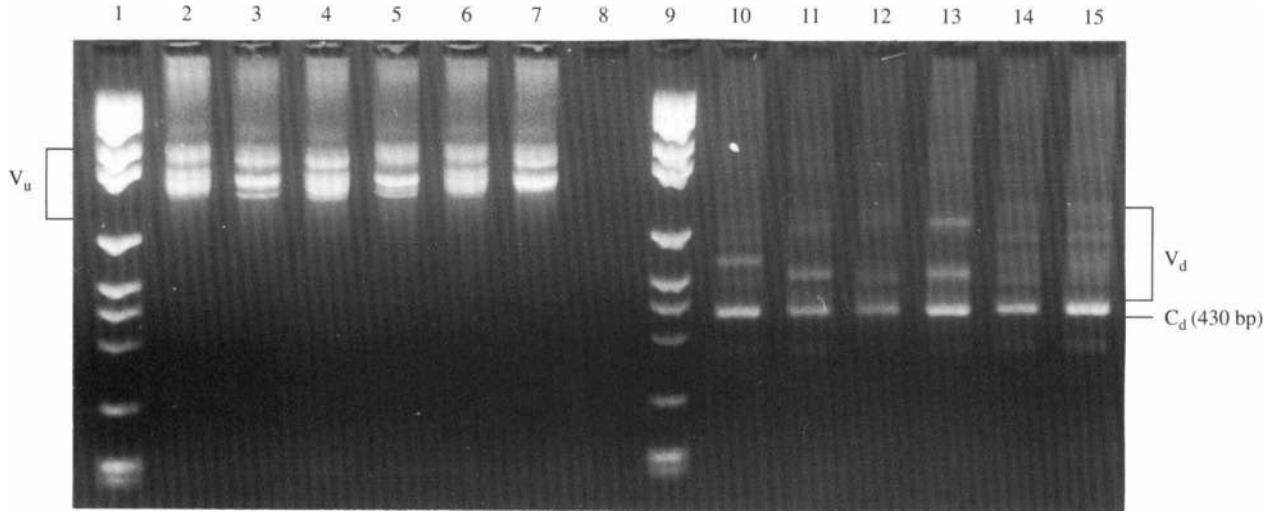

Fig. 5. Constant and variable length regions within PCR product C amplified from *C. difficile* strains. Agarose gel electrophoresis of undigested (lanes 2–7) and *Hin*dIII-digested (lanes 10–15) PCR product C. Lanes: 1, pBR328 DNA digested with *Hin*fI and *Bgl*I; 2–7, H15, H24, H28, H30, H31 and H33, respectively; 8, no DNA control; 9, pBR328 DNA digested with *Hin*fI and *Bgl*I; 10–15, H13, H14, H17, H19, H23 and 630, respectively. The size of the standards are (in bp) 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234 and 220. C_d, constant *Hin*dIII-digested; V_u, variable undigested; and V_d, variable *Hin*dIII-digested.

(1/10 of product B). When product B was hybridized to *C. bifermentans* genomic DNA digested with *Hin*dIII (Fig. 2) no Group I bands hybridized and an extra band appeared (Y) due to an extra *Hin*dIII site at position 675 of the 16S rRNA gene (Gürtler *et al.*, 1991). When PCR product A was used as a probe (Fig. 3), the Group I and II bands hybridized with equal intensity. The orientation of the Group I and II bands is as shown in Fig. 4 because (1) product B hybridized predominantly to Group II bands and (2) the *Hin*dIII site lies 62 bp downstream from the 5′ end of product B (Gürtler *et al.*, 1991).

From Figs 2, 3 and 4 it can be seen that the Group II bands are comprised of the spacer region and part of the 23S rRNA gene. These Group II bands were of variable length which could be explained by (1) a variable *Hin*dIII site or (2) an insertion within the spacer or the beginning of the 23S rRNA gene. To determine which possibility was correct, PCR products C and D were amplified, both of which include the spacer regions (Table 2). When the product C primer combinations were used (Table 2), several bands (V_u) of varying molecular masses were obtained from each *C. difficile* strain (Fig. 5). The presence of bands varied from strain to strain. When V_u bands were digested with *Hin*dIII, a band appeared at

Table 3. *Variable 16S–23S rRNA spacer regions in C. difficile strains*

PCR product C was amplified from various *C. difficile* strains and separated by denaturing PAGE (Fig. 6). The presence of variable length alleles (*rrn*A–P) is shown. The size of each allele is shown in Fig. 6. The outer limits of the constant regions is depicted by restriction enzyme-cut sites (see Figs 4 and 5). The strain numbers corresponding to the ribotype are listed in Table 1. The no. of isolates in each ribotype is listed below each letter. The constant length regions were collated from results obtained in Figs 2, 3 and 5 and Gürtler *et al.* (1991). The variable length regions were collated (using BioImage software) from Fig. 6 and three other denaturing polyacrylamide gels.

Allele	Restriction enzyme site (position, bp)	*C. difficile* ribotype Q 1	L 1	D 3	E 4	J 1	N 1	G 4	F 2	O 1	P 1	K 1	H 2	M 1	R 1	Band frequency*
							Constant region (16S rRNA)									
All	*Hin*dIII (1010)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
All	*Cfo*I (1100†)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
							Variable region (16S–23S spacer region)									
*rrn*A		−	−	−	−	−	−	−	−	−	−	−	+	−	−	8·3
*rrn*B		−	−	+	+	+	+	+	+	+	−	+	+	+	+	87·5
*rrn*C		−	−	−	−	−	−	+	−	−	−	−	−	−	−	16·7
*rrn*D		+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
*rrn*E		−	−	−	−	−	−	−	−	−	+	−	−	−	−	4·2
*rrn*F		+	+	−	+	+	−	−	−	−	−	−	−	+	−	33·3
*rrn*G		−	−	−	−	−	−	−	+	+	−	+	−	−	−	16·7
*rrn*H		−	−	−	−	−	−	−	−	−	−	+	−	−	−	4·2
*rrn*I		+	−	+	−	−	−	−	−	−	−	−	−	−	−	16·7
*rrn*J		+	+	+	−	−	−	−	−	+	−	+	−	−	−	29·2
*rrn*K		−	−	−	−	−	−	−	+	−	−	−	−	−	−	8·3
*rrn*L		+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
*rrn*M		+	−	−	−	−	−	+	+	+	+	+	+	−	−	50
*rrn*N		−	+	+	+	+	+	−	−	−	+	+	−	−	−	50
*rrn*O		+	+	+	−	−	−	+	+	+	+	−	+	+	+	70·8
*rrn*P		−	+	+	−	+	+	+	−	+	+	+	+	+	+	70·8
							Constant region (23S rRNA)									
All	*Hin*dIII (80)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

* Calculated by dividing the no. of isolates with allele *rrn* by the total no. of isolates.
† Enzyme site reported previously (Gürtler *et al.*, 1991).

430 bp (C_d) which was of higher intensity than the digested variable length bands, V_d. Band C_d appeared in all the strains listed in Table 1 (not shown). When the product D primer combination was used, the same *Hin*dIII band (C_d) was present (not shown) demonstrating that this band contains the 23S rRNA gene from position 80–507 (Table 3). The demonstration of band C_d shows that this region is of constant length between alleles. Taken with the Southern hybridization data (Figs 2 and 3) these results show that the variable length regions lie between the 16S and 23S rRNA genes. The exact base pair location of the spacer regions may only be determined when the separate alleles have been sequenced.

The resolution of the variable length bands was low (Fig. 5) and so it was decided to increase the resolution with long denaturing polyacrylamide gels. When this was done, the same amplification products (V_u; Fig. 5) separated into between 5 and 9 bands per strain, with the presence of bands variable between strains (Fig. 6). Each band was assigned as an allele resulting in a total of 16 alleles (A–P) of variable length (Fig. 6). The constant length regions within the 16S and 23S genes were partially characterized (Table 3). The variability in length was due to variable length 16S–23S spacer regions between alleles.

When all of the *C. difficile* strains listed in Table 1 were analysed, 24 strains were divided into 14 ribotypes (Table 3). A dendrogram is shown in Fig. 7 showing 3 clusters (*a*, *b*, *c*) found in all trees analysed. Within ribotype G, 2 isolates were cultured from one patient at different times; within ribotype E, 3 isolates were cultured from one patient at different times. All other isolates which had identical patterns (ribotypes D, E, F, G and H) were from different patients.

The stability of band V_o sizes and patterns was

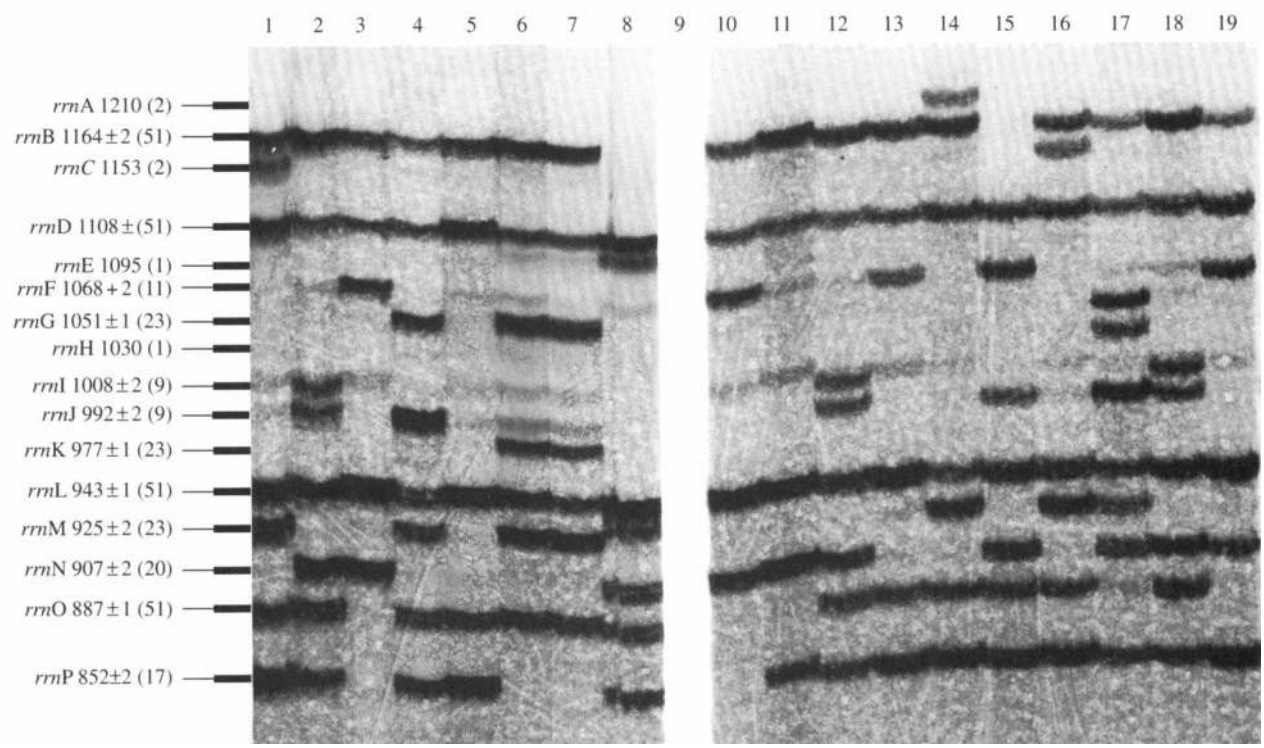

Fig. 6. Denaturing PAGE of PCR product C amplified from strains of *C. difficile*. Lanes: 1–8, H17, H15, H14, H13, 9689, 630, H23 and H19, respectively; 9, no DNA PCR control; 10–19, H33, H32, H31, H30, H29, H28, H27, H26, H25 and H24, respectively. The sizes of the respective alleles are shown on the left [mean ± SEM (number of determinations)]. The molecular mass markers used (not shown) were λ DNA digested with *Hin*dIII and *Eco*RI (947 and 831 bp bands only) and SPPI DNA digested with *Eco*RI (1150 and 1000 bp bands only).

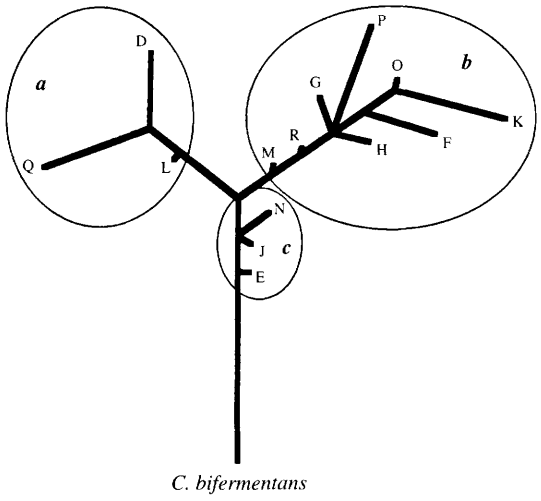

Fig. 7. Dendrogram showing the relationships of *C. difficile* ribotypes. Using maximum parsimony, 50 equally parsimonious trees were found, one of which is shown. The same ribotypes were found in each of the circled branches (*a*, *b*, *c*) for all 50 trees. The root of the tree (*C. bifermentans*) had no bands in common with any of the ribotypes.

investigated in detail by passaging five strains over a 5 week period (Table 4). The alleles were scored as positive or negative by appearing visually identical and by having similar calculated molecular masses (Fig. 6 and Table 4). The results show that both the band sizes and patterns were highly reproducible in five *C. difficile* strains. The band sizes and patterns of strains H23 and 630 were reproducibly identical.

Discussion

The main finding of the present study was that the presence or absence of specific variable length rDNA spacer regions varied between *C. difficile* strains. The patterns obtained were stable within strains upon repeated testing (with passaging *in vitro* and *in vivo*), allowed the designation of strains to specific types, discriminated within and between species, and allowed for the easy testing of large numbers of strains. With these criteria met, the novel molecular typing method described here may be applied to epidemiological studies of *C. difficile*.

Since 500 bp of the 5′ end of the 23S gene was amplified, it was possible that the observed heterogeneity of PCR products was due to an insertion within the first 500 bp of the 23S gene. In support of this possibility are the following studies: (1) at least one extra cleavage site has been reported in the large rRNA subunit of

Table 4. *Stability of product C bands from C. difficile strains*

Product C was amplified from various *C. difficile* strains and separated by denaturing PAGE. Accumulated values (mean ± SEM) taken from five separate electrophoresis runs are shown. The data include runs (PCR, DNA preparations and electrophoresis) done over a 9 month period, as well as a stability testing experiment with the no. of single colony passages per strain shown.

	Strain				
Allele	630	9689	H14 (type E)	H15 (type D)	H23 (type F)
*rrn*A					
*rrn*B	1166±3	1163±4	1164±4	1163±5	1161±4
*rrn*D	1108±2	1110±4	1106±3	1108±4	1106±3
*rrn*E					
*rrn*F			1068±2		
*rrn*G	1050±2				1052±2
*rrn*H					
*rrn*I				1008±2	
*rrn*J				992±2	
*rrn*K	978±2				975±1
*rrn*L	945±3	937±2	948±3	943±3	938±2
*rrn*M	928±3				922±2
*rrn*N			908±3	906±3	
*rrn*O	890±3	884±1		887±3	885±1
*rrn*P		851±1		853±3	
Statistics					
n	14	8	11	9	9
DNA preparations	5	5	4	4	5
Passages	14	14	10	10	14
PCR runs	10	5	4	4	6

Leptospira interrogans (Hsu *et al.*, 1990) and *Salmonella* spp. (Hsu *et al.*, 1992), producing several fragments smaller than 23S; and (2) a 90 bp intervening sequence has been shown to be excised during large subunit rRNA maturation (Burgin *et al.*, 1990). The results presented in this report show that in *C. difficile*, 430 bp 3′ from position 507 of the 23S rRNA gene was of constant length and the 16S–23S spacer DNA was of variable length between alleles.

The 16S–23S spacer regions of *B. subtilis* (Vold, 1985) and *E. coli* (Fournier & Ozeki, 1985) contain tRNA genes which vary in length from 75–90 bp. Of the 7 *rrn* operons in *E. coli* all contain from 1–3 tRNA genes (Brosius *et al.*, 1981), while in *B. subtilis*, of the 10 *rrn* operons, two out of the three analysed sets have been shown to contain tRNA genes (Loughney *et al.*, 1982). It is possible that the 16S–23S spacer regions in *C. difficile* characterized herein may contain tRNA genes.

Of the numerous typing methods which have been used in the epidemiology of *C. difficile* all have some disadvantages. The separation of [^{35}S]methionine-labelled proteins by PAGE (Tabaqchali *et al.*, 1984) has divided 245 strains of *C. difficile* into 15 distinct types (Tabaqchali, 1990). The current method has the potential to separate strains into more types with 24 strains divided into 14 types. The bacteriophage/bacteriocin typing scheme of Sell *et al.* (1983) has been used to type 114 *C. difficile* isolates producing 31 typing patterns (Mahony *et al.*, 1991), however, only 16–40% were typeable. The detection of plasmids has been used to type *C. difficile* isolates; however, the percentages of isolates which have plasmids varies from 18% (Muldrow *et al.*, 1982) to 59% (Mahony *et al.*, 1991). The present study found that three strains were not plasmid-typeable since no plasmids were detected (Table 1). The detection of cytotoxin (Wust *et al.*, 1982) is of limited value since only positive or negative production is measured; the present study also demonstrates this drawback. The large number of bands obtained with *Hin*dIII restriction endonuclease analysis of total DNA (Wren & Tabaqchali, 1987) makes this method difficult to interpret. Finally, Southern hybridization of rRNA genes (Bowman *et al.*, 1991; and this study) lacks resolution and is time consuming.

The present study has demonstrated a typing approach for *C. difficile* based on variable length 16S–23S rRNA spacer regions. The variability of the spacer regions between strains makes this method potentially useful in epidemiological studies. It does not have any of the disadvantages of the above methods making it the most promising method available.

I would like to thank Dr B. C. Mayall and Ms V. A. Wilson for helpful discussions. The technical assistance of Mrs H. D. Barrie is appreciated. I thank Drs R. Wilkinson and H. Hächler for supplying me with bacterial strains. I would like to thank the Department of Veterans' Affairs for permission to publish this paper.

References

ALTWEGG, M., HICKMANN-BRENNER, F. W. & FARMER, J. J., III (1989). Ribosomal RNA gene restriction patterns provide increased sensitivity for typing *Salmonella typhi* strains. *Journal of Infectious Diseases* **160**, 145–149.

BARTLETT, J. G., CHANG, T. W., GURWITH, M., GORBACH, S. L. & ONDERDONK, A. B. (1978). Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. *New England Journal of Medicine* **298**, 531–534.

BINGEN, E. H., DENAMUR, E., LAMBERT-ZECHVOSKY, N. Y., BOURDOIS, A., MARIANI-KURKDJIAN, P., CEZARD, J.-P., NAVARRO, J. & ELION, J. (1991). DNA restriction fragment length polymorphism differentiates crossed from independent infections in nosocomial *Xanthomonas maltophilia* bacteremia. *Journal of Clinical Microbiology* **29**, 1348–1350.

BOONDEEKHUN, H. S., GÜRTLER, V., ODD, M. L., WILSON, V. A. & MAYALL, B. C. (1993). Detection of the *Clostridium difficile* enterotoxin gene in clinical specimens by the polymerase chain reaction. *Journal of Medical Microbiology* **38**, 384–387.

BOWMAN, R. A., O'NEILL, G. L. & RILEY, T. V. (1991). Non-radioactive restriction fragment length polymorphism (RFLP) typing of *Clostridium difficile*. *FEMS Microbiology Letters* **63**, 269–272.

BROSIUS, J., PALMER, M. L., KENNEDY, P. J. & NOLLER, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from

Escherichia coli. *Proceedings of the National Academy of Sciences of the United States of America* **72**, 4801–4805.

BROSIUS, J., DULL, T. J., SLEETER, D. D. & NOLLER, H. F. (1981). Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *Journal of Molecular Biology* **148**, 107–127.

BURGIN, A. B., PARODOS, K., LANE, D. J. & PACE, N. R. (1990). The excision of intervening sequences from *Salmonella* 23S ribosomal RNA. *Cell* **60**, 405–414.

CATO, E. P., GEORGE, W. L. & FINEGOLD, S. M. (1986). Genus *Clostridium* Prazmowski 1880, 23^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1141–1200. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.

CLABOTS, C., LEE, S., GERDING, D., MULLIGAN, M., KWOK, R., SCHABERG, D., FEKETY, R. & PETERSON, L. (1988*a*). *Clostridium difficile* plasmid isolation as an epidemiologic tool. *European Journal of Clinical Microbiology and Infectious Diseases* **7**, 312–315.

CLABOTS, C. R., PETERSON, L. R. & GERDING, D. N. (1988*b*). Characterization of a nosocomial *Clostridium difficile* outbreak by using plasmid profile typing and clindamycin susceptibility testing. *Journal of Infectious Diseases* **158**, 731–736.

DE BUYSER, M.-L., MORVAN, A., AUBERT, S., DILASSER, F. & EL SOHL, N. (1992). Evaluation of a ribosomal RNA gene probe for the identification of species and subspecies within the genus *Staphylococcus*. *Journal of General Microbiology* **138**, 889–899.

DELMEE, M. & AVESANI, V. (1988). Correlation between serogroup and susceptibility to chloramphenicol, clindamycin, erythromycin, rifampicin and tetracycline among 308 isolates of *Clostridium difficile*. *Journal of Antimicrobial Chemotherapy* **22**, 325–331.

FOURNIER, M. J. & OZEKI, H. (1985). Structure and organization of the transfer ribonucleic acid genes of *Eschericia coli* K-12. *Microbiological Reviews* **49**, 379–397.

GARNIER, T., CANARD, B. & COLE, S. T. (1991). Cloning, mapping, and molecular characterization of the rRNA operons of *Clostridium perfringens*. *Journal of Bacteriology* **173**, 5431–5438.

GEORGE, R. H. S., SYMONDS, J. M., DIMOCK, F., BROWN, J. D., ARABI, Y., SHINAGAWA, N., KEIGHLEY, M. R. B., ALEXANDER-WILLIAMS, J. & BURDON, D. W. (1978). Identification of *Clostridium difficile* as a cause of pseudomembranous colitis. *British Medical Journal* **1**, 695.

GÜRTLER, V., WILSON, V. A. & MAYALL, B. C. (1991). Classification of medically important clostridia using restriction endonuclease site differences of PCR-amplified 16S rDNA. *Journal of General Microbiology* **137**, 2673–2679.

GUTTELL, R. R. & FOX, G. E. (1988). A compilation of large sub-unit RNA sequences presented in a structural format. *Nucleic Acids Research* **16**, r175–r269.

HARRISON, T. G., SAUNDERS, N. A., HATHTHOTUWA, A., DOSHI, N. & TAYLOR, N. G. (1992). Further evidence that genotypically closely related strains of *Legionella pneumophila* can express different serogroup specific antigens. *Journal of Medical Microbiology* **37**, 155–161.

HEARD, S. R., RASBURN, B., MATTHEWS, R. C. & TABAQCHALI, S. (1986). Immunoblotting to demonstrate antigenic and immunogenic differences among nine standard strains of *Clostridium difficile*. *Journal of Clinical Microbiology* **24**, 384–387.

HSU, D., PAN, M.-J., ZEE, Y. C. & LE FEBVRE, R. B. (1990). Unique ribosome structure of *Leptospira interrogans* is composed of four rRNA components. *Journal of Bacteriology* **172**, 3478–3480.

HSU, D., ZEE, Y. C., INGRAHAM, J. & SHIH, L.-M. (1992). Diversity of cleavage patterns of *Salmonella* 23S rRNA. *Journal of General Microbiology* **138**, 199–203.

JOHNSON, S., CLABOTS, C. R., LINN, F. V., OLSON, M. M., PETERSON, L. R. & GERDING, D. N. (1990). Nosocomial *Clostridium difficile* colonisation and disease. *Lancet* **336**, 97–100.

LIPUMA, J. J., STULL, T. L., DASEN, S. E., PIDCOCK, K. A., KAYE, D. & KORZENIOWSKI, O. M. (1989). DNA polymorphisms among *Escherichia coli* isolated from bacteriuric women. *Journal of Infectious Diseases* **159**, 526–532.

LOUGHNEY, K., LUND, E. & DAHLBERG, J. E. (1982). tRNA genes are found between the 16S and 23S rRNA genes in *Bacillus subtilis*. *Nucleic Acids Research* **10**, 1607–1624.

MAHONY, D. E., CLOW, J., ATKINSON, L., VAKHARIA, N. & SCHLECH, W. F. (1991). Development and application of a multiple typing system for *Clostridium difficile*. *Applied and Environmental Microbiology* **57**, 1873–1879.

MCFARLAND, L. V., MULLIGAN, M. E., KWOK, R. Y. & STAMM, W. E. (1989). Nosocomial acquisition of *Clostridium difficile* infection. *New England Journal of Medicine* **320**, 204–210.

MULDROW, L. L., ARCHIBOLD, E. R., NUNEZ, M. O. & SHEEHY, R. J. (1982). Survey of the extrachromosomal gene pool of *Clostridium difficile*. *Journal of Clinical Microbiology* **16**, 637–640.

MULLIGAN, M. E., PETERSON, L. R., KWOK, R. Y., CLABOTS, C. R. & GERDING, D. N. (1988). Immunoblots and plasmid fingerprints compared with serotyping and polyacrylamide gel electrophoresis for typing *Clostridium difficile*. *Journal of Clinical Microbiology* **26**, 41–46.

NEEFS, J.-M., VAN DE PEER, Y., HENDRIKS, L. & DE WACHTER, R. (1990). Compilation of small ribosomal sub-unit RNA sequences. *Nucleic Acids Research* **18**, r2237–r2317.

SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

SELL, T. L., SCHABERG, D. R. & FEKETY, F. R. (1983). Bacteriophage and bacteriocin typing scheme for *Clostridium difficile*. *Journal of Clinical Microbiology* **17**, 1148–1152.

SUTTER, V. L., CITRON, D. M., EDELSTEIN, M. A. C. & FINEGOLD, S. M. (1985). *Wadsworth Anaerobic Bacteriology Manual*, 4th edn. Belmont, California: Star Publishing Company.

SWOFFORD, D. L. (1985). Phylogenetic analysis using parsimony (PAUP) version 2.4. University of Illinois.

TABAQCHALI, S. (1990). Epidemiologic markers of *Clostridium difficile*. *Reviews of Infectious Diseases* **12** (suppl. 2), 192–199.

TABAQCHALI, S., HOLLAND, D., O'FARRELL, S. & SILMAN, R. (1984). Typing scheme for *Clostridium difficile*: its application in clinical and epidemiological studies. *Lancet* **1**, 935–938.

VANEECHOUTTE, M., ROSSAU, R., DE VOS, P., GILLIS, M., JANSSENS, D., PAEPE, N., DE ROUCK, A., FIERS, T., CLAYES, G. & KERSTERS, K. (1992). Rapid identification of bacteria of the *Comamonadaceae* with amplified ribosomal DNA-restriction analysis (ARDRA). *FEMS Microbiology Letters* **93**, 227–234.

VOLD, B. S. (1985). Structure and organization of genes for transfer ribonucleic acid in *Bacillus subtilis*. *Microbiological Reviews* **49**, 71–80.

WREN, B. W. & TABAQCHALI, S. (1987). Restriction endonuclease DNA analysis of *Clostridium difficile*. *Journal of Clinical Microbiology* **25**, 2402–2404.

WUST, J., SULLIVAN, N. M., HARDEGGER, U. & WILKINS, T. D. (1982). Investigation of an outbreak of antibiotic-associated colitis by various typing methods. *Journal of Clinical Microbiology* **16**, 1096–1101.