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NOTES

Molecular Analysis of the Pathogenicity Locus and Polymorphism in the Putative Negative Regulator of Toxin Production (TcdC) among *Clostridium difficile* Clinical Isolates

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The pathogenicity locus (PaLoc) of *Clostridium difficile* contains toxin A and B genes and three accessory genes, including *tcdD* and *tcdC*, which are supposed to code for the positive and negative regulators of toxin expression, respectively. Different studies have described variations in *C. difficile* toxin A and B genes, but little is known about *C. difficile* variants for the accessory genes. The PaLoc of several *C. difficile* clinical isolates was investigated by three different PCR methods with the aim to identify variant strains. Of the toxinogenic *C. difficile* strains examined, 25% showed variations. No correlation between *C. difficile* variant strains and key patient groups was found. Interestingly, all of these strains showed a variant *tcdC* gene. Three different *tcdC* alleles were identified, and one of these had a nonsense mutation which reduced the TcdC protein from 232 to 61 amino acids. It is possible that different TcdC variants affect toxin production differently, a hypothesis with important implications for the pathogenic potential of variant *C. difficile* strains.

C. difficile is the etiologic agent of pseudomembranous colitis and the most common cause of nosocomial antibiotic-associated diarrhea (3, 14, 15). Toxins A and B, *C. difficile* virulence factors, belong to the large clostridial cytotoxins, and both disrupt the actin cytoskeleton (1, 12, 13, 31). The toxin A and B genes (*tcdA* and *tcdB*) are part of the pathogenicity locus (PaLoc), a 19.6-kb genetic locus that also includes three additional *tcd* open reading frames (ORFs), *tcdD*, *tcdE*, and *tcdC*, and the ORFs for the insertion sequences, cdu-2, cdu-2′, cdd-2 cdd-3, and cdd-4 (5, 9). Sequencing and transcription analysis suggest that TcdD and TcdC are involved in the positive and negative regulation of TcdA and TcdB expression, respectively (10, 11).

Different studies have described variations in *C. difficile* toxin A and B genes (4, 8, 16, 20, 21, 22, 23). Despite the fact that variant strains can still be associated with clinical diseases, few epidemiological data on their circulation are reported (2, 19, 22) and little is known about PaLoc accessory gene variants (7, 25).

We analyzed several *C. difficile* clinical isolates to investigate their PaLocs, identify variant strains, and determine possible correlations with a particular patient population. Three different PCR-based methods were used to detect the PaLoc accessory genes (7, 29), the variations in the toxin A and B genes (21), and the presence of the binary toxin genes (17, 27). The majority of *C. difficile* strains with dramatic variations in toxin A and B genes harbor the binary toxin genes, so their detection is suggested as a method for a quick identification of these strains (19).

Bacterial strains, DNA extraction, and PCR primers. A total of 51 *C. difficile* strains, isolated in different Italian hospitals from 1986 to 1999, were examined. Six strains were representative of six different outbreaks, whereas 24 strains were isolated from sporadic cases and 21 were isolated from asymptomatic patients. *C. difficile* VPI 10463 and *C. difficile* ATCC 43597 were used as toxinogenic and nontoxinogenic control strains, respectively. *C. difficile* strains 51377 and 57267 were used as controls for toxinotypes VI and VII, respectively (a description of the toxinotyping method can be viewed online at http://www.uni-lj.si/~bfbcdiff). The in vitro production of toxins B and A was assayed by cytotoxicity testing (6) and an enzyme immunoassay method (Immunocard-Toxin A; Meridian Diagnostics, Cincinnati, Ohio), respectively.

Five microliters of crude extracts of DNA was used for multiplex PCRs and binary toxin gene detection. One microliter of purified DNA, extracted using a Nucleobond AXG100 kit (Macherey-Nagel, Düren, Germany), was the template for toxinotyping and amplification of the entire *tcdC* gene.

PaLoc PCR primers and their locations are shown in Fig. 1. Oligonucleotides were synthesized by M-Medical, Florence, Italy.

PaLoc genes detection and sequencing of the *tcdC* gene. Toxin A and B genes, *tcdA* and *tcdB*, were amplified by a multiplex PCR assay. The reaction mixture contained 1× buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$), 200 pmol of each deoxynucleoside triphosphate, 100 pmol of TA1 and TA2 primers, 25 pmol of TB1 and TB2 primers, and 2.5 U of Takara rTaq (Takara Shuzo Co., Ltd., Shiga, Japan). The template was denatured for 5 min at 94°C, and DNA was amplified for 30

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A

Gene	Primer	Sequence	Product (bp)
cdu-2	Tim5	5' CCA CAG ATG CTT TTA GCAGGA A 3'	162
	Struppi5	5' TCC AT CAC TGC TCC AGC TAT 3'	
*tcd*D	Tim3	5' AAA AGC GAT GCT ATT ATA GTC AAA 3'	300
	Struppi3	5' CCT TAT TAA CAG CTT GTC TAG AT 3'	
*tcd*B	TB1	5' GAG CTG CTT CAA TTG GAG AGA 3'	412
	TB2	5' GTA ACC TAC TTT CAT AAC ACC AG 3'	
	B1C	5' AGA AAA TTT TAT GAG TTT AGT TAA TAG AAA 3'	3100
	B2N	5' CAG ATA ATG TAG GAA GTA AGT CTATAG 3'	
*tcd*E	Tim1	5' GTT TAA GTG CAA TAA AAA GTC GTA 3'	262
	Struppi 1	5' GGT AAT CCA CAT AAG CAC ATA TT 3'	
*tcd*A	TA1	5' ATG ATA AGG CAA CTT CAG TGG 3'	624
	TA2	5' TAA GTT CCT CCT GCT CCA TCA A 3'	
	A3C	5' TAT TGA TAG CAC CTG ATT TAT ATA CAA G 3'	3100
	A4N	5' TTA TCA AAC ATA TAT TTT AGC CAT ATA TC 3'	
*tcd*C	Tim2	5' GCA CCT CAT CAC CAT CTT CAA 3'	345
	Struppi2	5' TGA AGA CCA TGA GGA GGT CAT 3'	
	C1	5' TTA ATT AAT TTT CTC TAC AGC TAT CC 3'	718
	C2	5' TCT AAT AAA AGG GAG ATT GTA TTA TG 3'	
cdd-3	Tim6	5' TCC AAT ATA ATA AAT TAG CAT TCC A 3'	622
	Struppi6	5' GGC TAT TAC ACG TAA TCC AGA TA 3'	

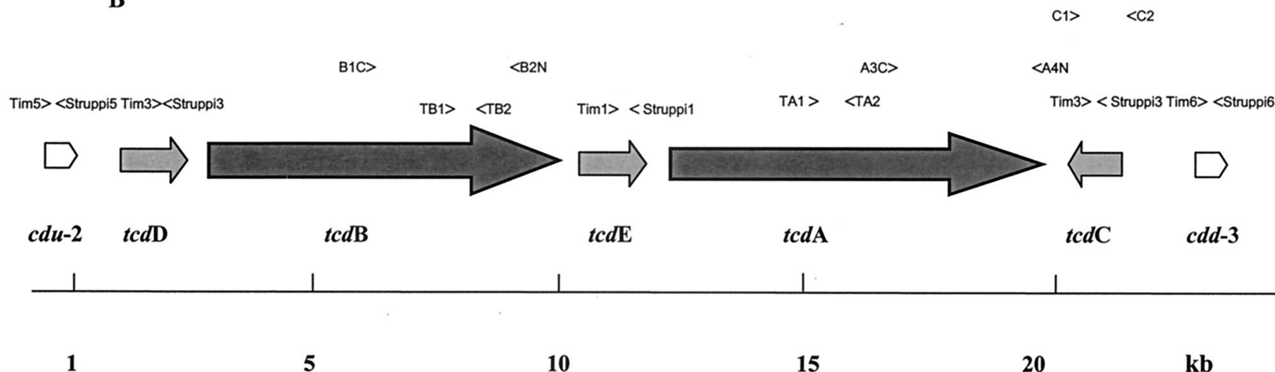

FIG. 1. Primers used in the PaLoc analysis. (A) Specificity and nucleotide sequences of primers and molecular sizes of the PCR products obtained for each pair of primers. (B) Location of PCR primers on a schematic representation of the PaLoc region. The small arrowheads indicate the orientation of primers.

cycles consisting of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C.

Multiplex PCR for the PaLoc accessory genes detection was performed as described by Cohen et al. (7). The same cycling conditions were employed to amplify the entire *tcdC* gene by using primers C1 and C2 (Fig. 1). The reaction mixture was prepared as described for toxin A and B detection. Sequencing was performed with a Perkin-Elmer ABI373A DNA sequencer. The deduced amino acid sequence was obtained by the ORF Finder program, whereas the nucleotide and amino

TABLE 1. Molecular characteristics of *C. difficile* strains with variant PaLoc genes analyzed in this study

C. difficile strain	Source	Year of isolation	PaLoc gene							Toxinotype[b]	Nucleotide *tcdC* type sequence	Binary toxin gene	
			cdu2	*tcdD*	*tcdB*	*tcdE*	*tcdA*	*tcdC*	*cdd3*			*cdtA*	*cdtB*
Pd 7	Sporadic case-child	1998	+	+	+[a]	+	+[a]	+[a]	+	V	A	+	+
Pd 13	Sporadic case-adult	1998	+	+	+[a]	+	+[a]	+[a]	+	V	A	+	+
Pd 16	Sporadic case-adult	1997	+	+	+[a]	+	+[a]	+[a]	+	V	A	+	+
Pd 55	Sporadic case-adult	1998	+	+	+[a]	+	+[a]	+[a]	+	V	A	+	+
Pd 5	Outbreak-adult	1999	+	+	+[a]	+	+[a]	+[a]	+	VI	A	+	+
Pd 53	Sporadic case-child	1999	+	+	+[a]	+	+[a]	+[a]	+	VI	A	+	+
Pd 3	Sporadic case-child	1999	+	+	+	+	+	+[a]	+	0	B	−	−
M7	Carrier-adult	1983	+	+	+[a]	+	+[a]	+[a]	+	0	C	+	+

[a] Variant gene (variations in PaLoc genes were detected by multiplex PCR assay or PCR-RFLP method).
[b] Toxinotypes were determined by PCR-RFLP analysis of fragment A3 from toxin A and fragment B1 from toxin B. 0, *C. difficile* strain belonging to a new toxinotype by PCR-RFLP on the entire PaLoc.

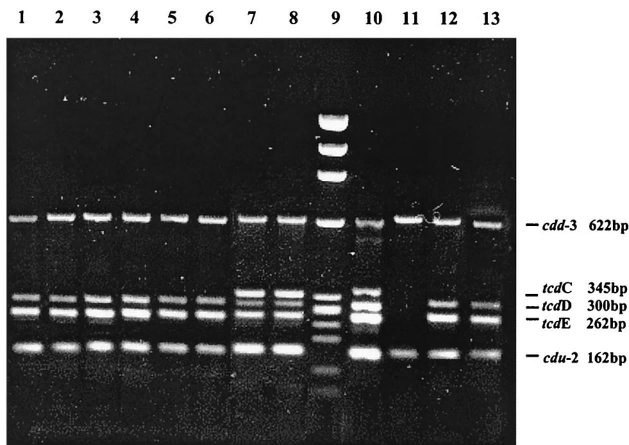

FIG. 2. Detection of PaLoc accessory genes *cdd-3*, *tcdC*, *tcdD*, *tcdE*, and *cdu-2* by multiplex PCR in eight *C. difficile* strains with variant PaLoc genes identified in this study. Lane 1, Pd 5; lane 2, Pd7; lane 3, Pd13; lane 4, Pd16; lane 5, Pd53; lane 6, Pd 55; lane 7, M7; lane 8, Pd3; lane 9, DNA molecular weight marker IX; lane 10, *C. difficile* VPI10463; lane 11, ATCC 43597; lane 12, *C. difficile* 51377; lane 13, *C. difficile* 57267.

acid sequences were compared with database entries by using the BLAST program.

Toxinotyping. Toxinotyping is a PCR-restriction fragment length polymorphism (RFLP) method consisting of the amplification of two toxin fragments, B1 from *tcdB* and A3 from *tcdA*, and of their digestion by specific restriction enzymes to obtain patterns characterizing the different variants of the toxin genes (20, 21). We followed the method reported in the toxinotyping home page (http://www.uni-lj.si/~bfbcdiff), with some minor modifications. The PCR mixture contained 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 400 pmol of each deoxynucleoside triphosphate, 100 pmol of primers, and 2.5 U of Takara ExTaq (Takara Shuzo Co., Ltd.). After a denaturation of 5 min at 94°C, the DNA was amplified by 30 cycles of 1 min at 94°C, 1 min at 47°C, and 5 min at 72°C. At the end, samples were held at 72°C for 10 min. PCR fragments were purified with a QIAquick PCR purification kit (Qiagen) and digested.

Binary toxin gene detection. Internal regions of binary toxin genes were detected as previously described (27). Two specific primers, BIN5 (5′ AAT ATT GGG AGG GAG AAT AAA TG 3′) and BIN6 5′ (TGT ATT TTC ATT GTT TCT CCT CC 3′), were designed to amplify the entire *ctdA* gene, which codes for the enzymatic toxin component, and two other primers, BIN7 (5′ ATT GTT GAT GCA ACA TTG ATA CC 3′) and BIN8 (5′ AAT ATA TAT TGT ATT GAG GGG AC 3′), were designed to amplify the entire *cdtB* gene, which codes for the binding toxin component. The reaction mixture and the cycling conditions were the same as described for toxin A and B detection.

Different studies have demonstrated a great heterogeneity in *C. difficile* toxin A and B genes (4, 8, 16, 20, 21, 22, 23). This characteristic has been successfully used for *C. difficile* strain typing, in addition to the other methods already known (24, 26, 28). On the other hand, there are few data on *C. difficile* strains with variant PaLoc accessory genes (7, 25) and on their circulation among patients. We examined the PaLoc of several *C. difficile* strains isolated from clinical samples to acquire further information.

A total of 51 *C. difficile* strains were examined, and 32 tested as toxinogenic. The analysis of the PaLoc accessory genes demonstrated that 8 (25%) of the 32 toxigenic strains showed a different pattern than that of the control strain, VPI 10463 (Table 1; Fig. 2). Six strains, Pd5, Pd7, Pd13, Pd16, Pd53, and Pd55, showed a pattern with only four bands, 622, 300, 262, and 162 bp in size, apparently corresponding to the genes *cdd3*, *tcdD*, *tcdE*, and *cdu2*, respectively. Two single PCRs, for *tcdC*

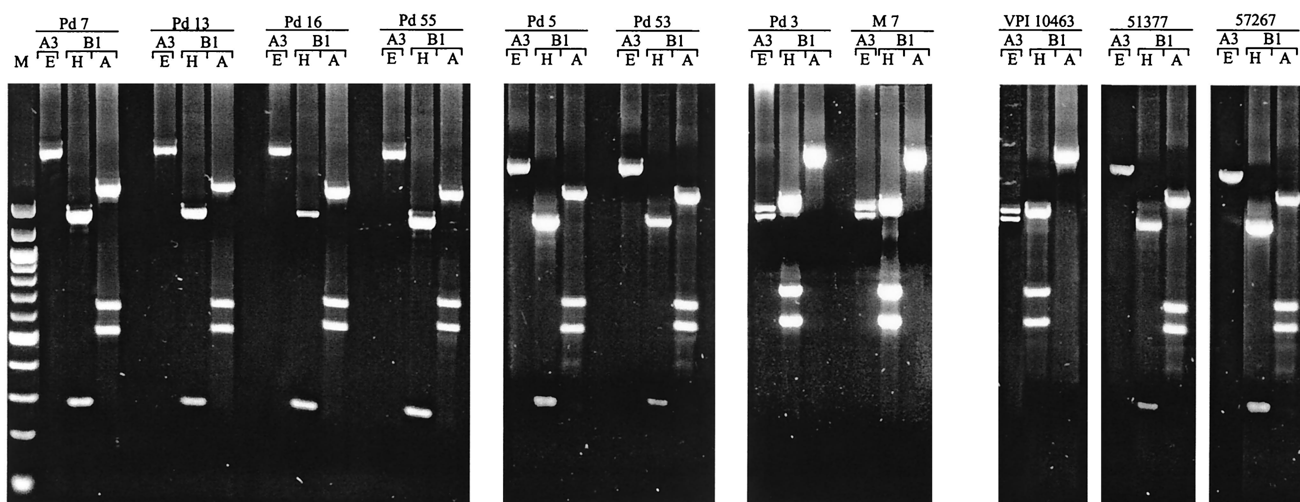

FIG. 3. Toxinotyping of eight *C. difficile* strains with variant PaLoc genes and of the reference strains *C. difficile* VPI 10463, *C. difficile* 51377, and *C. difficile* 57267. (For descriptions of the other toxinotypes, see Rupnik et al. [21, 22]). The PCR-RFLP patterns of A3 and B1 fragments are shown for each strain. A3 fragments were digested with *Eco*RI (E), and B1 fragments were digested with *Hin*cII (H) and *Acc*I (A). M, 100-bp DNA ladder (BioLabs). Pd7, Pd13, Pd16, and Pd55 (*C. difficile* strains), toxinotype V; Pd5 and Pd53, toxinotype VI; Pd3 and M7, toxinotype 0 (*C. difficile* M7 represents a new toxinotype, as demonstrated by PCR-RFLP analysis of the entire PaLoc). *C. difficile* VPI 10463, *C. difficile* 51377, and *C. difficile* 57267 are the reference strains for toxinotypes 0, VI, and VII, respectively.

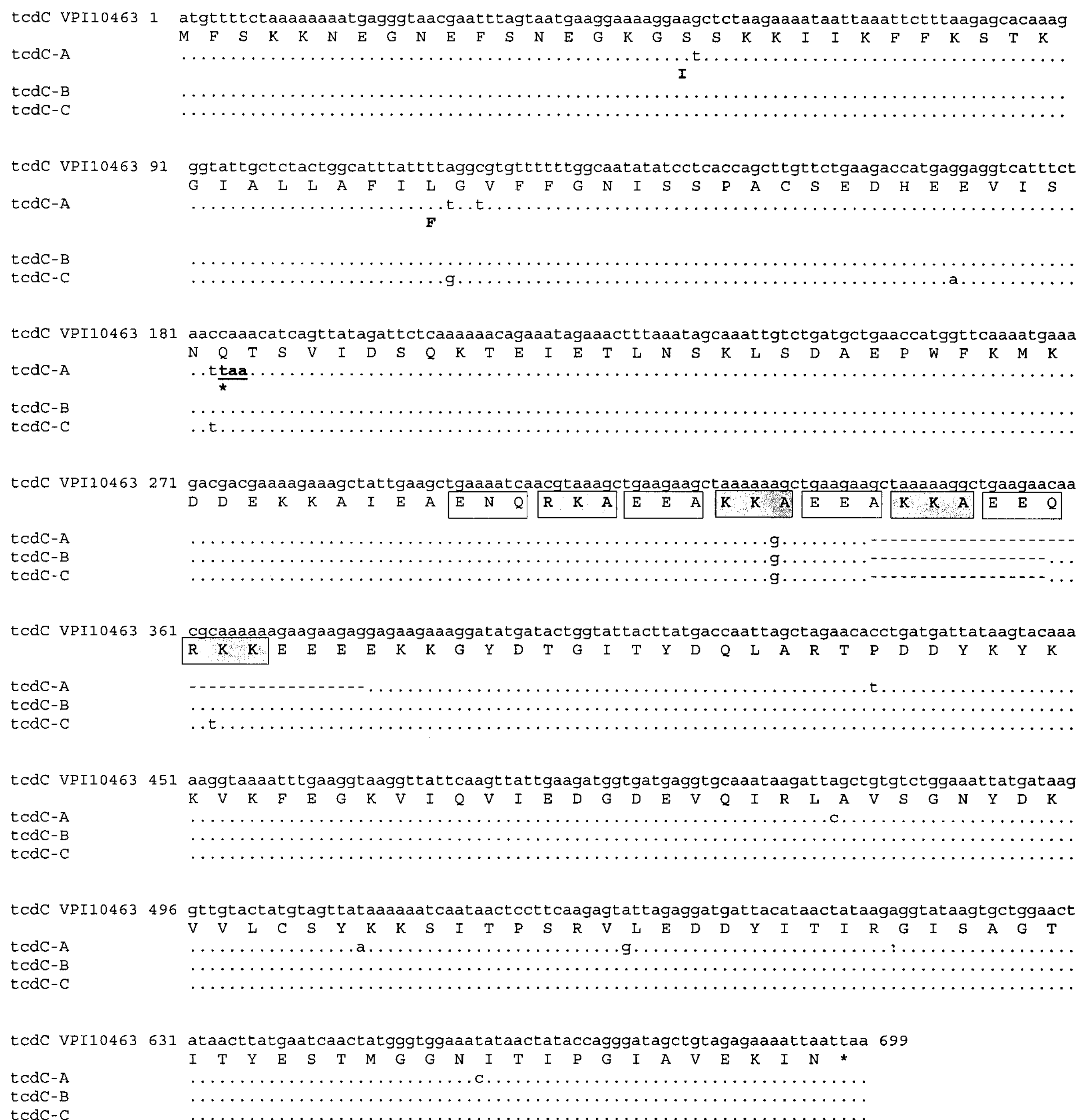

FIG. 4. Comparison of the TcdC nucleotide and amino acid sequences of the *C. difficile* reference strain VPI 10463 with those of the variant TcdC proteins identified in *C. difficile* clinical isolates examined in this study. Dots and dashes indicate identical bases and gaps, respectively, for the different *tcdC* alleles (*tcdC-A*, *-B*, and *-C*). The termination codon in *tcdC-A* is underlined. Only the amino acid changes are indicated for each TcdC variant. The eight 3-amino-acid repeats of the VPI 10463 TcdC are indicated by open (acidic in nature) and grey (basic in nature) boxes.

and *tcdD* internal fragments, were performed separately on these strains. Unexpectedly, we obtained a product of approximately 300 bp in both PCR assays from all the strains analyzed (data not shown). A deletion in the *tcdC* fragment explained its comigration with the *tcdD* fragment. The same deletion was also observed in the *tcdC* gene of control *C. difficile* strains 51377 and 57267. Two strains, M7 and Pd3, showed a *tcdC* fragment smaller than expected (Fig. 2).

Six *C. difficile* strains were also recognized as variant strains for the toxin A and B genes (Table 1) (Fig. 3). Four strains could be classified as toxinotype V, and two strains could be classified as toxinotype VI. It has already been suggested that toxinotypes VI and V are closely related (21). In this study, all the strains belonging to these two toxinotypes were isolated from cases which occurred in different departments of the same hospital from 1997 to 1999, suggesting persistent circulation of these variant strains. Few data are reported on the role of variant *C. difficile* strains in causing severe disease (2, 23); therefore, it is interesting that these *C. difficile* variant strains were responsible both for sporadic cases of antibiotic-associated diarrhea and for an outbreak. The infections caused by this particular group of *C. difficile* variant strains were not

age related, and there was no correlation with a particular patient population.

Both genes encoding the binary toxin were detected in seven of the eight variant strains (data not shown), including *C. difficile* M7, which was recognized as toxinotype 0 (Table 1). On the contrary, Pd3, the other strain belonging to toxinotype 0 and showing a variant *tcdC* gene, did not have the binary toxin genes. Specific PCRs for *cdtA* and *cdtB* confirmed the absence of the binary toxin genes in all the other toxigenic *C. difficile* strains. The entire PaLocs of *C. difficile* M7 and Pd3 were analyzed by PCR-RFLPs (21) to verify the absence of variations in other regions of the toxin A and B genes and in the rest of the genetic unit. *C. difficile* Pd3 did not show further variations, whereas three fragments of the *C. difficile* M7 PaLoc (A1, encoding the catalytic domain of toxin A; B2, encoding the translocation domain of toxin B; and PL2, located upstream the *tcdB* gene) showed different patterns than the reference strain *C. difficile* VPI 10463 after digestion with specific enzymes (21) (data not shown). These results demonstrate that *C. difficile* M7 represents a new toxinotype and indicate, in contrast to the data already known (27), that it is also possible to detect the binary toxin genes in strains with minor modifications in *tcdA* and *tcdB* genes.

In this study, we demonstrated genetic variability of the *tcdC* gene, which codes for the supposed negative regulator of toxin A and B gene transcription. Three different *tcdC* nucleotide sequences were identified and denominated types A, B, and C (Fig. 4). The deletions characterizing the *tcdC* variant genes are located in a DNA region featuring repeated sequences that code for eight 3-amino acid repeats of an acidic or basic nature (11). These DNA regions show higher mutation frequencies, due to recombination events between repeats (18). *tcdC* type A shows a deletion of 39 bp, whereas types B and C show a deletion of 18 bp. A transition from cytosine to thymine in *tcdC-A* determines a nonsense mutation, so the *tcdC* protein has only 61 amino acids compared to the 232 expected. Type B and C *tcdC* genes, in spite of the different nucleotide sequences, code for an identical amino acid sequence. This protein of 226 amino acids is characterized by the deletion of 6 amino acids, determining the loss of the basic repeat KKA and the partial loss of the acidic repeats EEA and EEQ (Fig. 4).

It is noteworthy that all the strains belonging to toxinotypes V, VI, and VII that were examined in this study showed a TcdC of only 61 amino acids. A truncated protein, with a sequence of 22 amino acids, has been previously observed in *C. difficile* strain 8864 (25). It has been hypothesized that this variant TcdC probably lacks its function and that it contributes to the extreme cytotoxicity of strain 8864 (4, 16, 30). All the *C. difficile* strains with major variations in toxins A and B examined in this study showed high levels of cytotoxicity (data not shown). This result seems to confirm the possibility that the dramatic modifications observed in TcdC could also lead to an altered function of the protein in these strains, contributing to the high level of toxin expression. A second TcdC variant was identified both in *C. difficile* Pd3 and in *C. difficile* M7. The level of cytotoxicity in vitro of M7 was significantly lower than that observed for the other variant TcdC strains (data not shown). Further studies should be performed to determine the influence of the variant TcdC on M7 toxin gene transcription and to investigate the functionality of the mutated toxins of this strain. It is possible that different TcdC variants have a different functionality and diversely affect toxin production, a hypothesis with important implications for the pathogenic potential of *C. difficile* strains. Therefore, it could be very interesting to extend these studies to all the other *C. difficile* toxinotypes and to investigate the influence of variant TcdC proteins on the virulence of this pathogenic microorganism.

Nucleotide sequence accession numbers. The nucleotide sequences of the *tcdC* genes of *C. difficile* strains Pd5, Pd3, and M7 were assigned EMBL numbers AJ428941, AJ428942, and AJ428943, respectively.

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REFERENCES

1. **Aktories, K., J. Selzer, F. Hofmann, and I. Just.** 1997. Molecular mechanisms of action of *Clostridium difficile* toxins A and B, p. 393–407. *In* J. I. Rood, B. A. McClane, J. G. Songer, and R. W. Titball (ed.), The clostridia: molecular biology and pathogenesis. Academic Press, London, United Kingdom.
2. **Alfa, M. J., A. Kabani, D. Lyerly, S. Moncrief, L. M. Neville, A. Al-Barrak, G. K. H. Harding, B. Dyck, K. Olekson, and J. M. Embil.** 2000. Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile* responsible for a nosocomial outbreak of *Clostridium difficile*-associated diarrhea. J. Clin. Microbiol. **38:**2706–2714.
3. **Bartlett, J. G.** 1992. Antibiotic-associated diarrhea. Clin. Infect. Dis. **15:** 573–581.
4. **Borriello, S. P., B. W. Wren, S. Hyde, S. V. Seddon, P. Sibbons, M. M. Krishna, S. Tabaqchali, S. Manek, and B. Price.** 1992. Molecular, immunological, and biological characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile.* Infect. Immun. **60:**4192–4199.
5. **Braun, V., T. Hundsberger, P. Leukel, M. Sauerborn, and C. von Eichel-Streiber.** 1996. Definition of the single integration site of the pathogenicity locus in *Clostridium difficile*. Gene **181:**29–38.
6. **Chang, T. W., M. Lauermann, and J. G. Bartlett.** 1979. Cytotoxicity assay in antibiotic-associated colitis. J. Infect. Dis. **140:**765–770.
7. **Cohen, S. H., Y. J. Tang, and J. Silva, Jr.** 2000. Analysis of the pathogenicity locus in *Clostridium difficile* strains. J. Infect. Dis. **181:**659–663.
8. **Deprite, C., M. Delmée, V. Avesani, R. L'Haridon, A. Roels, M. Popoff, and G. Corthier.** 1993. Serogroup F strains of *Clostridium difficile* produce toxin B but not toxin A. J. Med. Microbiol. **38:**434–441.
9. **Hammond, G. A., and J. L. Johnson.** 1995. The toxigenic element of *Clostridium difficile* strain VPI 10463. Microb. Pathog. **19:**203–213.
10. **Hammond, G. A., D. M. Lyerly, and J. L. Johnson.** 1997. Transcriptional analysis of the toxigenic element of *Clostridium difficile*. Microb. Pathog. **22:**143–154.
11. **Hundsberger, T., V. Braun, M. Weidmann, P. Leukel, M. Sauerborn, and C. von Eichel-Streiber.** 1997. Transcription analysis of the genes *tcd*A-E of the pathogenicity locus of *Clostridium difficile*. Eur. J. Biochem. **244:**735–742.
12. **Just, I., J. Selzer, M. Wilm, C. von Eichel-Streiber, M. Mann, and K. Aktories.** 1995. Glucosylation of Rho proteins by *Clostridium difficile* toxin B. Nature (London) **375:**500–503.
13. **Just, I., M. Wilm, J. Selzer, G. Rex, C. von Eichel-Streiber, M. Mann, and K. Aktories.** 1995. The enterotoxin from *Clostridium difficile* (ToxA) monoglucosylates the Rho proteins. J. Biol. Chem. **270:**13932–13936.
14. **Knoop, F. C., M. Owens, and I. C. Crocker.** 1993. *Clostridium difficile*: clinical disease and diagnosis. Clin. Microbiol. Rev. **6:**251–265.
15. **Lyerly, D. M., H. C. Krivan, T. D. Wilkins.** 1988. *Clostridium difficile*: its diseases and toxins. Clin. Microbiol. Rev. **1:**1–18.
16. **Lyerly, D. M., L. A. Barroso, T. D. Wilkins, C. Deprite, and G. Corthier.** 1992. Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile.* Infect. Immun. **60:**4633–4639.
17. **Perelle, S., M. Gibert, P. Bourlioux, G. Corthier, and M. R. Popoff.** 1997. Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196. Infect. Immun. **65:**1402–1407.
18. **Robertson, B. D., and T. F. Meyer.** 1992. Genetic variation in pathogenic bacteria. Trends Genet. **8:**422–427.
19. **Rupnik, M.** 2001. How to detect *Clostridium difficile* variant strains in a routine laboratory. Clin. Microb. Infect. Dis. **7:**417–420.
20. **Rupnik, M., V. Braun, F. Soehn, M. Janc, M. Hofstetter, R. Laufenberg-Feldmann, and C. von Eichel-Streiber.** 1997. Characterization of polymor-

phisms in the toxin A and B genes of *Clostridium difficile.* FEMS Microbiol. Lett. **148:**197–202.

21. **Rupnik, M., V. Avesani, M. Janc, C. von Eichel-Streiber, and M. Delmée.** 1998. A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. J. Clin. Microbiol. **36:**2240–2247.
22. **Rupnik, M., J. S. Brazier, B. I. Duerden, M. Grabnar, and S. L. J. Stubbs.** 2001. Comparison of toxinotyping and PCR ribotyping of *Clostridium difficile* strains and description of novel toxinotypes. Microbiology **147:**439–447.
23. **Sambol, S. P., M. M. Merrigan, D. Lyerly, D. N. Gerding, and S. Johnson.** 2000. Toxin gene analysis of a variant strain of *Clostridium difficile* that causes human clinical disease. Infect. Immun. **68:**5480–5487.
24. **Samore, M. H., M. Kristjansson, L. Venkataraman, P. C. Degirolami, and R. D. Arbeit.** 1996. Comparison of arbitrarily-primed polymerase chain reaction, restriction enzyme analysis and pulsed-field gel electrophoresis for typing *Clostridium difficile*. J. Microbiol. Methods **25:**215–224.
25. **Soehn, S., A. Wagenknecht-Wiesner, P. Leukel, M. Kohl, M. Weidmann, C. von Eichel-Streiber, and V. Braun.** 1998. Genetic rearrangements in the pathogenicity locus of *Clostridium difficile* strain 8864—implications for transcription, expression and enzymatic activity of toxin A and B. Mol. Gen. Genet. **258:**222–232.
26. **Spigaglia, P., R. Cardines, S. Rossi, M. G. Menozzi, and P. Mastrantonio.** 2001. Molecular typing and long-term comparison of *Clostridium difficile* strains by pulsed-field gel electrophoresis and PCR-ribotyping. J. Med. Microbiol. **50:**407–414.
27. **Stubbs, S., M. Rupnik, M. Gibert, J. Brazier, B. Duerden, and M. Popoff.** 2000. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. FEMS Microbiol. Lett. **186:**307–312.
28. **Stubbs, S. L. J., J. S. Brazier, G. L. O'Neill, and B. I. Duerden.** 1999. PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. J. Clin. Microbiol. **37:**461–463.
29. **Titov, L., N. Lebedkova, A. Shabanov, Y. J. Tang, S. H. Cohen, and J. Silva, Jr.** 2000. Isolation and molecular characterization of *Clostridium difficile* strains from patients and the hospital environment in Belarus. J. Clin. Microbiol. **38:**1200–1202.
30. **Torres, J. F.** 1991. Purification and characterisation of toxin B from a strain of *Clostridium difficile* that does not produce toxin A. J. Med. Microbiol. **35:**40–44.
31. **von Eichel-Streiber, C., C. Boquet, M. Sauerborn, and M. Thelestam.** 1996. Large clostridial cytotoxins—a family of glycosyltransferases modifying small GTP-binding proteins. Trends Microbiol. **4:**375–382.