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Genetic Characterization of Toxin A-Negative, Toxin B-Positive *Clostridium difficile* Isolates by PCR

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Toxin-specific enzyme immunoassays, cytotoxicity assays, and PCR were used to analyze 48 toxin A-negative, toxin B-positive *Clostridium difficile* isolates from various geographical sites around the world. All the isolates were negative by the TOX-A TEST and positive by the TOX A/B TEST. A deletion of approximately 1.7 kb was found at the 3′ end of the *toxA* gene for all the isolates, similar to the deletion in toxinotype VIII strains (e.g., *C. difficile* serotype F 1470). Additional PCR analysis indicated that the toxin B encoded by these isolates contains sequence variations downstream of the active site compared to the sequence of reference strain VPI 10463. This variation may extend the glucosylation spectrum to Ras proteins, as observed previously for closely related lethal toxin from *Clostridium sordellii* and toxin B from toxin A-negative, toxin B-positive strain F 1470. Toxin A-negative, toxin B-positive isolates have recently been associated with disease in humans, and they may be more common than was previously supposed.

Clostridium difficile is a major cause of nosocomial antibiotic-associated diarrhea (AAD) and colitis (22, 24). Symptoms of the disease range from mild diarrhea to fulminant pseudomembranous colitis (PMC). *C. difficile* is responsible for about 25% of cases of AAD diagnosed in the United States, although nearly all cases of the severe form of the disease are caused by *C. difficile*. The organism produces two toxins, termed A and B, that are responsible for the intestinal damage that occurs during infection. Strains of *C. difficile* that do not produce the toxins are not pathogenic (11).

Until recently, it was thought that all toxigenic strains associated with disease produced both toxins and that toxin A was required to produce the initial damage to the intestine (23). In 1991 and 1992 a strain that produced toxin B but no detectable toxin A was characterized (3, 21, 27). The strain, CCUG 8864, was shown to carry a large deletion in the *toxA* gene. Despite the absence of toxin A, CCUG 8864 causes disease in animal models. Furthermore, toxin B from this strain is weakly enterotoxic in rabbit intestinal loops and 10-fold more lethal than toxin B from strain VPI 10463. These findings suggested that strains that do not produce toxin A may still be capable of causing AAD. In 1993, serotype F strains were characterized as a second type of toxin A-negative, toxin B-positive (A−/B+) *C. difficile* (7). They were negative by toxin A-specific enzyme immunoassays (EIAs) but produced toxin B. Studies with serotype F strains (e.g., strain F 1470) indicated that they were not virulent in animal models. Serotype F strains are commonly isolated from asymptomatic children, further suggesting that they do not cause disease.

Rupnik et al. (26) recently characterized 10 toxinotypes (toxinotypes I to X) on the basis of deletions or additions within various regions of the toxin genes or other regions of the toxigenic element (also termed PaLoc) of *C. difficile* (14, 25). Of the 219 isolates characterized by Rupnik et al. (26), 47 contained variations in the toxin genes compared to the genes of reference strain VPI 10463. All the toxinotypes except toxinotype VIII and toxinotype X (of which CCUG 8864 is the only known strain) reacted in a toxin A-specific EIA. Of significance, 25 of the 47 defective strains were toxinotype VIII. A similar frequency of isolates with a deletion similar to that found in the *toxA* gene of toxinotype VIII was reported by Kato et al. (20). Others have also reported a relatively high frequency of *C. difficile* isolates with this characteristic deletion (4). Recently, A−/B+ isolates of *C. difficile* were implicated in an outbreak of AAD in Canada, and some patients developed PMC (1; M. Alfa, D. Lyerly, L. Neville, S. Moncrief, A. Al-Barrak, A. Kabani, B. Dyck, K. Olekson, and J. Embil, Abstr. 99th Annual Meeting of the American Society for Microbiology 1999, abstr. L-7, p. 440, 1999). Although further epidemiological studies are needed, it now appears that A−/B+ strains may be more common than was initially thought.

Toxic activity in a culture filtrate from toxinotype VIII strain F 1470 produces a cytopathic effect more closely resembling the effect of *Clostridium sordellii* lethal toxin (LT) (13; Alfa et al., Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999). In addition, toxin B from F 1470 has a spectrum of glucosylation activity similar to that of LT, which, in addition to the Rho proteins, includes the Ras proteins as substrates (13, 16, 17, 18). In the case of LT, a region of the toxin just downstream of the active site has been associated with the ability to recognize Ras as a substrate (16). In our study, we show that isolates from around the world that are negative by toxin A-specific EIAs but that are positive for toxin B are genetically similar and resemble toxinotype VIII. In addition to a large deletion in the repeat region of the toxin A gene, PCR analysis revealed that the region that encodes the toxin B substrate recognition domain is similar among all toxinotype VIII isolates and may extend the spectrum of glucosylation to the Ras proteins.

C. difficile isolates were grown in dialysis sac cultures in brain heart infusion (BHI) medium for 72 h as described previously (22). Culture filtrates of *C. difficile* A−/B+ isolates (Table 1) were analyzed for immunoreactivity and the presence of cytotoxicity. Immunoreactivity was measured by an EIA specific for toxin A (TOX-A TEST; TechLab, Inc., Blacksburg, Va.) and a second EIA that detects toxin B in addition to toxin A (TOX A/B TEST; TechLab, Inc.) according to the manufacturer's protocol. Titers were recorded as the reciprocal of the highest dilution with an A_{450} of 0.2 or greater. *C. difficile* strains of various phenotypes with respect to toxin production were in-

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TABLE 1. *C. difficile* A−/B+ isolates used in the study

Source	Location	Isolate(s)
M. Alfa and J. Embil	Canada	A 35533-9, A 35352-6, A 36060-9, A 35757-5, A 35089-1, A 36285-2, A 33479-8G, A 29090, A 30102, A 30103, A 30107, A 30112, A 35473
J. Brazier	Wales, United Kingdom	R 7404, R 8721, R 9117, R 9624, R 10214, R 10627, R 10726, R 11092
D. Craft	Chapel Hill, N.C.	TL 1321, TL 1334
M. Delmee	Belgium	F 1470 (control), F 5768, F 6058, F 22484, IS F 37, IS F 73, F 43853, X 5036, X 20822, X 23682, X 23747, X 34084
S. Johnson and S. Sambol	Chicago, Ill.	CF 2
N. Kato	Japan	GAI 95600, GAI 95601, GAI 95602, GAI 95603, GAI 95604, GAI 95605, GAI 95606
D. Turgeon	Seattle, Wash.	101 C
TechLab	Various locations in the United States	TL 457, MLR, CRW, J 1165 W

cluded in the study as controls. Most previous biological and molecular characterization of the toxins has been done with reference strain VPI 10463, which produces large amounts of both toxins. VPI 11186 is a nontoxigenic strain and is completely missing the toxigenic element that encodes toxins A and B. F 1470 and CCUG 8864 represent the two known toxinotypes (VIII and X, respectively) that produce toxin B but that are not reactive in toxin A-specific EIAs. Culture filtrates from all A−/B+ isolates failed to react in the TOX-A TEST, whereas VPI 10463 had a titer of 10^5. CCUG 8864 also did not react in the TOX-A TEST. Culture filtrates from all the A−/B+ isolates reacted in the TOX A/B TEST, with titers ranging from 10^1 to 10^3. F 1470 had a titer of 10^2. CCUG 8864 and VPI 10463 each had a titer of 10^5. The culture filtrate of nontoxigenic strain VPI 11186 failed to react in either test.

Culture filtrates from all 48 A−/B+ isolates had cytotoxic activity against CHO-K1 cells, with titers ranging from 10^3 to 10^5. F 1470 had a cytotoxicity titer of 10^5. VPI 10463 and CCUG 8864 had cytotoxicity titers of 10^5 and 10^6, respectively. The cytotoxic activities of A−/B+ isolates were neutralized by *C. difficile* VPI 10463 antisera as well as VPI 10463 toxin B-specific antibody.

The primers used to amplify regions of the toxin genes, along with their locations and the predicted sizes of the amplicons, are shown in Table 2. PCRs were performed with Ready to Go PCR Beads purchased from APBiotech (Piscataway, N.J.). Primers were from Gibco BRL Life Technology (Rockville, Md.). *C. difficile* isolates were grown on BHI plates overnight at 37°C in an anaerobic atmosphere by using oxygen-absorbent AnaeroGen (Oxoid, Ogdensburg, N.Y.) in a plastic anaerobic jar. For each reaction a single colony was placed into 100 μl of sterile deionized water. The tube was placed at 95°C for 5 min. Lysed cells were stored at −20°C until use. PCRs were performed in 25-μl volumes containing 10 μl of template DNA and each primer at a concentration of 1 μM. The annealing temperature varied according to the melting temperatures of the primers. PCRs were performed with denaturation at 94°C for 30 s, followed by 30 to 45 cycles with

TABLE 2. Primers used for PCR analysis of *C. difficile* toxin genes

PCR	Primers	Target gene	Location	Sequence	Amplicon size (kb)
CdB1	B1F	*toxB*	1–24	ATGAGTTTAGTTATTAGAAAACAG	1.79
	B1789R		1769–1789	CTAATTTTATCTCCTTGTAAC	
CdB2	TYBsrF	*toxB*	927–948	GATTTTTGGGAAATGACAAAG	0.56
	TYBsrR		1467–1487	GCTTCTATCAAATGGATATTC	
CdB3	FBsrF	*toxB*	931–948	GTAGATTGGGAAGAGATG	0.56
	FBsrR		1475–1494	CTCAGATGACAATATAGAAG	
CdB4	B4537F	*toxB*	4537–4559	TTGAAAGATGTCAAAGTTATAAC	0.86
	B5394R		5374–5394	CAGGTACATCTTGTTTATCAC	
CdB5	B4162F	*toxB*	4162–4183	GAGATTAATTTTTCTGGTGAGG	1.82
	B5984R		5962–5984	TCCTTTTTGCATAACTCCATCAG	
CdA1	A1F	*toxA*	1–24	ATGTCTTTAATATCTAAAGAAGAG	1.77
	A1769R		1749–1769	TCATCTCCTTGTAACTGTATG	
CdA2	A4570F	*toxA*	4570–4591	TAACAGGAAAATACTATGTTG	0.81
	A5382R		5362–5382	CATTATATATCCTAATGATAG	
CdA3	A5514F	*toxA*	5514–5534	TTCATTATTCTATTTTGATCC	2.66
	AE5DR		8158–8178	TAATTTCTTAGTAGCACAGGA	
CdA4	A6007F	*toxA*	6007–6027	GCTATTGCCTTTAATGGTTAT	1.18
	A7186R		7166–7186	CATTAATACTTGTATAACCAG	

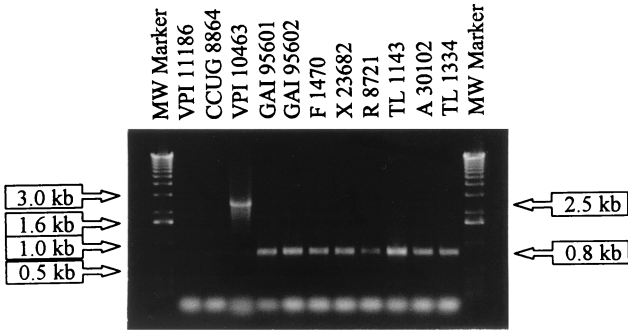

FIG. 1. CdA3 PCR analysis of the repeating units of representative A−/B+ isolates GAI 95601, GAI 95602, F 1470, X 23682, R 8721, TL 1143, A 30102, and TL 1334. Results for control strains VPI 11186 (nontoxigenic), CCUG 8864 (toxinotype X, A−/B+ strain), and VPI 10463 (toxin A-positive and toxin B-positive reference strain) are also shown. Faint bands (primer dimers) at the bottom of the gel were present in all PCRs with these primers. MW marker, molecular size marker.

annealing for 1 min and elongation for 1 to 2 min at 72°C. All of the A−/B+ isolates and VPI 10463 were positive for CdB1, CdB4, CdB5, CdA1, and CdA2 reactions. Collectively, the results indicate that the entire *toxB* gene, as well as the region of the *toxA* gene upstream of the repeating units, is present in all of the A−/B+ clinical isolates. CCUG 8864 was negative for the CdA2 reaction.

Primers for the CdA3 PCR flank the repeating units and were designed to analyze this region of the *toxA* gene. PCR CdA3 of VPI 10463 yielded an amplicon of the predicted size (approximately 2.5 kb) (Fig. 1). In contrast, all A−/B+ isolates yielded smaller identical products of 0.8 kb. The reaction products from representative isolates are shown in Fig. 1. These findings demonstrate that for all the isolates approximately 1.7 kb of DNA in the repeating units region has been deleted compared to the sequence of VPI 10463. To further verify that this region was missing from the variant isolates, primers designed to anneal to points that flank the two major PCG-4-binding epitopes were used for the CdA4 PCR (12). VPI 10463 but none of the A−/B+ isolates yielded the predicted PCR product of 1.2 kb (data not shown). CCUG 8864 did not react with either CdA3 or CdA4.

The primers for the CdB2 and CdB3 PCRs were designed to amplify a region of the *toxB* gene just downstream of the region that encodes the DXD (amino acids 286 to 288, VPI 10463 toxin B) active-site motif (5, 15). This region has been implicated in the recognition of Ras proteins by *C. sordellii* toxin LT that is closely related to toxin B (16). The sequence of strain F 1470 contains considerable divergence from VPI 10463 toxin B in this region (2, 9, 25). CdB2 primers were based on the sequence of VPI 10463, while CdB3 primers were based on the sequence of F 1470 (2, 9). Primers with sequences that matched the VPI 10463 sequence amplified a fragment of the predicted size for VPI 10463 but did not yield any product for any of the A−/B+ isolates (Fig. 2A). Conversely, the primers whose sequences were based on the F 1470 sequence amplified all the variant isolates (representative PCRs are shown in Fig. 2A), whereas no reaction was produced with VPI 10463. As predicted from the sequences of VPI 10463 and F 1470, digestion of PCR products with the restriction enzyme *Psi*I resulted in a pattern for the CdB2 PCR fragment from VPI 10463 different from that for CdB3 PCR fragments from A−/B+ isolates (Fig. 2B). Toxinotype X strain CCUG 8864 produced the same results as toxinotype VIII isolates with the CdB2 and CdB3 reactions and *Psi*I digestion.

In this study, we showed that a number of A−/B+ isolates from around the world were identical by PCR analysis with a series of primers designed to analyze various regions of both toxin genes. In particular, similar to the results of Rupnik et al. (26) and Kato et al. (19). a large region, was of toxin A repeating units from missing all A−/B+ isolates. This region encodes the epitopes for the monoclonal antibody used in toxin A-specific EIAs, suggesting a possible reason for the lack of reactivity (8, 12). Eichel-Streiber et al. (10), on the other hand, recently identified a nonsense mutation introduced at amino acid position 47 of the toxin A gene of strain F 1470 and two other A−/B+ strains that abrogated production of a functional toxin A. Furthermore, despite the large deletion, the repeating-unit region of F 1470 reacted with monoclonal antibody TTC8 (specific for the repetitive region of toxin A) when the region was expressed as a recombinant protein in *Escherichia coli*. This suggests that the lack of immunoreactivity in toxin A-specific EIAs is due to truncation by the nonsense mutation near the beginning of the toxin A gene.

The enzymatic domain at the 5′ end of the toxin B genes from A−/B+ strains F 1470 and CCUG 8864 has been sequenced (9, 25). Each of the strains is nearly identical to the other strain; however, their toxin B sequences vary considerably from the VPI 10463 toxin B sequence. The sequence variations are most striking in the region just downstream from the putative glucosylation active-site (DVD) motif (5). This region is associated with the extended spectrum of glucosylation by related toxin LT produced by *C. sordellii* (16). In addition to the Rho protein, LT glucosylates the Ras proteins (13, 17, 18). Toxins B from F 1470 and CCUG 8864 also glucosylate Ras, and it has been suggested that they represent hybrid toxins (6). In our study, we used primers based on the F 1470 *toxB* sequence to amplify this region in A−/B+ isolates. All the A−/B+ isolates yielded amplification products of sim-

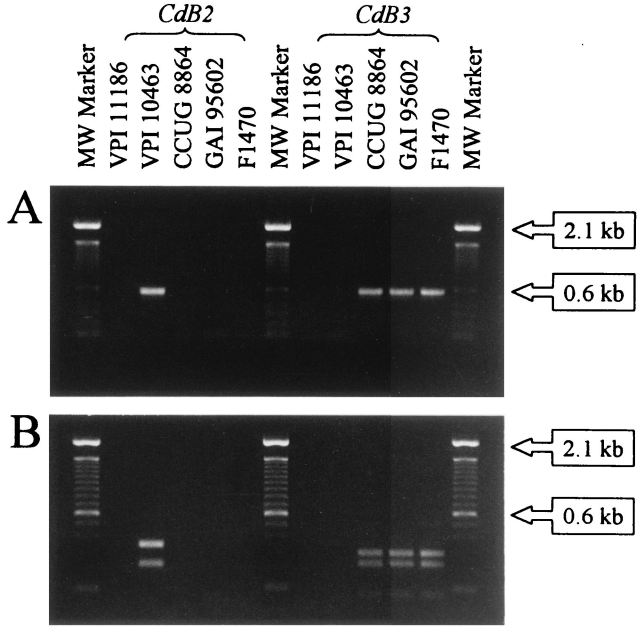

FIG. 2. (A) CdB2 and CdB3 PCRs of the toxin B gene downstream of the active-site motif. Representative A−/B+ isolates GAI 95602 and F 1470 are shown, along with control strains VPI 11186 (nontoxigenic), VPI 10463 (toxin A-positive, toxin B-positive reference strain), and CCUG 8864 (toxinotype X, A−/B+ strain). (B) CdB2 and CdB3 PCR products digested with *Psi*I. MW marker, molecular size marker.

ilar sizes and gave similar patterns by restriction digestion with *Psi*I. VPI 10463, on the other hand, did not yield a PCR product with these primers. Conversely, primers whose sequences are based on a similar region of VPI 10463 *toxB* amplified VPI 10463 but none of the A−/B+ isolates. The sequence variations of the A−/B+ isolates suggest that they may glucosylate Ras proteins in addition to the Rho protein, as observed for F 1470. The effect of this variation on the pathogenesis of infections with A−/B+ isolates remains to be determined. Toxin B from CCUG 8864 is weakly enterotoxic in rabbit ileal loop assays. Studies on the enterotoxic activities of toxins B from toxinotype VIII strains have not been reported. Further studies on the biological properties of toxins B from toxinotype VIII strains, particularly their enterotoxic activities, are needed.

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REFERENCES

1. **Al-Barrak, A., J. Embil, B. Dyck, K. Olekson, M. Alfa, and A. Kabani.** 1999. An outbreak of toxin A-negative, toxin B-positive *Clostridium difficile*-associated diarrhea in a Canadian tertiary-care hospital. Can. Communic. Dis. Rep. **25:**65–69.
2. **Barroso, L. A., S. Z. Wang, C. J. Phelps, J. L. Johnson, and T. D. Wilkins.** 1990. Nucleotide sequence of *Clostridium difficile* toxin B gene. Nucleic Acids Res. **18:**4004.
3. **Borriello, S. P., B. W. Wren, S. Hyde, S. V. Seddon, P. Sibbons, M. M. Krishna, S. Tabaqchali, S. Manek, and A. 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.
4. **Brazier, J. S., S. L. Stubbs, and B. I. Duerden.** 1999. Prevalence of toxin A negative/toxin B positive *Clostridium difficile* strains. J. Hosp. Infect. **42:**248.
5. **Busch, C., F. Hofmann, J. Selzer, S. Munro, D. Jeckel, and K. Aktories.** 1998. A common motif of eukaryotic glycosyltransferases is essential for the enzyme activity of large clostridial toxins. J. Biol. Chem. **273:**19566–19572.
6. **Chaves-Olarte, E., P. Low, E. Freer, T. Norlin, M. Weidmann, C. von Eichel-Streiber, and M. Thelestam.** 1999. A novel cytotoxin from *Clostridium difficile* serotype F is a functional hybrid between two other large clostridial toxins. J. Biol. Chem. **274:**11046–11052.
7. **Depetrie, C., M. Delmee, 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.
8. **Dove, C. H., S.-Z. Wang, S. B. Price, C. J. Phelps, D. M. Lyerly, T. D. Wilkins, and J. L. Johnson.** 1990. Molecular characterization of the *Clostridium difficile* toxin A gene. Infect. Immun. **58:**480–488.
9. **Eichel-Streiber, C. V., D. Meyer zu Heringdorf, E. Habermann, and S. Sartingen.** 1995. Closing in on the toxic domain through analysis of a variant *Clostridium difficile* cytotoxin B. Mol. Microbiol. **17:**313–321.
10. **Eichel-Streiber, C. V., I Zec-Pirant, M. Grabnar, and M. Rupnik.** 1999. A nonsense mutation abrogates production of a functional enterotoxin A in *Clostridium difficile* toxinotype VIII strains of serogroups F and X. FEMS Microbiol. Lett. **178:**163–168.
11. **Fluit, A. D. C., M. J. H. M. Wolfhagen, G. P. H. T. Verdonk, M. Janse, R. Torensma, and J. Verhoef.** 1991. Nontoxigenic strains of *Clostridium difficile* lack the genes for both toxin A and toxin B. J. Clin. Microbiol. **29:**2666–2667.
12. **Frey, S., and T. D. Wilkins.** 1992. Localization of two epitopes recognized by monoclonal antibody PCG-4 on *Clostridium difficile* toxin A. Infect. Immun. **60:**2488–2492.
13. **Genth, H., F. Hofmann, J. Selzer, G. Rex, K. Aktories, and I. Just.** 1996. Difference in protein substrate specificity between hemorrhagic toxin and lethal toxin from *Clostridium sordellii*. Biochem. Biophys. Res. Commun. **229:**370–374.
14. **Hammond, G. A., and J. L. Johnson.** 1995. The toxigenic element of *Clostridium difficile* strain 10463. Microb. Pathog. **19:**203–213.
15. **Hofmann, F., C. Busch, U. Prepens, I. Just, and K. Aktories.** 1997. Localization of the glucosyltransferase activity of *Clostridium difficile* toxin B to the N-terminal part of the holotoxin. J. Biol. Chem. **272:**11074–11078.
16. **Hofmann, F., C. Busch, and K. Aktories.** 1998. Chimeric clostridial cytotoxins: identification of the N-terminal region involved in protein substrate recognition. Infect. Immun. **66:**1076–1081.
17. **Hofmann, F., G. Rex, K. Aktories, and I. Just.** 1996. The *ras*-related protein Ral is monoglucosylated by *Clostridium sordellii* lethal toxin. Biochem. Biophys. Res. Commun. **227:**77–81.
18. **Just, I., J. Selzer, F. Hofmann, G. A. Green, and K. Aktories.** 1996. Inactivation of Ras by *Clostridium sordellii* lethal toxin-catalyzed glucosylation. J. Biol. Chem. **271:**10149–10153.
19. **Kato, H., N. Kato, S. Katow, T. Maegawa, S. Nakamura, and D. M. Lyerly.** 1999. Deletions in the repeating sequences of the toxin A gene of toxin A-negative, toxin B-positive *Clostridium difficile* strains. FEMS Microbiol. Lett. **175:**197–203.
20. **Kato, H., N. Kato, K. Wantabe, N. Iwai, H. Nakamura, T. Yamamoto, K. Suzuki, S.-M. Kim, Y. Chong, and E. Wasito.** 1998. Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. J. Clin. Microbiol. **36:**2178–2182.
21. **Lyerly, D. M., L. A. Barroso, T. D. Wilkins, C. Depitre, and G. Corthier.** 1992. Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile*. Infect. Immun. **60:**4633–4639.
22. **Lyerly, D. M., H. C. Krivan, and T. D. Wilkins.** 1998. *Clostridium difficile*: its disease and toxins. Clin. Microbiol. Rev. **1:**1–18.
23. **Lyerly, D. M., K. E. Saum, D. K. MacDonald, and T. D. Wilkins.** 1985. Effects of *Clostridium difficile* toxins given intragastrically to animals. Infect. Immun. **47:**349–352.
24. **Lyerly, D. M., and T. D. Wilkins.** 1995. *Clostridium difficile*, p. 867–891. *In* M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg, and R. L. Guerrant (ed.), Infections of the gastrointestinal tract, Raven Press, New York, N.Y.
25. **Rupnik, M., V. Braun, F. Soehn, M. Janc, M. Hofstetter, R. Laufenberg-Feldmann, and C. von Eichel-Streiber.** 1997. Characterization of polymorphisms in the toxin A and B genes of *Clostridium difficile*. FEMS Microbiol. Lett. **148:**197–202.
26. **Rupnik, M., V. Avesani, M. Janc, C. von Eichel-Streiber, and M. Delmee.** 1998. A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. J. Clin. Microbiol. **36:**2240–2247.
27. **Torres, J. F.** 1991. Purification and characterization of toxin B from a strain of *Clostridium difficile* that does not produce toxin A. J. Med. Microbiol. **34:**40–44.