JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1991, p. 2666–2667
0095-1137/91/112666-02$02.00/0

Vol. 29, No. 11

Nontoxigenic Strains of *Clostridium difficile* Lack the Genes for Both Toxin A and Toxin B

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Received 29 March 1991/Accepted 29 July 1991

A total of 39 toxigenic and 20 nontoxigenic strains of *Clostridium difficile* were tested for the presence of either toxin A or toxin B by the polymerase chain reaction (PCR). All toxigenic strains produced cytotoxin as assayed by using highly sensitive fetal lung fibroblasts and were positive for toxin A as well as toxin B in the PCR assay. All nontoxigenic strains failed to produce toxin and were negative in the PCR assay. This study shows that nontoxigenic strains of *Clostridium difficile* lack the toxin A as well as the toxin B gene.

Clostridium difficile, recognized as a major cause of antibiotic-associated pseudomembranous enterocolitis, produces two toxins. Toxin A, an enterotoxin, causes hemorrhagic fluid accumulation in rabbit ileal loops and is cytotoxic for cultured fibroblasts. Toxin B is an extremely potent cytotoxin (for a review, see reference 7). Recently it has been showed that the toxin A gene encodes for a 308-kDa protein, in agreement with the molecular mass estimated from polyacrylamide gel electrophoresis (3, 9). The toxin B gene has also been located and sequenced (1, 5). The molecular mass of toxin B, based on this sequence, is 270 kDa. (1). Toxin A displays several repeated amino acid sequence patterns. When primers from this part of the genome were used to amplify a fragment with the polymerase chain reaction (PCR), positive amplification was also seen with DNA of *Clostridium sordellii* (10). Recently, Kato et al. (6) used several primer sets to amplify a fragment of toxin A. They distinguished toxigenic *C. difficile* strains from nontoxigenic strains by amplifying segments of repeating and nonrepeating sequences of the *C. difficile* toxin A gene. No amplification of fragments was observed with purified DNA of *C. sordellii* and other *Clostridium* spp. Nontoxigenic strains of *C. difficile* lacked the total toxin A gene. The present note reports that noncytotoxic strains of *C. difficile* lack at least a part of both the toxin A and toxin B gene.

The *C. difficile* strains investigated were obtained from several institutes: 11 strains, including the now-known 10 serotypes of *C. difficile* (2), were obtained from the American Type Culture Collection (ATCC 9689, 43593, 43594, and 43596 through 43603); 6 strains were a gift from L. Peterson (VA Hospital, Minneapolis, Minn.); 24 strains were clinical isolates obtained from the Dutch National Institute for Public Health and Environmental Protection, Bilthoven, The Netherlands (strains were isolated at several hospitals in The Netherlands over a period of 10 years); 16 strains were obtained from the Wilhelmina Children's Hospital, Utrecht, The Netherlands; and 2 strains were isolated at the University Hospital, Utrecht, The Netherlands. Strains were identified according to smell (horse stable odor), morphology, latex slide agglutination (Mercia Diagnostics, Guildford, Surrey, United Kingdom), and prereduced anaerobically sterilized biochemical testing as described by Holdeman et al (4).

Cytotoxicity was determined by incubating culture supernatants on fetal lung fibroblasts. Cytotoxicity was defined as greater-than-50% cell rounding after 18 h of incubation.

PCRs were performed in a mixture containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.01% (wt/vol) gelatin, 100 μM (each) deoxynucleoside triphosphates, 50 pmol of each appropriate primer, and 2.5 U of *Taq* polymerase (Cetus, Emeryville, Calif.). The sequences of the primers used for amplification of the toxin A fragment were 5′-CTACTTCACTAGATTTTTCC and 5′-TCCTAAATGAT CTCTATCTA, resulting in an amplification product of 434 bp (3, 9). The sequences of the primers for toxin B were 5′-TAATAGAAAACAGTTAGAAA and 5′-TCCAATCCA AACAAAATGTA, which produced a fragment of 301 bp (1). The toxin A gene is located downstream from the toxin B gene (3). The primers for toxin B are located at the 5′ end of the toxin B gene. The primers for toxin A were chosen just before the repeating units in this gene (1, 3). Samples were subjected to 30 cycles of amplification. Amplification was carried out in a DNA Thermal Cycler (Perkin-Elmer, Norwalk, Conn.) with each cycle consisting of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C. Amplified DNA was detected by agarose gel electrophoresis (8) after staining with ethidium bromide and was confirmed by dot blotting using ^{32}P-labeled oligonucleotides specific for toxin A (5′-GTCTACA GAAGGAAGTGACT) and toxin B (5′-GTCAGAGAATAC TGTAGTCG).

In total, we have processed 59 *C. difficile* strains in the PCR for amplification of both the toxin A and B gene. Of these strains, 39 were positive in the cytotoxicity assay and 20 were negative. In toxigenic strains, the expected 434-bp DNA fragment of toxin A and the 301-bp fragment of toxin B were found. No strains missing only one of either toxin gene were found. Johnson et al. (5) showed that the toxin B gene is located upstream of the toxin A gene and that both genes are separated by a small open reading frame. Therefore, it can be concluded that in nontoxigenic strains of *C. difficile*, at least a part of both the toxin A and toxin B genes is absent. A similar observation has been made for *Clostridium perfringens* (10). The results presented in this paper show that cytotoxic strains always are in the possession of at least a part of both the toxin A and toxin B genes. This suggests that

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simultaneous expression of these two proteins is due to the presence of both genes. These genes are apparently strongly linked. However, for each gene, putative promoter and ribosome binding sites are described elsewhere (5). The genes for toxin A and toxin B are not likely to form an operon. There is no clear explanation for either the absence or the presence of these genes in individual strains of *C. difficile*.

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