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Characterization of Cross-Reactive Proteins Detected by Culturette Brand Rapid Latex Test for *Clostridium difficile*

DAVID M. LYERLY,* DONALD W. BALL, JULIANNA TOTH, AND TRACY D. WILKINS

Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

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***Clostridium sporogenes*, *Peptostreptococcus anaerobius*, and *Bacteroides asaccharolyticus* have been reported to react in the Culturette Brand Rapid Latex Test (Marion Scientific, Div. Marion Laboratories, Inc., Kansas City, Mo.) for *Clostridium difficile*. From the results of this study we showed that *C. sporogenes* and *P. anaerobius* produce a protein which is very similar biochemically and immunologically to the protein of *C. difficile* that is detected by the test. Thus, the positive latex reactions observed with *C. sporogenes* and *P. anaerobius* are due to a cross-reactive protein. We did not detect this cross-reactive protein in filtrates from *B. asaccharolyticus*, indicating that this bacterium reacts with the latex reagent by some other mechanism. We cloned the *C. difficile* gene that codes for the cross-reactive protein and showed that the protein produced by the recombinant organism is nontoxic and distinct from toxin A, thus confirming our earlier findings.**

Recently, a commercial latex test (the Culturette Brand Rapid Latex Test; Marion Scientific, Div. Marion Laboratories, Inc., Kansas City, Mo.) became available for the clinical detection of *Clostridium difficile*, the etiologic agent of pseudomembranous colitis. Initially, the test was marketed for the detection of toxin A. Results from our laboratory and other laboratories have shown, however, that the test detects an antigen that is distinct from the toxins (1, 5, 11, 14). The latex-reactive antigen is produced by all of the toxigenic and nontoxigenic strains of *C. difficile* which we have examined (D. Lyerly and T. Wilkins, unpublished data).

Further evaluation of the latex test has revealed that *Clostridium sporogenes* and proteolytic *Clostridium botulinum*, which are indistinguishable with the exception of neurotoxin production (17), react in the test (5; B. L. Miles and J. A. Siders, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, C4, p. 324; C. A. Gaydos, B. E. Laughon, L. M. Mundy, R. G. Bennett, and L. Bobo, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, B216, p. 613). In addition, *Peptostreptococcus anaerobius* (S. Allen and B. Miles, personal communication) and *Bacteroides asaccharolyticus* (1) have been reported to give positive reactions in the test. It has not been determined whether the reaction of these bacteria in the latex text is nonspecific or whether the bacteria produce an antigen which cross-reacts with the latex-reactive antigen of *C. difficile*. In this study we prepared monospecific antiserum against the latex-reactive antigen of *C. difficile* and used the antiserum to show that *C. sporogenes* and *P. anaerobius* produce an antigen which is similar to the latex-reactive antigen of *C. difficile*. *B. asaccharolyticus* does not produce this antigen, and the mechanism by which it reacts with the latex reagent is still not known. We cloned the gene which codes for the latex-reactive antigen of *C. difficile* and showed that the cloned gene product is nontoxic. These findings confirm our earlier results which demonstrated that the latex-reactive antigen is a protein that is not associated with either toxin A or toxin B of *C. difficile*.

* Corresponding author.

MATERIALS AND METHODS

Preparation of culture filtrates. Bacterial strains were obtained from the culture collection of the Department of Anaerobic Microbiology at the Virginia Polytechnic Institute and State University (Blacksburg, Va.) and were identified by L. V. Moore and W. E. C. Moore by the method described by Holdeman et al. (4). Each strain was grown anaerobically in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) for 48 to 72 h at 37°C. The cultures were centrifuged, and the supernatants were filtered through 0.45 μm-pore-size membranes and stored at 4°C.

Preparation of toxin A. Toxin A was purified from culture filtrates of *C. difficile* VPI 10463 by affinity chromatography on thyroglobulin-Affi Gel 10, as described previously (7).

Preparation of *C. difficile* antiserum. Goat antiserum against the culture filtrate of *C. difficile* VPI 10463 was obtained as described previously (2).

Latex agglutination assay. Culture filtrates were analyzed by using the Culturette Brand Rapid Latex Test for *Clostridium difficile* (Marion Scientific), as recommended by the manufacturer.

Preparation of monospecific antiserum against the latex-reactive antigen. Culture filtrate (1 ml) from *C. difficile* VPI 10463 and 1 ml of the positive latex reagent from the Culturette Brand Rapid Latex Test were mixed for 30 min at room temperature. The suspension was centrifuged, and the latex particles were washed three times with phosphate-buffered saline (PBS; pH 7.4). After the final wash, the beads were suspended in 5 ml of PBS and injected subcutaneously into a New Zealand White rabbit. Ten weeks later, the rabbit was injected a second time with 5 ml of the vaccine. Serum was obtained from the rabbit and analyzed for precipitating antibody by crossed immunoelectrophoresis.

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was done in 1.2% low electroendosmotic agarose (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M Tris–Tricine (Sigma) buffer (pH 8.6), as described previously (10). Samples were concentrated 10 times on Minicon B-15 units (Amicon Corp., Lexington, Mass.) prior to analysis.

Polyacrylamide gel electrophoresis and immunoblot analysis. Samples containing 2.5% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol were heated at 100°C for 5 min, mixed with glycerol-bromphenol blue, and analyzed by

SDS-polyacrylamide gel electrophoresis, as described by Laemmli (8). Prestained SDS molecular weight markers and electrophoresis reagents were purchased from Sigma.

After electrophoresis, proteins were transferred to nitrocellulose (Bio-Rad Laboratories, Richmond, Calif.) by using the general procedures described by Towbin et al. (16). The transfer was done in 0.025 M Tris–0.192 M Glycine buffer (pH 8.3) containing 20% (vol/vol) methanol at 100 V for 1.5 h. After the transfer was completed, the nitrocellulose membranes were blocked in 0.05 M Tris hydrochloride buffer (pH 7.4)–0.2 M NaCl (TBS) containing 0.5% sodium caseinate for 30 min at room temperature. A 1/1,000 dilution (in TBS) of the monospecific antiserum against the latex-reactive antigen was added for 3 h at room temperature. The membranes were washed three times (20 min per wash), and a 1/1,000 dilution (in TBS) of goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase (Sigma) was added. After overnight incubation at room temperature, the membranes were washed with TBS and the substrate 4-chloro-1-naphthol (Sigma) was added for color development.

Ouchterlony double immunodiffusion. Culture filtrates were concentrated 10-fold on a concentrator (Minicon; Amicon) and analyzed by double immunodiffusion in 1.2% agarose gels prepared in 0.1 M Tris–Tricine buffer (pH 8.6), by using the monospecific antiserum described above.

Cloning of the gene which codes for the latex-reactive antigen. *C. difficile* VPI 10463 was grown in brain heart infusion broth overnight at 37°C, and the cells were collected by centrifugation. The cells were suspended in 0.01 M Tris hydrochloride buffer (pH 8.0), incubated with achromopeptidase (Wako Chemicals, Dallas, Tex.) to make the cells more susceptible to lysis, and lysed with SDS. The DNA was isolated from the lysed cells by the method described by Marmur (13).

The high-molecular-weight DNA was digested with the restriction endonuclease *Dra*I, and the fragments were ligated to the *Hin*cII restriction site in M13mp19(pUC19) with T4 DNA ligase, as described elsewhere (12). The ligation mixture was subsequently used to transform *Escherichia coli* chi 1776 cells that were made competent by the $CaCl_2$ procedure (12). The restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories (Gaithersburg, Md.). All of the work involving recombinant DNA procedures was done in a Biosafety Laboratory-2 containment facility.

Recombinant clones were selected on Luria-Bertani agar (12) containing ampicillin and were screened by an immunoassay by using the monospecific antiserum against the latex-reactive antigen described above. Briefly, transformants were replica plated, and the replicate colonies were transferred onto nitrocellulose membranes (diameter, 82 mm; Bio-Rad Laboratories). The transferred colonies were lysed with chloroform vapors in a glass chamber for 30 min, and screened for antigen by the procedure described by Helfman et al. (3). Briefly, the membranes were blocked with PBS-Triton X-100 (PBS-T) containing 0.5% sodium casein and incubated in a 1/1,000 dilution of the monospecific antiserum (in PBS-T) for 3 h at room temperature. The membranes were then rinsed and incubated in a 1/1,000 dilution of goat anti-rabbit IgG-horseradish peroxidase (in PBS-T; Sigma) overnight at room temperature. After incubation, the membranes were rinsed and developed in 4-chloro-1-naphthol substrate solution. Positive clones were grown in 5 ml of Luria-Bertani broth overnight at 37°C. The cells were collected by centrifugation, suspended in 5 ml of 0.01 M Tris hydrochloride buffer (pH 7.5), and lysed with a French pressure cell. The lysate was analyzed with the Culturette Brand Rapid Latex kit. In addition, the lysate was screened for cytotoxic activity, lethal activity in mice, and enterotoxic activity in rabbit ileal loops by using previously described procedures (9, 15).

The M13mp19(pUC19) vector containing the gene for the latex-reactive antigen was subsequently cloned into host *E. coli* JM109 by using the methods described above. The host *E. coli* JM109 was used to obtain larger amounts of the gene product.

RESULTS

Initial screening studies. We initially screened 39 species of clostridia and anaerobic cocci using the Culturette Brand Rapid Latex Test. Positive reactions were obtained with strains of *C. difficile*, *C. sporogenes*, *P. anaerobius*, and *B. asaccharolyticus*. All of the strains of *C. difficile* (56 strains) and *C. sporogenes* (9 strains) gave strong positive reactions. The reaction in the *P. anaerobius* group was more variable. Five of the eight strains of *P. anaerobius* gave strong reactions, and two strains, including ATCC type strain 27337 (VPI 4330), gave weak reactions. One strain consistently gave negative reactions, even though it grew as well as the other strains of *P. anaerobius*. In the *B. asaccharolyticus* group, VPI 10630F (the only strain which reacted of nine strains tested) gave a strong, easily visible reaction.

Characterization of the latex-reactive antigen. We produced monospecific antiserum against the latex-reactive antigen of *C. difficile* VPI 10463 (Fig. 1A) and used the antiserum to analyze culture filtrates from *C. sporogenes*, *P. anaerobius*, and *B. asaccharolyticus* for the presence of a similar antigen. Analysis by crossed immunoelectrophoresis showed that *C. sporogenes* and strains of *P. anaerobius* which reacted in the latex test produced an antigen which reacted with the monospecific antiserum (Fig. 1B and C). No immunoprecipitin arcs were observed with filtrate from the strain of *B. asaccharolyticus* which reacted in the test. As can be seen in the crossed immunoelectrophoretic patterns, the antigen of *P. anaerobius* migrated faster in an electric field than the antigen of *C. difficile*, whereas the antigen of *C. sporogenes* migrated more slowly. This difference in migration was confirmed by showing that three different arcs were present when mixtures of filtrates from the three species were analyzed by crossed immunoelectrophoresis (Fig. 1D).

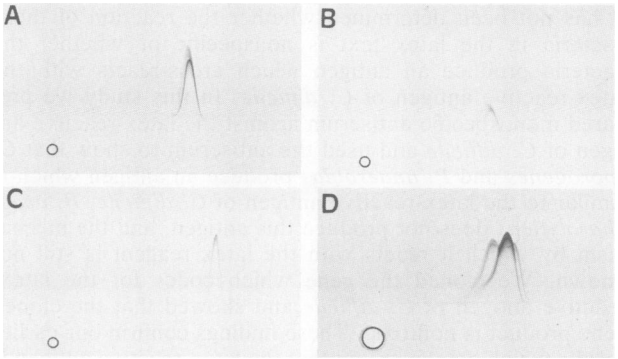

FIG. 1. Analysis by crossed immunoelectrophoresis of culture filtrate from *C. difficile* VPI 10463 (A); *C. sporogenes* VPI 9743 (B); *P. anaerobius* VPI 6116 (C); and a mixture containing equal amounts of filtrates from *C. difficile* VPI 10463, *C. sporogenes* VPI 9743, and *P. anaerobius* VPI 6116. The second dimension of agarose in each plate contained 50 μl of monospecific antiserum (4 μl/cm^2) against the latex-reactive antigen of *C. difficile* VPI 10463.

Analysis by nondenaturing electrophoresis, followed by immunoblotting with the monospecific antiserum, showed the presence of a single band in filtrates from *C. difficile*, *C. sporogenes*, and *P. anaerobius* (Fig. 2A). Again, the filtrate from *B. asaccharolyticus* VPI 10630F was negative. When the filtrates from *C. difficile*, *C. sporogenes*, and *P. anaerobius* were denatured and analyzed by immunoblotting, a major band corresponding to a 43,000-dalton subunit was observed (Fig. 2B). Two minor bands which were not consistently produced were also detected in many of the filtrates from strains of *C. difficile* (Fig. 2B, lanes 1 and 2).

When we analyzed filtrates of the three species by Ouchterlony double immunodiffusion, we observed spur formation, indicating that there is partial immunological identity between the latex-reactive antigen of *C. difficile* and the antigens of *C. sporogenes* and *P. anaerobius* (Fig. 3A). The reaction of partial identity between the latex-reactive antigens of these three species was also evident from the immunoelectrophoretic profile observed in Fig. 1D, as noted by the fusion of the immunoprecipitin arcs from *C. sporogenes* and *P. anaerobius* with the arc from *C. difficile*. The antigens of *C. sporogenes* and *P. anaerobius* showed partial identity with each other. All of the strains of *C. difficile* showed a single band of complete identity (Fig. 3B).

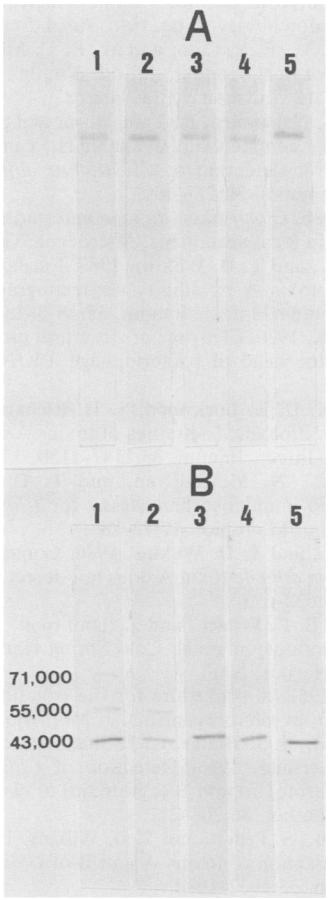

FIG. 2. Immunoblot analysis of culture filtrate from *C. difficile* VPI 10463 (lanes 1), *C. difficile* 237 (lanes 2), *C. difficile* 1145 (lanes 3), *C. sporogenes* VPI 9743 (lanes 4), and *P. anaerobius* VPI 6116 (lanes 5). (A) Samples were subjected to electrophoresis under native conditions. (B) Samples were denatured with SDS and 2-mercaptoethanol prior to electrophoresis. The immunoblot was developed by using monospecific antiserum against the latex-reactive antigen of *C. difficile* VPI 10463. Estimated molecular weights are listed on the left side of the panel.

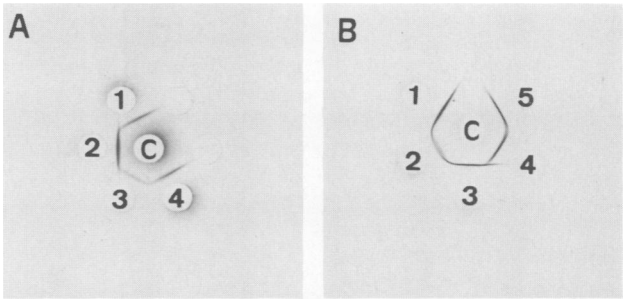

FIG. 3. Analysis of the latex-reactive antigen by Ouchterlony double immunodiffusion. (A) Well 1, *P. anaerobius* VPI 6116 filtrate; well 2, *C. difficile* VPI 10463 filtrate; well 3, *C. sporogenes* VPI 9743 filtrate; well 4, *P. anaerobius* VPI 6116 filtrate. (B) Well 1, *C. difficile* VPI 10463 filtrate; well 2, *C. difficile* 237 filtrate; well 3, *C. difficile* 1145 filtrate; well 4, *C. sporogenes* VPI 1963 filtrate; well 5, *C. sporogenes* VPI 9743 filtrate. The center (C) wells in each plate contained monospecific antiserum against the latex-reactive antigen of *C. difficile* VPI 10463.

Cloning of the gene which codes for the latex-reactive antigen. A recombinant *E. coli* clone which expressed the latex-reactive antigen was identified by enzyme-linked immunosorbent assay by using the monospecific antiserum and the Culturette Brand Rapid Latex Test. The antigen reacted specifically with the monospecific antiserum (Fig. 4A) and showed complete immunological identity with the latex-reactive antigen of *C. difficile* VPI 10463 (Fig. 4B). In addition, the expressed antigen behaved identically to the latex-reactive antigen from *C. difficile* when analyzed by electrophoresis under native conditions (Fig. 4C, lane 1). Under denaturing conditions, the 43,000-dalton subunit was present (Fig. 4C, lane 2). Concentrates of the filtrate from the recombinant strain were not cytotoxic or lethal in mice and were negative in rabbit ileal loops. The cloned antigen did not show any lines of identity with toxin A when analyzed by Ouchterlony double immunodiffusion with *C. difficile* antiserum containing antibodies against both antigens (data not shown).

DISCUSSION

Results of this study indicate that *C. sporogenes* and *P. anaerobius* produce a protein which is immunologically similar to the latex-reactive protein of *C. difficile*, thus explaining why these organisms react in the Culturette Brand Rapid Latex Test. The protein from each species contains a 43,000-dalton subunit, indicating that they are very similar. Kohno (6) also reported an estimated molecular weight of 43,000 for the latex-reactive antigen. It was reported in that article, however, that the latex-reactive antigen was toxin A, and it has now been shown that this is not the case (1, 5, 11). There are some minor differences among the latex-reactive antigens of *C. difficile*, *C. sporogenes*, and *P. anaerobius*. The reactions of partial immunological identity which we observed when we compared the proteins by immunoelectrophoresis and immunodiffusion indicate that the proteins from these species are not identical. In addition, they exhibit slightly different charge properties, as shown by crossed immunoelectrophoresis.

It appears that we cloned the entire gene for the latex-reactive protein. This is based on the findings that the

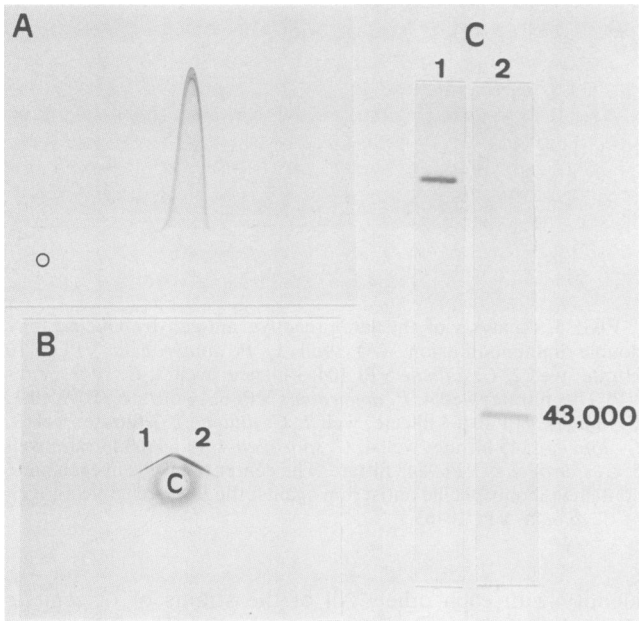

FIG. 4. Analysis of the recombinant latex-reactive protein. (A) Analysis by crossed immunoelectrophoresis. The well initially contained lysate from *E. coli* JM109, which contains the gene for the latex-reactive protein. The second dimension of agarose contained 50 μl of monospecific antiserum (4 μl/cm^2) against the latex-reactive antigen of *C. difficile* VPI 10463. (B) Analysis by Ouchterlony double immunodiffusion. Well 1, *C. difficile* VPI 10463 filtrate; well 2, lysate from *E. coli* JM109 containing the gene for the latex-reactive antigen. The center (C) well contained monospecific antiserum against the latex-reactive antigen of *C. difficile* VPI 10463. (C) Analysis by polyacrylamide gel electrophoresis followed by immunoblotting. Lane 1, The sample was subjected to electrophoresis under native conditions; lane 2, the sample was denatured with SDS and 2-mercaptoethanol prior to electrophoresis. The immunoblot was developed by using monospecific antiserum against the latex-reactive antigen of *C. difficile* VPI 10463. The estimated molecular weight is listed on the right side of the panel.

recombinant latex-reactive protein consists of the 43,000-dalton subunit and is identical to the native protein by nondenaturing electrophoresis and immunoblotting, as well as by immunodiffusion. The cloned protein was negative in a number of biological assays and did not show any immunological relatedness to toxin A. These findings support our previous observations which show that the latex-reactive antigen is nontoxic and is not a fragment or subunit of toxin A. These findings are supported further by our observations that nontoxigenic strains of *C. difficile* produce as much of the latex-reactive antigen as do highly toxigenic strains and that filtrates from nontoxigenic strains are consistently negative in the toxin A enzyme-linked immunosorbent assay with polyclonal and monoclonal antibodies against toxin A (10).

The similarity of the latex-reactive proteins from *C. difficile*, *C. sporogenes*, and *P. anaerobius* indicates that the gene has been conserved among these three species. The function of the protein remains to be determined. It is intriguing, however, that these two distinct *Clostridia* species and a species in a completely separate genus each produce this protein. It should be possible to develop antibodies which are directed toward the species-specific epitopes on each of the antigens and use the antibodies to learn more about the structure of the antigen. In addition, the antibodies against the *C. difficile*-specific epitopes may be useful in developing a more specific clinical test.

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