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Purification and Characterization of *Clostridium sordellii* Hemorrhagic Toxin and Cross-Reactivity with *Clostridium difficile* Toxin A (Enterotoxin)

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Hemorrhagic toxin (toxin HT) was purified from *Clostridium sordellii* culture filtrate. The purification steps included ultrafiltration through an XM-100 membrane filter and immunoaffinity chromatography, using a monoclonal antibody to toxin A of *Clostridium difficile* as the ligand. Toxin HT migrated as a major band with a molecular weight of 525,000 and a minor band at 450,000 on nondenaturing gradient polyacrylamide gel electrophoresis. The molecular weight was estimated at 300,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Isoelectric focusing indicated an apparent pI of 6.1. Toxin HT was cytotoxic for cultured cells and lethal for mice by intraperitoneal injection, and it elicited an accumulation of hemorrhagic fluid in rabbit ileal loops. Immunodiffusion analysis revealed a reaction of partial identity between toxins A and HT. Immunological cross-reactivity between these toxins was further demonstrated by immunoblotting and by neutralization of toxin HT biological activity with antibodies to toxin A. A sensitive indirect enzyme-linked immunosorbent assay was used to examine the affinity involved in homologous and heterologous antigen-antibody interactions. Our findings show that toxin HT has biological activities and immunological properties similar to those of toxin A; however, the toxins are not identical.

Clostridium sordellii is one of six clostridia associated with clostridial myonecrosis (gas gangrene) in humans (15) as well as in domestic animals (9). More recently, it has been recognized as a causal agent of diarrhea and enterotoxemia in cattle and sheep (2–4, 30). *C. sordellii* also was once suspected to be the causative agent of pseudomembranous colitis (PMC) in humans. This was because the cytotoxicity of stool filtrates from PMC patients was neutralized by *C. sordellii* antitoxin (1, 18, 36). However, researchers were unable to isolate *C. sordellii* from stool samples of patients with PMC. *Clostridium difficile* isolated from these patients was later shown to be the cause of PMC in humans (8, 13). The cross-neutralization of *C. difficile* toxins by *C. sordellii* antitoxin was a fortuitous cross-reaction that led to this discovery. Two toxins, A and B, have been purified from culture supernatants of toxigenic strains of *C. difficile* (7, 40, 41). Both toxins are cytotoxic to tissue-cultured mammalian cells, although toxin B is at least 1,000-fold more cytotoxic. Toxin A is an enterotoxin, causing fluid accumulation with hemorrhage in the small intestine and cecum of animals (19, 22, 26). Both of the toxins produced by *C. difficile* are neutralized by *C. sordellii* antitoxin. This suggests that *C. sordellii* produces toxins immunologically related to the toxins produced by *C. difficile* (1, 11, 35, 36).

The production of two toxins with distinctive biological activity by *C. sordellii* was first described by Arseculeratne et al. (5), who noted that intradermal injections of cell-free extracts into guinea pigs caused edema and hemorrhage. The edematous and hemorrhagic activities were shown to be independent, with the toxin which caused the edema (lethal toxin [toxin LT]) being more lethal than the hemorrhagic toxin (toxin HT). It has been reported that culture supernatants are toxic to cultured cells and induce a hemorrhagic response in rabbit ileal loops (28, 29, 43, 44). Toxin HT can be separated from the highly cytotoxic toxin (toxin LT) by ion-exchange chromatography (43). However, the relatedness of these two toxins to the toxins of *C. difficile* was not studied by these authors. Popoff recently purified toxin LT and showed that it is similar to toxin B of *C. difficile* (31).

We report here on the purification of *C. sordellii* toxin HT. Our findings show that this toxin has physicochemical characteristics and biological activities similar to those of *C. difficile* toxin A (enterotoxin).

MATERIALS AND METHODS

Protein determination. Protein concentrations were estimated by the method of Bradford (10) with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.). Bovine gamma globulin (Bio-Rad) was the standard.

Bacterial strains. *C. sordellii* VPI 9048 (Tox^+) and VPI 7319 (Tox^-) were obtained from the anaerobe collection of the Department of Anaerobic Microbiology at Virginia Polytechnic Institute and State University (Blacksburg) and were identified by L. V. Moore, E. P. Cato, and W. E. C. Moore by methods in the Virginia Polytechnic Institute Anaerobe Laboratory Manual (14). Cells were grown in 2-liter brain heart infusion dialysis flasks for 96 h at 37°C as described for the production of *C. difficile* toxins (40). This method was originally described by Sterne and Wentzel for the production of *Clostridium botulinum* toxin (38).

***C. difficile* antiserum and toxin A antibodies.** Rabbit and goat antisera against crude *C. difficile* VPI 10463 culture filtrate were produced as previously described (12). Affinity-purified goat antibody and PCG-4 mouse monoclonal antibody (PCG-4 MAb) were also prepared as previously described (24).

Purification of toxin HT by immunoaffinity chromatography. MAb to toxin A (PCG-4 MAb) was partially purified from ascites by 45% $(NH_4)_2SO_4$ precipitation and was coupled to Affi-Gel 10 (Bio-Rad) as recommended by the manufacturer. About 15 mg of protein was bound per ml of gel.

Culture filtrate from *C. sordellii* VPI 9048 (500 ml) was

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concentrated to 100 ml by ultrafiltration on an XM-300 membrane (Amicon Corp., Lexington, Mass.) and washed with two volumes of 0.05 M Tris hydrochloride buffer, pH 7.5, containing 0.15 M NaCl (TBS). The sample was passed through a column (2.5 by 20 cm) containing 5 ml of PCG-4 MAb-gel, and the gel was washed with 100 bed volumes of 0.05 M Tris hydrochloride buffer, pH 7.5, containing 0.50 M NaCl to remove the unbound material. The bound toxin was eluted with 1.5 bed volumes of 5 M $MgCl_2 \cdot 6H_2O$ (in TBS), dialyzed against TBS, and concentrated by ultrafiltration to 0.5 ml with a Centriprep-30 concentrator (Amicon Corp.). The concentrated toxin preparation was stored at 4°C.

Crossed immunoelectrophoresis and immunodiffusion analysis. Crossed immunoelectrophoresis was performed as described before (40). Ouchterlony double-immunodiffusion analysis was done as described previously (16).

Analytical isoelectric focusing. Analytical isoelectric focusing was performed in thin-layer isoelectric focusing polyacrylamide gels with the LKB 2117 Multiphor apparatus (LKB Instruments, Inc., Rockville, Md.) and commercial polyacrylamide gel plates (pH 3.5 to 9.5) as recommended by the manufacturer (Pharmacia, Inc., Piscataway, N.J.). Isoelectric point standards were purchased from Pharmacia, Inc.

PAGE. Nondenaturing polyacrylamide gel electrophoresis (PAGE) was done in two types of gels. Gradient PAGE was done as previously described (16). Molecular weight was estimated with a high-molecular-weight calibration kit (Pharmacia). PAGE was also done in a discontinuous gel system according to procedures described before (23). Sodium dodecyl sulfate (SDS)-PAGE was done according to the general procedures of Laemmli (17) as described before (23).

Temperature stability. Immunoaffinity-purified toxin HT (0.15 mg/ml) and culture filtrate (1.30 mg/ml) were stored at −20, 4, 20 to 25, and 37°C. After 72 h, samples were examined for loss of cytotoxicity and lethality. The purified toxin and the crude culture filtrate were also incubated at 56°C, and samples were taken at intervals (0, 5, 10, 15, 30, and 60 min) and examined for loss of cytotoxic and lethal activities.

pH stability. Purified toxin HT (0.75 mg/ml) was diluted 1:10 in the following pH buffer systems and incubated for 2 h at room temperature: 0.2 M glycine hydrochloride (pH 2.0), 0.05 M acetate (pH 4.0), 0.05 M Tris hydrochloride (pH 7.5), and 0.2 M glycine-sodium hydroxide (pH 10.0). The samples then were diluted 1:1 in 0.2 M Tris hydrochloride buffer (pH 7.5) and examined for loss of cytotoxic and lethal activities.

Immunoblotting. Samples from polyacrylamide gels were blotted onto nitrocellulose membranes by procedures described previously (23).

Production of *C. sordellii* antiserum. Toxoid was prepared by incubating crude culture filtrate from *C. sordellii* VPI 9048 in formaldehyde (0.1% final concentration) for 3 h at 37°C. New Zealand White female rabbits (Hazleton Research Animals, Denver, Pa.) were injected subcutaneously each week with 2.5 ml of formalinized culture filtrate mixed with an equal volume of Freund incomplete adjuvant (Sigma Chemical Co., St. Louis, Mo.). Antitoxin was detected after injection 6 and reached maximum titers, as determined by crossed immunoelectrophoresis with crude culture filtrate in the first dimension, 24 weeks after the start of injections. At that time, the rabbit was bled out by cardiac puncture.

ELISA. An indirect enzyme-linked immunosorbent assay (ELISA) for the detection of toxin HT was done as previously described for the detection of toxin A of *C. difficile* (27). Affinity-purified goat antibodies or mouse ascites containing MAb PCG-4 to toxin A were used as the detecting antibody. The amount of toxin HT present in the culture filtrate was determined by comparison to a standard curve with known concentrations of homogeneous toxin HT.

Biological assays. (i) Cytotoxicity assay. The cytotoxicity assay was determined as described previously (6). The tissue culture dose (TCD_{100}) was defined as the reciprocal of the highest dilution which rounded 100% of the CHO-K1 cells.

Neutralization of toxin HT with *C. difficile* toxin A antibodies was performed as previously described (20). The neutralization titer was expressed as the reciprocal of the dilution which completely inhibited the rounding of all cells.

(ii) Lethality assay. Lethality in mice was determined as previously described (40). The LD_{100} was defined as the highest dilution of toxin which killed 100% of the mice within 36 h.

Neutralization was determined by mixing twofold dilutions of the antitoxins with twofold LD_{100} doses of the toxin. After incubation at room temperature for 1 h, 0.5 ml of each mixture was administered to mice by intraperitoneal injection. The titer of each antitoxin was defined as the reciprocal of the highest dilution of antitoxin at which all mice survived the LD_{100}.

(iii) Ligated intestinal loops. Ligated intestinal loops were prepared as described by Lyerly et al. (22). *C. difficile* toxin A (10 μg) served as a positive control. Ratios greater than 1 were considered positive. For the neutralization assay, 100 μg of toxin HT or 0.5 ml of *C. sordellii* 9048 culture filtrate (1:2.5 dilution in phosphate-buffered saline) was mixed with an equal volume of undiluted MAb PCG-4 (ascites) and incubated for 30 min at room temperature. After incubation, 1-ml samples of each mixture were injected into ileal loops. The enterotoxic response was determined as described above.

RESULTS

Purification of toxin HT. Toxin HT was specifically and quantitatively removed from the concentrated culture filtrate by the MAb to toxin A of *C. difficile* coupled to Affi-Gel 10 (Fig. 1). The toxin was eluted with 5 M $MgCl_2 \cdot 6H_2O$ and showed a single immunoprecipitin arc by crossed immunoelectrophoresis against antiserum to *C. sordellii* culture filtrate (Fig. 1c). Table 1 shows the percent recovery of toxin HT as determined by indirect ELISA.

We also attempted to remove the toxin HT from the crude culture filtrate by using bovine thyroglobulin immobilized on Affi-Gel 15 as described previously for the purification of *C. difficile* toxin A (16). Toxin HT did bind to the receptor column and could be eluted at 37°C; however, the yield was very low and the toxin was contaminated with degraded thyroglobulin (data not shown).

Characterization of toxin HT. The preparation of toxin HT which eluted from the PCG-4 MAb column showed one major band and one minor band on gradient PAGE. The major band had an estimated native molecular weight of 525,000, whereas the molecular weight of the minor band was 450,000 (Fig. 2). The toxin HT preparation migrated on SDS-PAGE as one major band with an estimated molecular weight of 300,000 and several faster-migrating bands (Fig. 3). By isoelectric focusing, the purified toxin preparation focused as a single band at pH 6.10 (Fig. 4).

To identify toxin HT in the crude culture filtrate, immunoblots were made with culture filtrate from a toxigenic *C. sordellii* strain (VPI 9048). Immunoblots were also done with

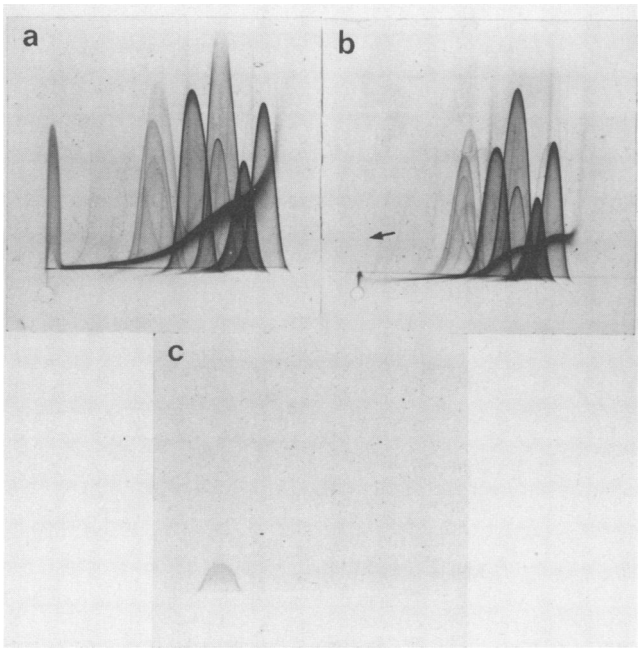

FIG. 1. Analysis by crossed immunoelectrophoresis of toxin HT purified by immunoaffinity chromatography on MAb PCG-4–Affi-Gel 10. Culture filtrate from *C. sordellii* VPI 9048 was adsorbed with the gel, and the bound toxin was eluted from the gel. (a) Culture filtrate before immunoadsorption (80 μg). (b) Culture filtrate after immunoadsorption (75 μg). The arrow indicates the disappearance of the toxin HT immunoprecipitin arc, demonstrating the removal of the toxin from culture filtrate. (c) Toxin HT (1.5 μg) eluted from the gel with 5 M $MgCl_2 \cdot 6H_2O$ in 0.05 M Tris hydrochloride–0.15 M NaCl, pH 7.5. The upper portion of each plate contained 250 μl of rabbit antiserum against culture filtrate from *C. sordellii* VPI 9048.

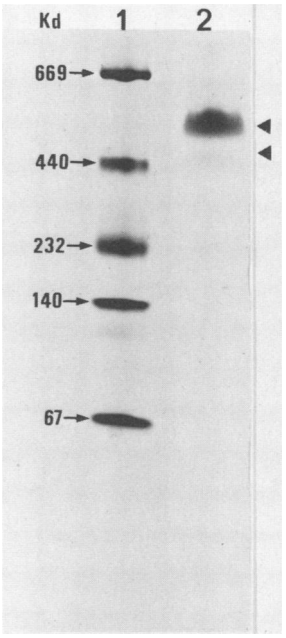

FIG. 2. Gradient (4 to 30%) PAGE of purified toxin HT. Lane 1, molecular size standards (Pharmacia); lane 2, purified toxin HT (25 μg). Arrows in lane 2 point to the major and minor bands. Kd, Kilodaltons.

culture filtrate of a nontoxigenic strain (VPI 7319) and with affinity-purified toxin HT. Under nondenaturing conditions, MAb PCG-4 reacted with both bands in the toxin HT preparation and in the culture filtrate of the toxigenic strain (Fig. 3, lanes 1 and 2). The MAb did not react with any antigens in the culture filtrate from the nontoxigenic strain. Affinity-purified antibodies to toxin A also reacted strongly with both bands in the toxin HT preparation (data not shown). The denatured toxin observed by SDS-PAGE was analyzed by immunoblotting to determine whether the minor bands were reactive with the MAb (PCG-4) and affinity-purified antibodies to toxin A. Each of the antibodies to toxin A reacted with the major band and also with the minor bands (Fig. 3).

Immunodiffusion analysis was performed to examine the relationship between toxins HT and A of *C. difficile*. Affinity-purified antibodies to toxin A reacted with toxin HT and formed a precipitin line that showed partial identity with the *C. difficile* toxin (Fig. 5).

TABLE 1. Purification of *C. sordellii* toxin HT

Purification step	Vol (ml)	Protein		% Recovery[a]
		mg/ml	Total mg	
Culture filtrate	500	1.30	650	100
Ultrafiltration	100	2.50	250	33
Concentrated affinity column eluate	0.5	0.70	0.35	8.5

[a] Percent of toxin HT recovered from starting material found in culture filtrate as determined by ELISA.

An indirect ELISA procedure was used to study the reaction of toxin A antibodies with toxin HT. The response observed with MAb PCG-4 as the detecting antibody is shown in Fig. 6. When equal concentrations of toxins HT and A were tested in the ELISA, the titers of toxin HT were lower than those obtained with toxin A. The results observed with the affinity-purified goat antibody to toxin A were similar to those seen with MAb PCG-4 (data not shown).

Stability of toxin HT at different temperatures and pHs. Toxin preparations were stable from −20 and 37°C. Purified toxin HT and culture filtrate were completely inactivated after incubation for 5 min at 56°C. Toxin HT was stable at pH 4.0 to 10.0 and completely inactivated at pH 2.0.

Biological activity. The biological activities of toxin HT are listed in Table 2. Purified HT was cytotoxic and caused the same type of cell-rounding response in CHO-K1 cells as toxin A of *C. difficile*. The specific activity of purified toxin HT in the tissue culture assay was 2.0×10^3/mg, whereas the specific activity of the culture filtrate was 1.0×10^3/mg. The specific activity in the mouse lethality assay was 8.3×10^3/mg, and the specific activity of the culture filtrate was 3.2×10^4/mg (because of the presence of toxin LT).

Injection of purified toxin HT in rabbit intestinal loops elicited an accumulation of a viscous hemorrhagic fluid (Table 3). Culture filtrate from the toxigenic *C. sordellii* strain also elicited a similar response in the intestinal loop assay. No accumulation of fluid or hemorrhage was observed in the intestinal loops injected with culture filtrate from the nontoxigenic strain.

Affinity-purified antibody to toxin A neutralized the cytotoxicity and lethality for mice (antibody titers of 128 and 1,024, respectively) of toxin HT. These results indicate that toxin LT was completely removed from the toxin HT preparation, since affinity-purified antibody to toxin A does

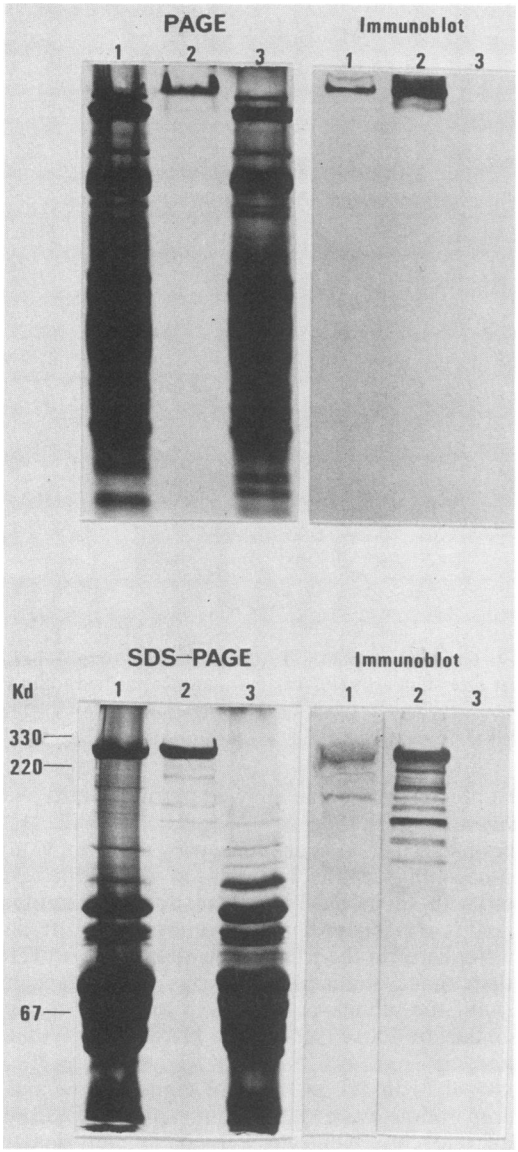

FIG. 3. Analysis by PAGE-SDS-PAGE, and immunoblots of toxigenic *C. sordellii* VPI 9048 culture filtrate (250 μg) (lanes 1), purified toxin HT (8 μg) (lanes 2), and nontoxigenic *C. sordellii* VPI 7319 culture filtrate (250 μg) (lanes 3). PAGE and SDS-PAGE were performed in 4% stacking–7.5% running gels. After electrophoresis, the proteins were either stained with Coomassie brilliant blue R-250 or blotted onto nitrocellulose for immunoblot analysis with an MAb specific to toxin A (MAb PCG-4). Molecular size markers appear to the left of lower panels. Kd, Kilodaltons.

not neutralize the cytotoxic and lethal activities of toxin LT. Affinity-purified antibody to toxin A also neutralized the enterotoxic activity of toxin HT. MAb PCG-4 did not neutralize the cytotoxicity or lethality of toxin HT but did neutralize the enterotoxicity (Table 3).

DISCUSSION

This study represents the first description of a purification scheme for toxin HT from *C. sordellii* and the first direct comparison of this toxin with toxin A of *C. difficile*. We purified toxin HT from culture filtrate of *C. sordellii*, using a single immunoaffinity step involving an MAb against toxin

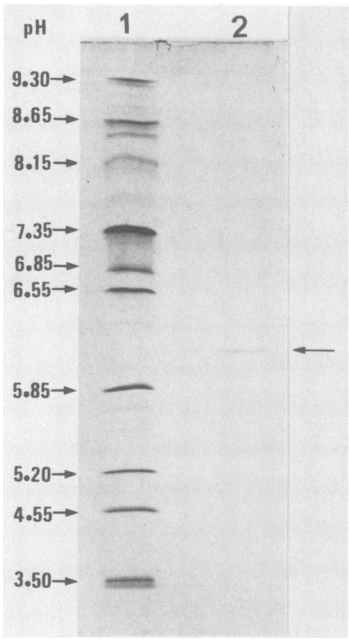

FIG. 4. Estimation of toxin HT pI by analytical thin-layer isoelectric focusing in polyacrylamide gel (LKB). Lane 1, pI markers (Pharmacia); lane 2, toxin HT (2 μg).

A. This antibody binds with less avidity to toxin HT than to toxin A, which accounts for the differences in elution of the toxins from the PCG-4 MAb column. Toxin HT readily eluted from the MAb-gel with $MgCl_2$, whereas toxin A requires harsher conditions such as pH extremes (23). The purified toxin HT was homogeneous by crossed immunoelectrophoresis and analytical isoelectric focusing. This preparation had a major and a minor band when analyzed by analytical PAGE under nondenaturing conditions. This minor band reacted with toxin A antibodies and was detected in the culture filtrate. Thus, it is not a contaminant and does not result from the purification procedure.

Toxins HT and A exhibit a number of similarities. Both toxins have estimated molecular weights in excess of 300,000 as determined by SDS-PAGE. The very large size of toxins HT and A is unusual, and this has led many investigators to

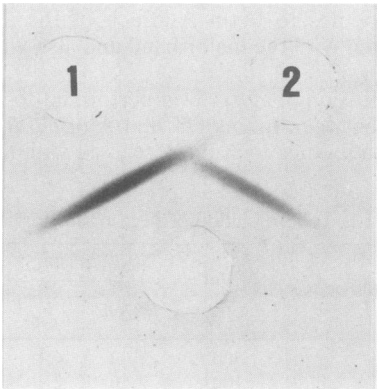

FIG. 5. Immunodiffusion analysis of toxins A and HT. Center well contained 20 μl of affinity-purified antibodies to toxin A. Well 1 contained 20 μg of toxin A; well 2 contained 20 μg of affinity-purified toxin HT.

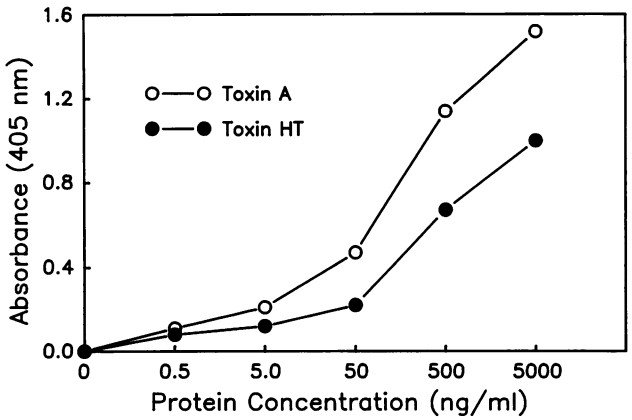

FIG. 6. Comparative quantitation of toxins A and HT in the indirect ELISA, using antibodies to *C. difficile* toxin A. The detecting antibody consisted of a 1/1,000 dilution of an MAb specific to toxin A (MAb PCG-4).

suggest that the toxins have subunit structures. The high molecular weight of toxin A is supported, however, by the finding that its gene has an open reading frame larger than 7.1 kilobases (J. L. Johnson, personal communication), which means that the resulting polypeptide is larger than 290,000 daltons.

Several faster-migrating bands are observed when toxins HT and A are subjected to denaturing conditions and analyzed by SDS-PAGE (23). These minor bands react with both MAbs and affinity-purified antibodies to toxin A in immunoblots. The appearance of these bands is not inhibited by protease inhibitors (R. D. Martinez and T. D. Wilkins, unpublished results), but it is still unclear whether they are the product of proteolysis. The fact that this occurs with both toxin HT and toxin A indicates that this is a common characteristic of these toxins.

Most strains of *C. sordellii* produce low amounts of toxins, and this makes purification by classical techniques extremely difficult. *C. sordellii* 3703 has been reported to produce large amounts of these toxins (28, 29, 43, 44), but this strain was unavailable to us for comparison.

We attempted to purify toxin HT by use of the receptor-affinity column procedure which has been used recently for the purification of toxin A (16). In this procedure, toxin A binds to the trisaccharide Galα1-3Galβ1-4GlcNAc on bovine thyroglobulin at 4°C and then is eluted by increasing the temperature to 37°C. When toxin HT was purified in this manner, the resulting preparation was contaminated with thyroglobulin. *C. sordellii* is considerably more proteolytic than *C. difficile* (14), and we suspect that the thyroglobulin present in the toxin HT preparation resulted from proteolytic cleavage of immobilized thyroglobulin. However, this work and the work by Price et al. (32) indicate that the two toxins may have similar receptors.

Affinity-purified toxin HT retained its biological activities. These activities are similar to those observed with toxin A (25, 40, 41) and included cytotoxicity (cell rounding), lethality, and enterotoxicity. The cytotoxic activity of toxin HT is significantly less than the cytotoxicity of toxin A. This may be due to partial inactivation of the toxin during the elution from the antibody column with $MgCl_2$, since toxin A purified by immunoaffinity chromatography also loses a significant amount of cytotoxic activity (23). However, the lethal activity of the affinity-purified toxin HT is comparable to that of toxin A. We could not follow the specific activity of the cytotoxicity or lethality through the purification scheme because the other *C. sordellii* toxin (toxin LT) has these activities and masks the action of toxin HT in such assays (31). Toxin HT elicited a positive response (with hemorrhage, mucus, and fluid accumulation) in the rabbit ileal loop assay that is identical to the type of reaction caused by toxin A (22). The hemorrhagic fluid response induced by toxins HT and A appears to be a common mode of action for both toxins and is not observed with cholera toxin and the heat-labile enterotoxin of *Escherichia coli*, which cause the accumulation of a watery fluid (34). The purified toxin HT appears to have lost more than 90% of its enterotoxic activity; we base this on the amount of toxin HT detected by

TABLE 3. Response in rabbit intestinal loops

Sample	Vol/length ratio[a]
C. sordellii VPI 9048 culture filtrate (1/10 dilution)	1.48 ± 0.40
C. sordellii VPI 9048 culture filtrate + MAb PCG-4	0.02 ± 0.01[b]
C. sordellii VPI 7319 culture filtrate	0.02 ± 0.01
Toxin HT (100 μg)	1.06 ± 0.18
Toxin HT (100 μg) + MAb PCG-4	0.02 ± 0.01[b]
Toxin A (10 μg)	1.81 ± 0.22

[a] The volume/length ratios were determined 16 h after injection and are expressed as the mean ± 1 standard error for four test loops injected per sample.

[b] Culture filtrate (1/2.5 dilution) or toxin HT was mixed with undiluted PCG-4 ascites. Ascites against another clostridial species did not have any effect on the enterotoxic activity of the culture filtrate from the toxigenic strain of *C. sordellii* or toxin HT.

TABLE 2. Comparison of toxins HT and A

Property	Toxin HT	Toxin A[a]
Native molecular weight[b]	525,000	550,000
Denatured molecular weight	300,000	300,000
Isoelectric point	6.10	5.2–5.7
Temperature stability	Stable at −20 to 37°C, inactivated at 56°C	Stable at −20 to 37°C, inactivated at 56°C
pH stability	Stable at pH 4.0 to 10.0	Stable at pH 4.0 to 10.0
TCD_{100}	500 ng	10 ng
LD_{100}	120 ng	90 ng
Enterotoxic dose[c]	100 μg (actual); 2 μg (corrected)	1 μg

[a] Toxin A was purified by sequential ammonium sulfate precipitation, ion exchange chromatography, and precipitation at pH 5.6 (21, 25, 40).

[b] A minor band with an estimated molecular weight of 450,000 was also observed in the toxin HT preparation.

[c] Minimum dose which induced a positive response in rabbit ileal loops. The corrected dose is based on the amount of toxin HT in the culture filtrate as determined by ELISA.

ELISA in the culture filtrate and comparison of its enterotoxicity to that of purified toxin HT. If this loss were taken into account, the specific activity of toxin HT would be similar to that of toxin A.

Antibodies to toxin A did not significantly neutralize the cytotoxic or lethal activities of the *C. sordellii* culture filtrate. This indicates that toxin LT is a more potent toxin, accounting for most of the cytotoxic and lethal activities present in the culture filtrate. This situation is analogous to that seen with culture filtrates of *C. difficile* treated with toxin A antibodies (20). In that instance, neutralization of toxin A does not neutralize the cytotoxic or lethal effects due to toxin B. On the other hand, the MAb (PCG-4) to toxin A did completely neutralize the enterotoxicity of the *C. sordellii* culture filtrate; this indicates that toxin HT is responsible for all the enterotoxicity, just as toxin A is responsible for all of the enterotoxicity of the *C. difficile* culture filtrates (26).

Previous investigators have documented the fact that immunological cross-reactivity exists between the toxins produced by *C. difficile* and by *C. sordellii* (1, 11, 33, 36). Antisera against either of the toxic culture filtrates neutralize the action of toxins present in the culture filtrate of the other organism, although usually in a less potent manner, suggesting that this is due to a lack of serological specificity (6, 12, 42). Analysis by immunodiffusion revealed a reaction of partial identity between toxin A and toxin HT. This supports the presence of common antigenic determinants between the two toxins. The antigenic differences between the toxins also were evident from the ELISA results showing that the toxin A antibodies did not react as strongly with toxin HT as with toxin A, indicating a lower binding affinity of the toxin A antibodies to cross-reactive epitopes present on toxin HT.

The production of similar toxins by different species and seven different genera of bacteria is not unusual. The oxygen-labile hemolysins, for example, are closely related proteins that are produced by members of the *Bacillus, Clostridium, Listeria,* and *Streptococcus* genera (37). This type of phenomenon is also seen with clostridial species; *Clostridium perfringens* and *Clostridium spiroforme* produce binary toxins that share many immunochemical and physiochemical features (39). Comparison of closely related toxins produced by different organisms can yield important information, since the portions of the molecules which are most conserved are normally the active and binding sites. Our future aim is to compare the nucleotide sequence of the genes coding for the toxins of *C. sordellii* to those of *C. difficile* so that the conserved areas can be identified and studied in detail. We are also interested in any differences in biological activity that may have occurred because of genetic changes.

In conclusion, our work and the work of Popoff (31) show that toxins HT and LT of *C. sordellii* correspond to toxins A and B of *C. difficile*. Although the toxins are not identical, they have retained remarkable immunological similarities, as well as physiochemical and biological properties.

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LITERATURE CITED

1. **Allo, M., J. Silva, R. Fekety, G. D. Rifkin, and H. Waskin.** 1979. Prevention of clindamycin-induced colitis in hamsters by *Clostridium sordellii* antitoxin. Gastroenterology **76**:351–355.
2. **Al Mashat, R. R., and D. J. Taylor.** 1983. Production of diarrhoea and enteric lesions in calves by the oral inoculation of pure cultures of *Clostridium sordellii*. Vet. Rec. **112**:141–146.
3. **Al Mashat, R. R., and D. J. Taylor.** 1983. *Clostridium sordellii* in enteritis in an adult sheep. Vet. Rec. **112**:19.
4. **Al Mashat, R. R., and D. J. Taylor.** 1983. Bacteria in enteric lesions of cattle. Vet. Rec. **112**:5–10.
5. **Arseculeratne, S. N., R. G. Panabokke, and S. Wijesundera.** 1969. The toxins responsible for the lesions of *Clostridium sordellii* gas gangrene. J. Med. Microbiol. **2**:37–53.
6. **Aswell, J. E., M. Ehrich, R. L. Van Tassell, C.-C. Tsai, L. V. Holdeman, and T. D. Wilkins.** 1979. Characterization and comparison of *Clostridium difficile* and other clostridial toxins, p. 272–275. *In* D. Schlessinger (ed.), Microbiology—1979. American Society for Microbiology, Washington, D.C.
7. **Banno, Y., T. Kobayashi, H. Kono, K. Watanabe, K. Ueno, and Y. Nozawa.** 1981. Two toxins (D-1 and D-2) of *Clostridium difficile* causing antibiotic-associated colitis: purification and some characterization. Biochem. Int. **2**:629–635.
8. **Bartlett, J. G., T.-W. Chang, M. Gurwith, S. L. Gorbach, and A. B. Onderdonk.** 1978. Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. N. Engl. J. Med. **298**:531–534.
9. **Blood, D. C., J. A. Henderson, and O. M. Radostits.** 1979. Veterinary medicine, p. 445–447. Balliere Tindal, London.
10. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
11. **Chang, T.-W., S. L. Gorbach, and J. G. Bartlett.** 1978. Neutralization of *Clostridium difficile* toxin by *Clostridium sordellii* antitoxins. Infect. Immun. **22**:418–422.
12. **Ehrich, M., R. L. Van Tassell, J. M. Libby, and T. D. Wilkins.** 1980. Production of *Clostridium difficile* antitoxin. Infect. Immun. **28**:1041–1043.
13. **George, R. H., J. M. Symonds, F. Dimock, J. D. Brown, Y. Arabi, N. Shinagawa, M. R. B. Keighley, J. Alexander-Williams, and D. W. Burdon.** 1978. Identification of *Clostridium difficile* as a cause of pseudomembranous colitis. Br. Med. J. **1**:695.
14. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
15. **Joklik, W. K., H. P. Willet, and D. B. Amos (ed.).** 1984. Zinsser microbiology, p. 698–707. Appleton-Century-Crofts, Norwalk, Conn.
16. **Krivan, H. C., and T. D. Wilkins.** 1987. Purification of *Clostridium difficile* toxin A by affinity chromatography on immobilized thyroglobulin. Infect. Immun. **55**:1873–1877.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Larson, H. E., and A. B. Price.** 1977. Pseudomembranous colitis: presence of clostridial toxin. Lancet **ii**:1312–1314.
19. **Libby, J. M., B. S. Jortner, and T. D. Wilkins.** 1982. Effects of the two toxins of *Clostridium difficile* in antibiotic-associated cecitis in hamsters. Infect. Immun. **36**:822–829.
20. **Libby, J. M., and T. D. Wilkins.** 1982. Production of antitoxins to two toxins of *Clostridium difficile* and immunological comparison of the toxins by cross-neutralization studies. Infect. Immun. **35**:374–376.
21. **Lyerly, D. M., H. C. Krivan, and T. D. Wilkins.** 1988. *Clostridium difficile*: its disease and toxins. Clin. Microbiol. Rev. **1**:1–18.
22. **Lyerly, D. M., D. E. Lockwood, S. H. Richardson, and T. D. Wilkins.** 1982. Biological activities of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**:1147–1150.
23. **Lyerly, D. M., C. J. Phelps, J. Toth, and T. D. Wilkins.** 1986. Characterization of toxins A and B of *Clostridium difficile* with monoclonal antibodies. Infect. Immun. **54**:70–76.
24. **Lyerly, D. M., C. J. Phelps, and T. D. Wilkins.** 1984. Monoclonal and specific polyclonal antibodies for immunoassay of *Clostridium difficile* toxin A. J. Clin. Microbiol. **21**:12–14.
25. **Lyerly, D. M., M. D. Roberts, C. J. Phelps, and T. D. Wilkins.**

1986. Purification and properties of toxins A and B of *Clostridium difficile*. FEMS Microbiol. Lett. **33**:31–35.

26. **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.
27. **Lyerly, D. M., N. M. Sullivan, and T. D. Wilkins.** 1983. Enzyme-lynked immunosorbent assay for *Clostridium difficile* toxin A. J. Clin. Microbiol. **17**:72–78.
28. **Nakamura, S., H. Ogura, J. Tanaka, N. Tanabe, K. Hamakawa, M. Hatano, and S. Nishida.** 1984. Difference in susceptibility of various cell cultures to cytotoxic culture filtrates of *Clostridium sordellii*. Microbiol. Immunol. **28**:493–497.
29. **Nakamura, S., N. Tanabe, K. Yamakawa, and S. Nishida.** 1983. Cytotoxin production by *Clostridium sordellii* strains. Microbiol. Immunol. **27**:495–502.
30. **Popoff, M. R.** 1984. Bacteriological examination in enterotoxaemia of sheep and lamb. Vet. Rec. **114**:324.
31. **Popoff, M. R.** 1987. Purification and characterization of *Clostridium sordellii* lethal toxin and cross-reactivity with *Clostridium difficile* cytotoxin. Infect. Immun. **55**:35–43.
32. **Price, S. B., C. J. Phelps, T. D. Wilkins, and J. J. Johnson.** 1987. Cloning of the carbohydrate-binding portion of the toxin A gene of *Clostridium difficile*. Curr. Microbiol. **16**:55–60.
33. **Rehg, J. E.** 1980. Cecal toxin(s) from guinea pigs with clindamycin-associated colitis, neutralized by *Clostridium sordellii* antitoxin. Infect. Immun. **27**:387–390.
34. **Richards, K. L., and S. D. Douglas.** 1978. Pathophysiological effects of *Vibrio cholerae* and entertoxigenic *Escherichia coli* and their exotoxins on eucaryotic cells. Microbiol. Rev. **42**:592–613.
35. **Rifkin, G. D., F. R. Fekety, and J. Silva.** 1978. Neutralization by *Clostridium sordellii* antitoxin of toxins implicated in clindamycin-induced cecitis in the hamster. Gastroenterology **75**:422–424.
36. **Rifkin, G. D., F. R. Fekety, J. Silva, and R. B. Sack.** 1977. Antibiotic-induced colitis. Implication of a toxin neutralised by *Clostridium sordellii* antitoxin. Lancet **ii**:1103–1106.
37. **Stephen, J., and R. A. Pietrowski.** 1986. Bacterial toxins, 2nd ed., p. 74–77. American Society for Microbiology, Washington, D.C.
38. **Sterne, M., and L. M. Wentzel.** 1950. A new method for the large scale production of high-titre botulinum formol-toxoid types C and D. J. Immun. **35**:1032–1040.
39. **Stiles, B. G., and T. D. Wilkins.** 1986. *Clostridium perfringens* iota toxi ı: synergism between two proteins. Toxicon **24**:767–773.
40. **Sullivan, N. M., S. Pellett, and T. D. Wilkins.** 1982. Purification and characterization of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**:1032–1040.
41. **Taylor, N. S., G. M. Thorne, and J. G. Barlett.** 1981. Comparison of two toxins produced by *Clostridium difficile*. Infect. Immun. **34**:1036–1043.
42. **Wilkins, T., M. Ehrich, R. Van Tassell, and E. Balish.** 1980. Studies on the clostridial toxin associated with antibiotic-associated pseudomembranous colitis, p. 411–417. *In* D. Eaker and T. Wadstrom (ed.), Natural toxins. Pergamon Press, Inc., Elmsford, N.Y.
43. **Yamakawa, K., S. Nakamura, and S. Nishida.** 1985. Separation of two cytotoxins of *Clostridium sordellii* strains. Microbiol. Immunol. **29**:553–557.
44. **Yamakawa, K., N. Tanabe, Y. Okada, S. Nishida, and S. Nakamura.** 1983. Rabbit ileal loop responses to *Clostridium sordellii* strains. Microbiol. Immunol. **27**:807–809.