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Enterotoxin Formation by Different Toxigenic Types of *Clostridium perfringens*

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Sixty-nine strains of *Clostridium perfringens* of different toxigenic types were investigated for enterotoxin production. Enterotoxin was definitively detected only in strains of types A and C. This is the first report where enterotoxin production has been demonstrated in a toxigenic type other than type A. The enterotoxin-positive type C strains were isolated from cases of enteritis necroticans ("pig bel") in New Guinea. The major enterotoxin from type C showed a reaction of complete identity with enterotoxin from type A in immunodiffusion using anti-enterotoxin serum prepared against the latter; it induced erythema when injected intradermally into depilated guinea pigs and caused fluid accumulation in the rabbit ileal loop. The results indicate that the major enterotoxin from type C was serologically and biologically similar to enterotoxin from type A. In some type C strains, an enterotoxin was detected that showed a reaction of partial serological identity. Spore coat proteins were extracted from 14 strains by alkaline dithiothreitol, and the extracts were assayed for enterotoxin-like spore protein. Enterotoxin could be extracted from type A and type C spores, and all positive strains showed a reaction of complete identity in immunodiffusion with enterotoxin obtained from cell extracts of type A. Disc immunoelectrophoresis demonstrated that two distinct components that reacted serologically with anti-enterotoxin serum were present in both the cell extract and in extracted spore protein from one type C strain. These distinct components differed in molecular weight.

Clostridium perfringens produces a multiplicity of toxins and, according to the kinds and amount of toxin formed, the species is subdivided into five toxigenic types, A to E. This classification is based on the ability to produce the four major toxins, alpha-, beta-, epsilon-, and iota-toxin as assayed by lethality test in mice or skin test in guinea pigs. Only certain strains of types A and C have been implicated in food-borne diseases in humans (14).

C. perfringens type A is one of the most common causes of human food poisoning (3). The course of the intoxication is usually mild, and fatal cases are relatively rare.

Since the first reports of *C. perfringens* type A food poisoning occurred (17, 19), a considerable amount of information has accumulated and progress in understanding the nature of this type of food-borne disease has been made in recent years. An enterotoxin has been isolated and purified, and the toxin has been characterized biologically, serologically, and chemically (13, 24, 25). Enterotoxin-positive strains of *C. perfringens* type A often are characterized by feebleness in the production of alpha-toxin and other soluble antigens (29), and the enterotoxin is the only active factor that has been shown to be responsible for the symptoms of the illness.

In contrast to the mild symptoms of *C. perfringens* type A food poisoning, the illness caused by *C. perfringens* type C is a severe disease accompanied by a grave hemorrhagic enteritis resulting in necrosis of the intestinal mucosa (enteritis necroticans).

In outbreaks of this kind, the mortality has been reported to be 35 to 40% despite intense medical and surgical treatment (20). The most comprehensive investigations of outbreaks of the severe form are reported from Germany (31), where the disease was referred to as "Darmbrand," and from the New Guinea highlands (21) referred to as "pig bel." In these illnesses the beta-toxin is reported to be primarily responsible for the symptoms and the pathological changes in the intestine.

Enterotoxin from *C. perfringens* has only been demonstrated in sporulating cultures, and the toxin can be detected intracellularly about 3 h after the inoculation of actively growing vegetative cells into a sporulation medium (4). However, not all sporulating strains produce detectable levels of enterotoxin. Recently

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Frieben and Duncan presented data indicating that the enterotoxin of *C. perfringens* type A is a structural component of the spore coat (12). They showed that an enterotoxin-like protein, serologically and biologically active, could be extracted from the spore coat by reducing agents.

Previously, little effort has been made to elucidate the occurrence and importance of *C. perfringens* enterotoxin in other toxigenic types than type A. This investigation was undertaken to obtain more information about the production of enterotoxin and enterotoxin-like spore coat protein in different toxigenic types.

MATERIALS AND METHODS

Cultures. Strains of *C. perfringens* used in this study included all the toxigenic types. Thirty-seven strains were made available by L. S. McClung, Indiana University, Bloomington, Ind. Eight strains, 5381 through 5388, isolated from pig bel cases in New Guinea were kindly provided by P. D. Walker, Wellcome Research Laboratories, Beckenham, England. Detailed information about these strains is given in Table 1. Four strains of type D, three type B, and one type E were obtained through the courtesy of L. D. S. Smith, Virginia Polytechnic Institute and State University, Blackburg, Va. The National Center for Disease Control, Atlanta, Ga., provided one strain of type B, five type C, two type D, and one type E. Organisms obtained in pure culture by single-colony isolation were typed according to the method of Oakley and Warrack (22). *C. perfringens* diagnostic serum was obtained from Wellcome Laboratories, Beckenham, England. Working stock cultures were maintained in cooked meat medium (Difco) and kept at room temperature.

Growth and sporulation condition. Fluid thioglycolate medium (BBL) and DS medium (8) were employed throughout the study to grow inocula and for sporulating cultures, respectively. Stock cultures from each strain (0.5 ml) were inoculated into 10 ml of fluid thioglycolate medium and heat shocked in a water bath at 75 C for 20 min followed by incubation at 37 C for 4 to 5 h. The cultures, if viable, were then transferred to new tubes of fluid thioglycolate medium and incubated at 37 C for 12 to 15 h. These actively growing cultures were inoculated (1% by volume of final sporulation medium) into DS sporulation medium and incubated for 7 to 10 h before harvesting.

Phase contrast microscopy. Samples were removed at various time intervals and examined by phase contrast microscopy for growth, sporulation, and formation of paracrystalline inclusions (5). For phase contrast photomicrography, cells were immobilized on a thin 1% agar layer on a glass slide and covered with a glass cover slip. The preparations were photographed with a Zeiss phase contrast microscope mounted with a Polaroid 545 Land film holder.

Enumeration of spores. Just before harvesting cells from the sporulation medium, a 10-ml sample was taken from each culture and heat shocked as described above. Viable counts of those heat-treated spores were made by means of pour plates using TSN agar without antibiotics (6) and 0.1% peptone water

TABLE 1. *Strains of C. perfringens of different origin*

Strain	Type	Comments
690	A	Isolated from chicken croquette—marked symptoms of food poisoning in volunteer.
1158	A	Isolated from food poisoning outbreak.
1168	A	Isolated from stool specimen from a person with diarrhea and abdominal pains after a dinner party.
2110 A	A	Received from hygiene laboratory as Hobbs type 2.
2117	A	Isolated from meat rissole—food poisoning.
2158	A	Isolated from stool specimen.
2159 A	A	Isolated from shrimp.
684	A	Isolated from raw chicken blood.
686	A	Isolated from chicken intestinal content.
688	A	Isolated from raw chicken meat.
1136	A	Isolated from a wound.
1171	A	Isolated from a wound.
1302	A	Isolated from cat feces.
1309	A	Isolated from gas gangrene.
1310	A	Isolated from gas gangrene.
146	A	Received from National Institutes of Health.
666	C	Received from I.C. Hall, England.
1015	A	Isolated from aborted equine fetus.
1205	A	Received from B. Hobbs, England.
1202	A	Isolated from vaccine virus.
1223	A	Isolated from leg biopsy.
1313	A	Received from G. Willish, Germany.
1331	C	Isolated from mouse feces.
1340	A	Isolated from pickled herring.
1423	C	Isolated from calves with hemorrhagic enteritis.
1434	A(C)[a]	Received from Wellcome Labs, England.
1485	A(D)[a]	Received from A.W. Rodwell.
1521	A	Isolated from sewage.
1581	C	Received from E.E. Schmidt, Australia.
2172	A	Isolated from Korean soil.
2174	B	Isolated from a case of lamb dysenteria.
2272	A	Isolated from intestine diseased rabbit.
2825	A	Isolated from cooked shrimp, food poisoning.
2831	A	Isolated from cooked salami.
3032	A	Received from B. Hobbs, England.
3344	A	Isolated from turkey gravy involved in food poisoning.

[a] In our laboratory, these strains were typed as toxigenic type A. The original type designation is given in parenthesis. All strains were obtained from L.S. McClung.

as dilution blanks. All dilutions were plated in duplicate and the results were averaged. Incubation was in anaerobic jars at 37 C for 24 h.

Cell extracts. After 7 to 10 h of incubation in DS medium, cells were harvested by centrifugation at 4 C and sonically disrupted as previously described (25). The sonically treated suspension was centrifuged at 12,000 × *g* for 15 min and the supernatant fluid was sterilized by using membrane filters (Gelman Instrument Co., Ann Arbor, Mich.). The sterile fluid was saved for use in different assay methods for *C. perfringens* enterotoxin. If enterotoxin was not detected from these preparations, cell extracts were concentrated 5 to 10 times by dialysis against polyethyleneglycol or by lyophilization. Protein was determined by the method of Lowry et al. (18), with bovine serum albumin as a standard.

Washing of spores and extraction of spore coat proteins. The pellets from cell extract preparations were saved and used as a source of spores. The pellets were subjected to further intervals of ultrasonic treatment until all vegetative cells were disrupted and then were washed 10 times at 4 C in distilled water. Spores produced by this method were free from sporangial remnants as determined by phase contrast microscopy. The clean spores were lyophilized and stored at −20 C.

For spore coat extraction, the spores were suspended at a concentration of 15 mg (dry weight) per ml in 0.05 M dithiothreitol in 0.025 M NaOH-glycine buffer at pH 10 as previously described (12). After incubation at 37 C for 2 h, the suspensions were centrifuged at 15,000 × *g* and 4 C for 15 min. The supernatants were dialyzed against 0.0125 M barbital buffer, pH 8.6, at 5 C for 20 h.

Neutralization of biological activity. Cell extracts and spore coat extracts at various concentrations in 0.85% NaCl were mixed 1:1 with immune sera of various dilutions. *C. perfringens* diagnostic antisera A, B, C, D, and E were diluted 1:4 before mixing 1:1 with various concentrations of the samples. Antiserum against purified enterotoxin from *C. perfringens* type A was produced by immunization of 3-month-old rabbits (New Zealand White) as described by Stark and Duncan (24). All antigen-antiserum mixtures were incubated at 37 C for 30 min before assay for biological activity by the guinea pig skin test.

Immunodiffusion. The immunological homology between purified enterotoxin from *C. perfringens* type A and toxins in crude cell extracts were established by precipitation reactions in agar gels using the double-diffusion technique (24). The immunodiffusion tests were performed in petri dishes having a diameter of 9 cm as described by Frieben and Duncan (12), and the plates were incubated in a humidity chamber at room temperature for 24 to 72 h.

Enterotoxin detection. Quantitative detection of the biologically active enterotoxin in erythemal units was based on its ability to produce erythema when injected intradermally into depilated guinea pigs as previously described (24). In control samples, the enterotoxin was neutralized by antiserum prepared against the purified enterotoxin.

Serological quantitation of enterotoxin antigen was by electroimmunodiffusion as described by Duncan and Somers (7).

Disc gel electrophoresis and disc gel immunodiffusion. Disc electrophoresis was performed on 7% acrylamide gel with pH 9.5 tris(hydroxymethyl)aminomethane-glycine as the running buffer as described (12). Of two gels loaded with the same protein solution, one gel was stained with Coomassie brilliant blue R-250 to reveal the protein bands. The other gel was subjected to immunodiffusion against anti-enterotoxin serum as described by Stark and Duncan (25). From the precipitin lines formed adjacent to the serologically active protein bands, the relative mobility (Rm) of the proteins were expressed relative to the mobility of the tracking dye bromophenol blue. Ferguson plots, i.e., log relative mobility versus acrylamide concentration, were constructed as described by Hedric and Smith (15) to obtain molecular weight estimates for enterotoxin in cell extracts and enterotoxin-like protein extracted from spore coats. At least six migration values at each gel concentration were used, and the ratio of *N,N'*-methylene bisacrylamide to acrylamide was 1:30 in all cases. Horse myoglobin, molecular weight 17,000, and α-chymotrypsinogen, molecular weight 25,000 (Calbiochem, Los Angeles, Calif.), and deoxyribonuclease I, molecular weight 31,000, and bovine serum albumin, molecular weight 68,000 (Sigma Chemical Co., St. Louis, Mo.), were used as marker proteins and were run electrophoretically under identical conditions.

Reversed passive hemagglutination test. Specific immunoglobulin was prepared from rabbit anti-enterotoxin serum with 35% $(NH_4)_2SO_4$ as described by Herbert et al. (16). The globulin was dialyzed against 0.85% NaCl solution, pH 8, until $(NH_4)_2SO_4$ free. The quality of the globulin was tested by immunoelectrophoresis in agar gel using sheep anti-rabbit globulin, and by electrophoresis on Sepharose strips (Gelman Instrument Co., Ann Arbor, Mich.). Formalin-treated sheep erythrocytes were prepared (Rokos and Duncan, unpublished data), and $CrCl_3$ was used as the sensitizing agent for the reversed passive hemagglutination test. For the batch of antiserum used in this investigation, a $CrCl_3$ concentration of 0.025% and a globulin concentration of 250 μg/ml gave the most reproducible results. The hemagglutination titration was carried out in plastic disposable U-bottom microtiter plates using 1% normal rabbit serum in phosphate-buffered saline (pH 7.4) as diluent.

Animal assay for enteropathogenicity. Cell extracts were tested for enteropathogenicity by their ability to induce fluid accumulation in ligated ileal loops of rabbit intestine. The operative procedure and techniques used have been described (9).

RESULTS

Sporulation and enterotoxin production. Table 2 gives the results of sporulation and enterotoxin production in the 37 strains received from L. S. McClung. Spores of the strains were heat shocked once before inoculation into DS sporulation medium.

Only four of the cell extracts were positive in

the immunodiffusion test (1168, 2110A, 2158, 2159A), but examination by electroimmunodiffusion revealed evidence for enterotoxin formation in seven of the samples. All the negative cell extracts were then concentrated 5- to 10-fold, depending on the protein content, and were reexamined in the immunodiffusion test. This gave positive reaction in 10 strains as indicated in Table 2. All the cell extracts positive in immunodiffusion gave a reaction of complete identity.

It is interesting to notice that five of the enterotoxin-positive type A strains (690, 1158, 1168, 2110A, 2117) have definitely been associated with food poisoning, and the association of two of the positive type A strains, 2158 and 2159A, is uncertain. However, three of the strains labeled as food poisoning-associated remained enterotoxin negative (1205, 2825, 3344).

The two strains 666 and 1434 originally designated as type C and 1485 originally designated as type D produced the toxin in very small amounts that could be detected only after concentration of the cell extracts. This was the first indication of enterotoxin production in other toxigenic types than type A. However, in our hands strains 1434 and 1485 did not produce other major toxins than the alpha-toxin and by definition were reclassified as type A. Strain 666 was a characteristic type C with alpha- and beta-toxin formation, but when single colonies were isolated and enterotoxin production was retested, we did not succeed in detecting enterotoxin formation. From Table 2 it can also be seen that the sporulation frequency is very poor for many strains in DS medium, and this may explain the failure to detect enterotoxin in their cell extracts.

It is also of some interest to notice that the first seven strains in Table 2 (enterotoxin-forming strains) produced very small amounts of alpha-toxin. In the skin test of guinea pigs, which is the most convenient and reliable method for typing of *C. perfringens*, all these strains failed to evoke the characteristic dermonecrotic reaction of alpha-toxin. When the same strains were tested for lecithinase activity on SFP-egg yolk agar (23), all gave precipitation reactions indicating the presence of alpha-toxin.

Cytoplasmatic paracrystalline inclusion bodies, previously reported by Duncan et al. (5) in enterotoxin-positive strains, were seen only in three of the strains. However, these were also producers of high amounts of enterotoxin. Strain 2158, which was the strongest enterotoxin producer, had detectable inclusion bodies in 90% of cells with refractile spores, and more than 95% sporulation occurred. Strain 2159A and 2110A had detectable inclusions in 65% and 14% of the sporulating cells, respectively, and produced also relatively high levels of enterotoxin. All the enterotoxin-positive strains induced erythemal reactions in the skin of depilated guinea pigs when cell extracts were injected intradermally, and these reactions were neutralized only by anti-enterotoxin serum.

Sporulation and enterotoxin formation in cells of *C. perfringens* type C isolated from cases of enteritis necroticans are presented in Table 3. These data were obtained after spores of the

TABLE 2. *Enterotoxin production by 37 C. perfringens isolates of different origin*

Strain	Spores/ml of DS medium	Enterotoxin (μg) per mg of cell extract protein (by electroimmunodiffusion)	Immunodiffusion of cell extracts		Sporulating cells containing paracrystalline inclusions (%)
			Before concn	After concn	
690	8.0×10^7	0.6	−	+	0
1158	3.2×10^3	0.4	−	+	0
1168	3.0×10^4	13.0	+	+	0
2110A	3.4×10^6	62.0	+	+	14
2117	6.0×10^2	0.4	−	+	
2158	1.5×10^7	200.0	+	+	90
2159A	3.5×10^7	125.4	+	+	65
146	8.0×10^5		−	−	0
666	<10		−	+	0
684	<10		−	−	0
686	1.1×10^5		−	−	0
688	3.2×10^4		−	−	0
1136	2.0×10^4		−	−	0
1171	8.0×10^6		−	−	0
1302	4.1×10^2		−	−	0
1309	6.5×10^6		−	−	0
1310	2.3×10^6		−	−	0
1015	1.2×10^6		−	−	0
1202	3.7×10^6		−	−	0
1205	2.5×10^4		−	−	0
1223	<10		−	−	0
1313	5.6×10^6		−	−	0
1331	2.5×10^5		−	−	0
1340	6.2×10^3		−	−	0
1423	<10		−	−	0
1434	1.8×10^2		−	+	0
1485	9.2×10^2		−	+	0
1521	8.5×10^3		−	−	0
1581	2.8×10^3		−	−	0
2172	8.3×10^5		−	−	0
2174	6.2×10^4		−	−	0
2272	<10		−	−	0
2825	4.3×10^4		−	−	0
2831	3.0×10^3		−	−	0
3032	8.2×10^2		−	−	0
3344	9.8×10^2		−	−	0

TABLE 3. *Enterotoxin production by C. perfringens type C strains isolated from cases of enteritis necroticans*

Strain	Spores/ml of DS medium	Enterotoxin (μg) per mg of cell extract protein (by electro-immunodiffusion)	Immunodiffusion of cell extracts[a]		Sporulating cells containing paracrystalline inclusions (%)
			Before concn	After concn	
5381	<10	0	+ (p.i.)	+ (c.i.)	0
5382	1.2×10^3	8.3	+ (c.i.)	+ (c.i.)	0
5383	4.8×10^3	0	+ (p.i.)	+ (p.i.)	0
5384	4.5×10^3	0	+ (p.i.)	+ (p.i.)	0
5385	1.4×10^2	0	–	+ (p.i.)	0
5386	1.6×10^4	21.6	+ (c.i.)	+ (c.i.)	0
5387	3.2×10^7	0	–	+ (p.i.)	0
5388	<10	0	–	+ (c.i.)	0

[a] +, Positive reaction; –, negative reaction; c.i., reaction of complete identity; p.i., reaction of partial identity.

isolates had been heat shocked once before inoculation into DS medium for enterotoxin production. Only two of the strains, 5382 and 5386, contained enterotoxin in their cell extracts as measured by electroimmunodiffusion. The same cell extracts were positive in the double immunodiffusion test and gave a reaction of complete identity with *C. perfringens* type A enterotoxin. The strains 5381, 5383, and 5384 gave faint precipitation lines which were interpreted as reactions of partial identity. The remaining strains were negative in immunodiffusion of the unconcentrated cell extracts. When the same cell extracts were tested in immunodiffusion after concentration, four of the strains showed reactions of complete identity with type A enterotoxin. The reaction of partial identity seen in unconcentrated cell extract from 5381 was not observed after concentration. Concentrated cell extracts from 5388 gave an immunodiffusion pattern interpreted as complete identity after 48 h incubation.

Typical reactions of partial and complete identity are shown in Fig. 1.

Paracrystalline inclusions could not be detected in any of these strains. However, enterotoxin formation was also low (less than 22 μg/mg of cell extract protein).

All the strains in Table 3 except 5387 sporulated at a very low level. The strains were therefore subjected to further heat shocking and selection for sporeforming cells. Before testing for enterotoxin production, the strains were heat shocked and passed through fluid thioglycolate medium and DS sporulation medium three times before finally growing sporulating cells to test for enterotoxin production. The results are presented in Table 4. It can be seen that the sporulation frequency has increased considerably for many of the strains; the most striking effect is observed in strain 5388. The amount of enterotoxin in the cell extracts also has increased considerably compared to the values in Table 3. Strains 5386 and 5388 have become strong enterotoxin producers and showed the ability to form paracrystalline inclusion bodies in a high percentage of the sporulating cells.

Figure 2 shows sporulating cells from strain 2158 (type A) and 5388 (type C) grown in DS medium for 10 h. The cells and spores from 5388 have a greater diameter than usually encountered in sporulating *C. perfringens* type A cultures. Inclusions can be seen in both types.

The cell extracts from type C strains (Table 4) were tested for erythemal activity in guinea pigs. These cell extracts contained alpha- and beta-toxins which had to be neutralized by antisera before testing for biological activity. The strains that showed complete identity in immunodiffusion (5381, 5382, 5386, and 5388) all gave erythemal reactions in the skin test of guinea pigs which could be neutralized by anti-enterotoxin serum against *C. perfringens* type A. Erythemal activity could not be demonstrated in the other strains.

To obtain more information about the toxins produced by the type C strains 5381 to 5388, the cell extracts were run electrophoretically on 7% polyacrylamide gels and subjected to disc gel immunodiffusion. The Rm values of the precipitated arcs are listed in Table 5. Four of the strains did not give a visible precipitation reaction in this test, and these strains corresponded to the ones that showed a reaction of partial identity in the immunodiffusion test. All the positive strains gave an average Rm of 0.53, which is essentially the same as the Rm value for enterotoxin from *C. perfringens* type A.

Strain 5386 and 5388 showed a second precipitation arc with an Rm value of 0.80, indicating

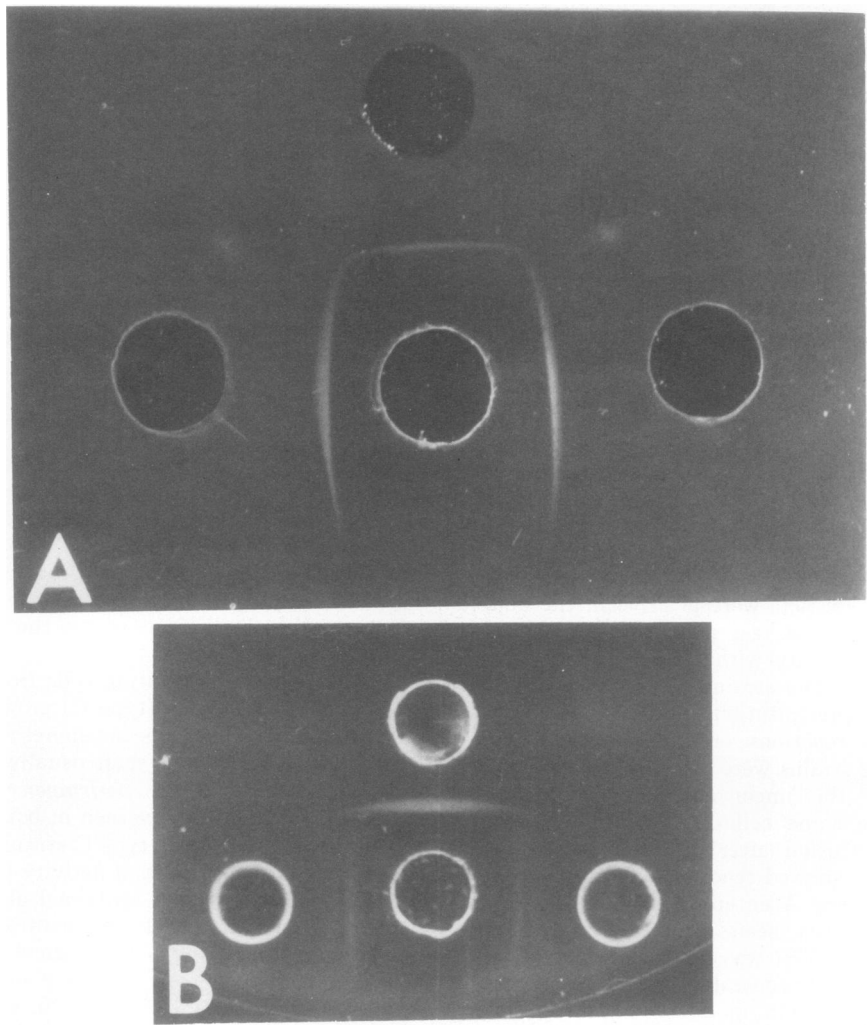

FIG. 1. *Immunodiffusion patterns obtained with cell extracts from C. perfringens type C, strains 5388 (A) and 5383 (B). The center wells contained antiserum against purified enterotoxin from C. perfringens type A. Upper wells contained purified enterotoxin from C. perfringens type A. Cell extracts from strains 5388 and 5383 were added to the lateral wells.*

the presence of a faster-migrating component which might be due to lower molecular weight or to differences in net charge or tertiary structure of the protein. This faster-migrating component makes up a very small part of the total serologically reacting protein (Fig. 3) and can easily be missed in the immunodiffusion test. A reaction of partial identity is indicated for this faster-migrating component.

Enteropathogenicity. Cell extract from *C. perfringens* type C strain 5388 was neutralized for alpha- and beta-toxin activity before rabbit ileal loops were injected with different dilutions of the cell extract. The injected loops responded with fluid accumulation, as do cell extracts from enterotoxin positive strains of *C. perfringens* type A.

Only one of the 17 other strains mentioned above produced enterotoxin in detectable amounts. Type D strain PS-53 received from the Center for Disease Control produced cell extract which gave a faint reaction of complete identity in immunodiffusion after concentration. When single colonies were isolated and typed and the enterotoxin production repeated, we detected no enterotoxin formation in the cell extracts. Subsequent heat shocking of the cultures had no effect upon enterotoxin production.

Extraction of spore protein. A recent report

TABLE 4. *Enterotoxin production by C. perfringens type C strains isolated from cases of enteritis necroticans*[a]

Strain	Spores/ml of DS medium	Enterotoxin (μg) per mg of cell extract protein (by electro-immunodiffusion)	Immunodiffusion of cell extracts[a]		Sporulating cells containing paracrystalline inclusions (%)
			Before concn	After concn	
5381	1.4×10^2	11.2	+ (c.i.)	+ (c.i.)	0
5382	3.8×10^4	23.9	+ (c.i.)	+ (c.i.)	0
5383	4.2×10^6	0	+ (p.i.)	+ (p.i.)	0
5384	9.3×10^4	0	+ (p.i.)	+ (p.i.)	0
5385	1.9×10^2	0	+ (p.i.)	+ (p.i.)	0
5386	8.3×10^6	125.2	+ (c.i.)	+ (c.i.)	70
5387	4.2×10^7	0	−	+ (p.i.)	0
5388	3.3×10^7	163.8	+ (c.i.)	+ (c.i.)	85

[a] Isolates heat shocked and passed through the spore state three times.

[b] +, Positive reaction; −, negative reaction; c.i., reaction of complete identity; p.i., reaction of partial identity.

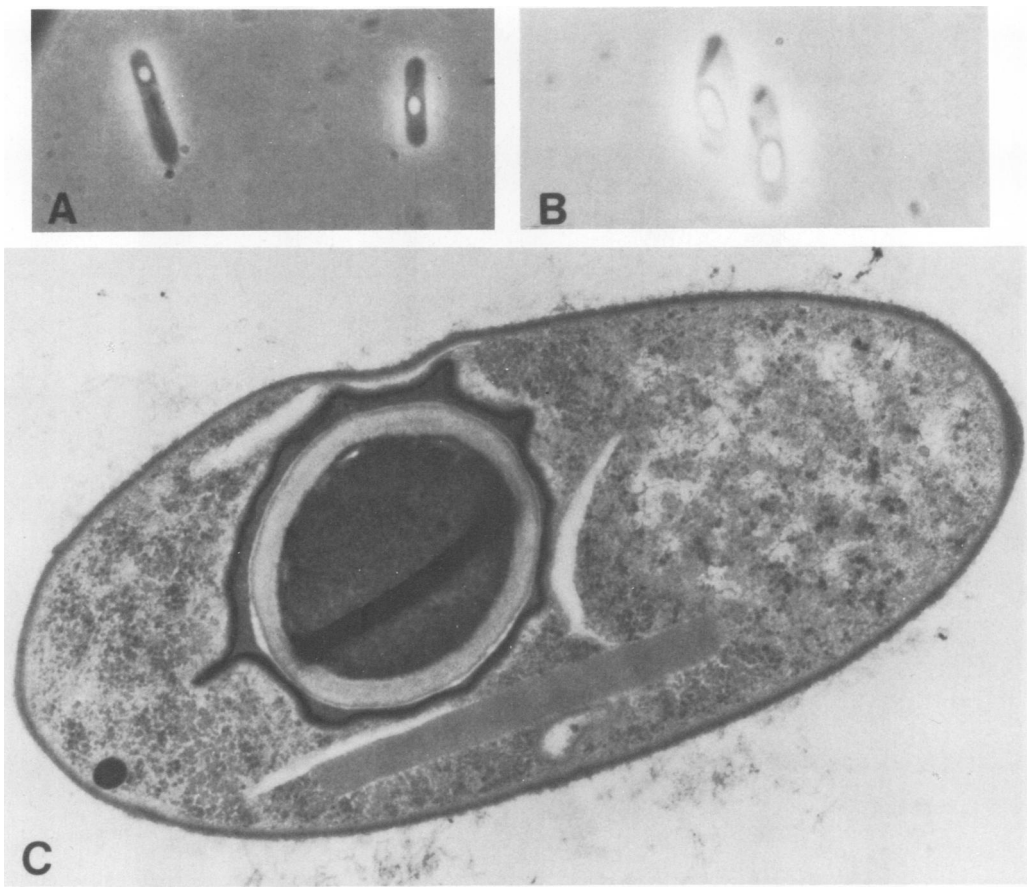

FIG. 2. *(A) Spores and paracrystalline inclusion formation in C. perfringens type A, strain 2158. (B) Spores and paracrystalline inclusion formation in C. perfringens type C, strain 5388. (C) Electron micrograph of a thin section of a sporulating cell of strain 5388 with inclusion.*

from our laboratory (12) demonstrated that clean intact spores of *C. perfringens* type A could be treated with reducing agents such as dithiothreitol and mercaptoethanol under alkaline conditions which solubilized a spore coat protein fraction possessing biological and serological activity similar to that of enterotoxin. Altogether six strains were tested including

TABLE 5. *Rm values on 7% polyacrylamide gels of enterotoxin from C. perfringens type C isolated from cases of enteritis necroticans*

Strain	Rm[a]
5381	0.53
5382	0.53
5383	—
5384	—
5385	—
5386	0.53, 0.80
5387	—
5388	0.53, 0.80

[a] —, No reaction.

enterotoxin-positive and enterotoxin-negative ones, and all of them gave reactions of partial or complete identity with enterotoxin in immunodiffusion. These results indicated that an enterotoxin-like protein was present in the spore of *C. perfringens* type A, in general and possibly in other toxigenic types. Clean spores from a variety of enterotoxin-positive and enterotoxin-negative strains of *C. perfringens* types A and C were investigated (Table 6).

Of the 14 strains investigated, four gave a positive reaction in electroimmunodiffusion and double immunodiffusion. Three of the positive strains were type A and one was type C. All the

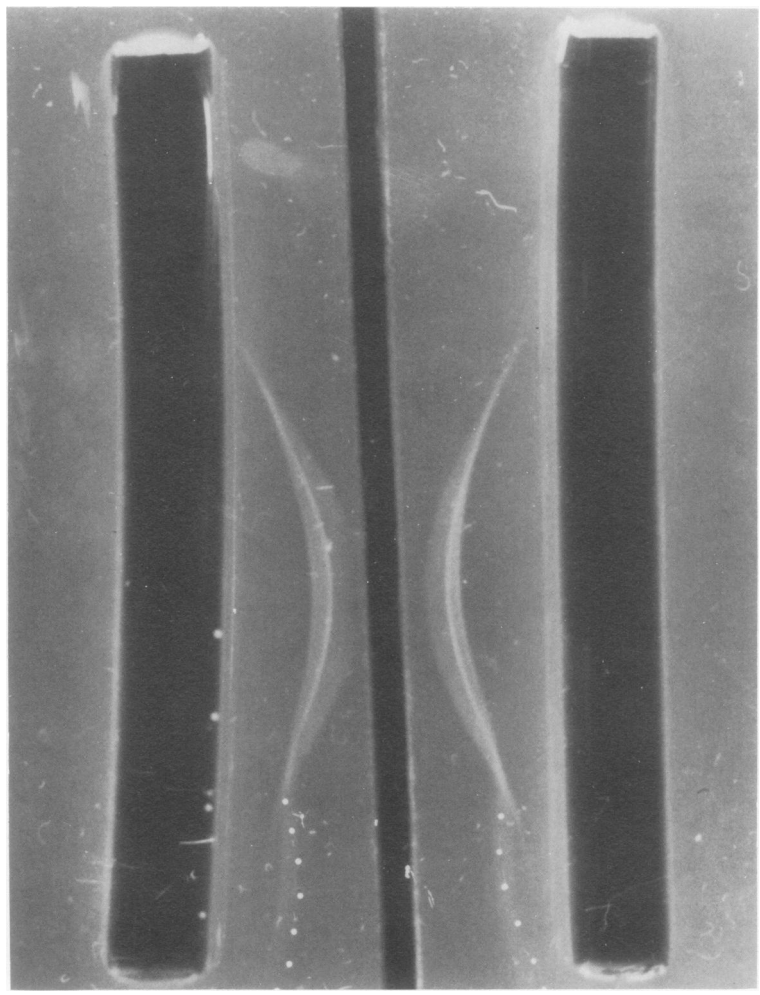

FIG. 3. *Acrylamide disc gel electrophoresis of cell extract from C. perfringens type C, strain 5388, followed by immunodiffusion against antiserum prepared against enterotoxin from type A. The strong arcs correspond to a relative mobility of 0.53, and the faint arcs (indicated by white dots) correspond to a relative mobility of 0.80. The two arcs show reaction of partial identity.*

TABLE 6. *Qualitative and quantitative assay of serologically detectable enterotoxin-like protein in the extracts of spores from various strains of C. perfringens type A and C*[a]

Strain	Type	Enterotoxin (μg) per mg of extracted spore protein (by electroimmunodiffusion)	Immunodiffusion of spore protein	Reversed passive hemagglutination test
690	A	0	–	–
2110A	A	8.3	+ (c.i.)	+
2158	A	24.2	+ (c.i.)	+
2159A	A	19.0	+ (c.i.)	+
1015	A	0	–	+
1171	A	0	–	–
1202	A	0	–	–
1205	A	NT	+ (c.i.)	NT
1310	A	0	–	–
1331	A	0	–	–
1340	A	0	–	–
3344	A	0	–	–
5387	C	0	–	–
5388	C	26.5	+ (c.i.)	NT

[a] Extraction procedure and assay methods for enterotoxin are as described. +, Positive reaction; –, negative reaction; c.i., reaction of complete identity; NT, not tested.

positive strains gave a reaction of complete identity with enterotoxin from type A. After concentration and retesting of the extracted spore proteins, one additional strain (1205) gave a reaction of complete identity. The samples were then assayed for enterotoxin by means of the reversed passive hemagglutination test, and by this method strain 1015 also gave a positive reaction.

Four of the strains which gave a reaction of complete identity in immunodiffusion (2110A, 2158, 2159A, and 5388) all had high levels of enterotoxin in their cell extracts (Table 2 and 4). However, extracted spore protein from strain 1205 also gave a reaction of complete identity in immunodiffusion despite the fact that the cell extract from the same strain was negative (Table 2). This is an interesting point because strain 1205 had been associated with food poisoning. Strain 690 had also been associated with food poisoning and produced low levels of enterotoxin in the cell extract (Table 2), but enterotoxin-like protein was not detectable in the spore extract by any of the methods used.

Spore protein from four of the positive strains was subjected to disc electrophoresis and subsequent immunodiffusion in agar. The Rm values obtained for enterotoxin are presented in Table 7. All the strains had an average Rm value of 0.53 to 0.54, but the extracted spore proteins from 5388 showed a faint arc of precipitation with an Rm value of 0.82 that showed partial identity to the stronger arc with an Rm of 0.53 (Fig. 4).

Extracted protein from the spores of strains 2110A, 2158, 2159A, and 5388 induced erythema in the skin of guinea pigs after intradermal injection and the erythemal activity was neutralized by anti-enterotoxin serum from type A.

Ferguson plots and molecular weight determination. As indicated in the results presented above, two components that reacted serologically with anti-enterotoxin serum could be obtained in both cell extracts and from spore coat extracts of strain 5388. These forms had relative mobilities in disc gels of 0.53 and 0.80 to 0.82. Ferguson plots were constructed to determine the molecular weight of these slow- and fast-migrating components. Figure 5 shows the effect of acrylamide concentration on the relative mobility of enterotoxin and enterotoxin-like spore protein from *C. perfringens* type C strain 5388.

When these samples were run on gels of different acrylamide concentrations, the main precipitation arc, i.e., that with a relative mobility of 0.53, was always distinct and could easily be visualized. The faster migrating component with a relative mobility of 0.80 to 0.82 gave faint precipitation arcs, and the Rm values on the different gel concentrations were difficult to read. The arcs corresponding to the faster migrating component were absent in many of the samples, and we were not able to obtain enough readings to construct Ferguson plots for this particular toxin form.

Estimates of the molecular weight of the 0.53 Rm enterotoxin from both cell and spore preparations were obtained by fitting the slopes of lines constructed in Fig. 5 to a standard plot (Fig. 6). Enterotoxin from cell extracts was found to have a molecular weight of 37,000, and the molecular weight of enterotoxin-like spore protein was estimated to be 35,000. The slope for the faster-migrating component was 2.4, and this value was too low to obtain a reading on the

TABLE 7. *Rm value of 7% acrylamide gels of enterotoxin-like protein extracted from spores of various strains of C. perfringens type A and C*

Strain	Type	Rm
2110A	A	0.54
2158	A	0.54
2159 A	A	0.53
5388	C	0.53, 0.82

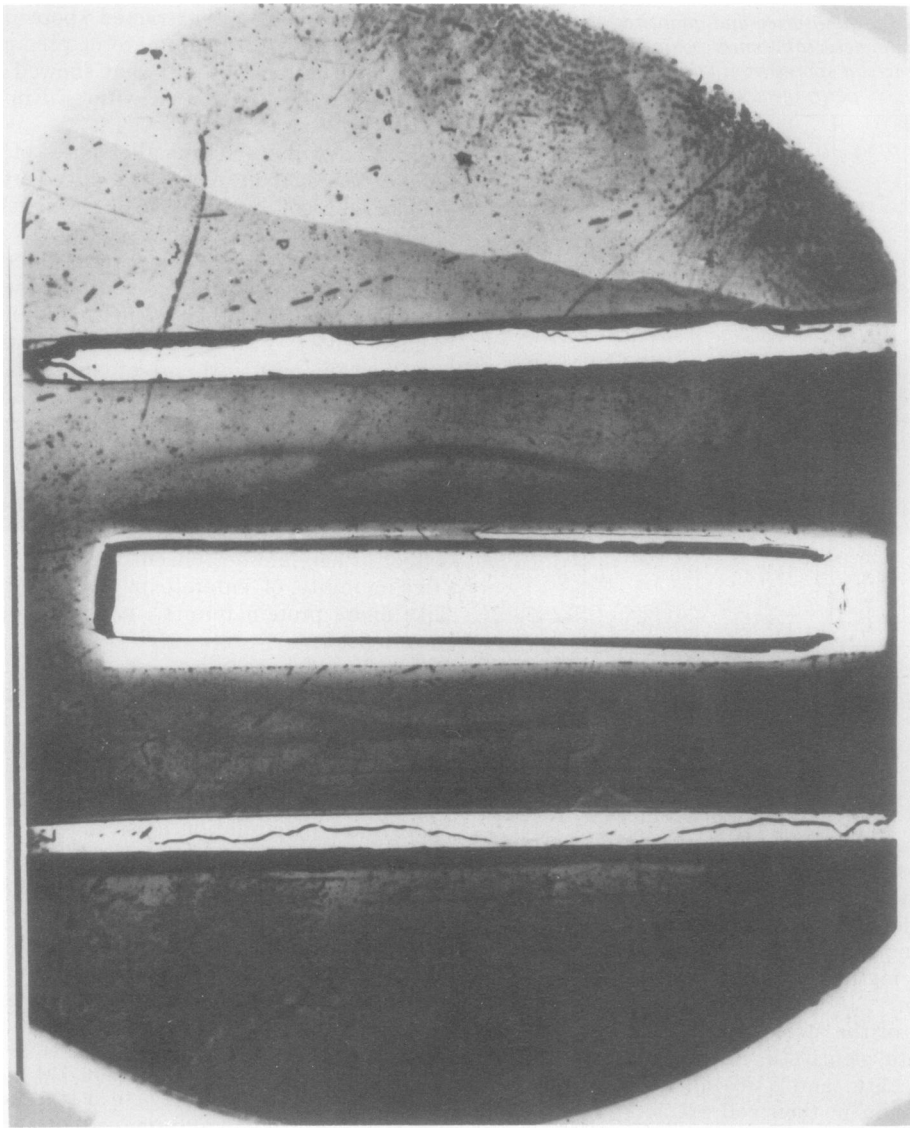

FIG. 4. *Disc gel electrophoresis of DTT-solubilized spore protein from type C, strain 5388 followed by immunodiffusion against antiserum prepared against enterotoxin from type A. Two precipitation arcs are formed showing reaction of partial identity.*

standard curve. The smallest protein used for constructing the standard curve had a molecular weight of 17,000, at which point the curve remains linear. We do not know the reliability of this technique for determination of molecular weight of small proteins. The low slope value of the fast-migrating component indicates a considerably lower molecular weight than the smallest standard used, most likely less than 15,000.

DISCUSSION

This is the first report where enterotoxin production in *C. perfringens* has been documented in a toxigenic type other than type A. Uemura et al. (28) assayed a number of strains of *C. perfringens* for enterotoxin production in vitro, including types A, B, C, D, and E. Of 14 type A strains, only five were enterotoxin positive, and the 16 strains of the other toxigenic types were all negative. Many of the strains

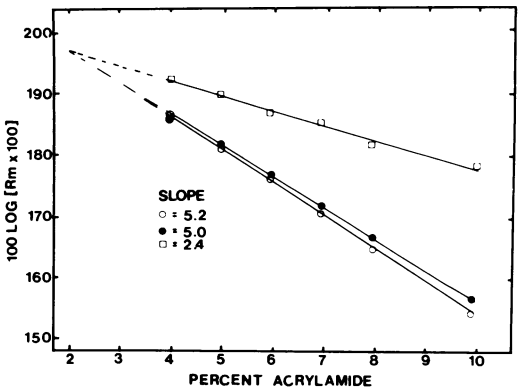

FIG. 5. *Influence of acrylamide concentration on the mobility of enterotoxin and enterotoxin-like spore protein from C. perfringens type C, strain 5388. Enterotoxin in cell extract* (○), *spore protein Rm 0.53* (●), *and spore protein Rm 0.82* (□).

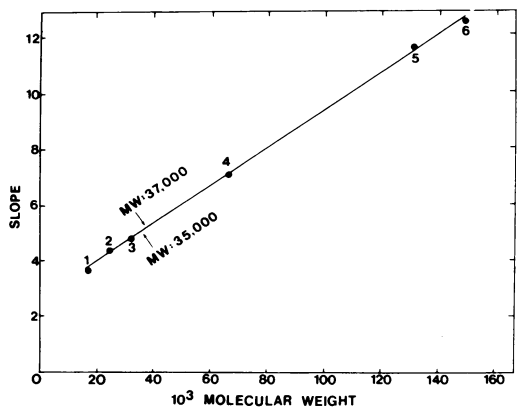

FIG. 6. *Molecular weight determination of enterotoxin and enterotoxin-like spore protein from C. perfringens type C, strain 5388. The molecular weights were determined from a plot of the slope of log mobility lines (see Fig. 5) versus the molecular weight of standard proteins of known molecular weight. Standard proteins are designated by numbers: 1, myoglobin; 2, α-chymotrypsinogen; 3, deoxyribonuclease I; 4, bovine serum albumin monomer; 5, bovine serum albumin dimer; 6, yeast alcohol dehydrogenase. The slope value for enterotoxin corresponded to molecular weight (MW) of 37,000, and the slope value for spore protein Rm 0.53 corresponded to MW of 35,000.*

sporulated poorly in DS medium or did not survive the heat shocking. The results of our study are similar except for the type C strains isolated from cases of enteritis necroticans. The frequency of enterotoxin-positive strains of type A is relatively low. A similar relationship may exist in other toxigenic types. No conclusion can therefore be drawn as to enterotoxin formation before a large number of sporulating strains of other toxigenic types has been investigated. Unfortunately, these are difficult to obtain. Sporulation is necessary for enterotoxin synthesis (10), but good sporulation can also be seen in enterotoxin-negative strains (Table 2). However, low sporulation frequency may explain the failure to detect enterotoxin in cell extracts from many of the strains investigated in this study. Most of the strains of types B, C, D, and E did not sporulate, or sporulated with a very low frequency in DS sporulation medium. To overcome this problem, different modifications of the DS medium were investigated. Enterotoxin-negative strains were grown in DS medium with varying concentrations of starch or without starch, and with or without addition of activated carbon which has been reported to increase sporulation (8). Experiments with growth in DS medium where the pH was maintained constant were also carried out, but these attempts to increase sporulation were less than successful. The sporulation media of Ellner (11), Angelotti et al. (1), and Taniguti (27) were also tried with some of the poorly sporulating strains, but without success.

The reason for enhancement of enterotoxin formation after several passages of a strain through the spore state as shown in this study with type C strains and previously by Uemura et al. (28) with type A strains is not clear. The heat treatment presumably selects for a spore-forming population with enhanced enterotoxin-forming ability.

The fact that enterotoxin is produced by other toxigenic types than type A indicates that typing of the causative organism should be done routinely when food poisoning outbreaks of *C. perfringens* occur. This apparently is not routinely done at the present time, and instead outbreaks are assumed to be caused by type A.

The ability of type C strains to produce enterotoxin raises the question of the importance of this toxin in enteritis necroticans caused by type C. Beta-toxin has been thought to be the principal toxin involved in type C intoxications. However, the possible role of enterotoxin in this type of food poisoning needs further clarification.

Strains 1434 and 1485 received as type C and type D, respectively, produced small amounts of enterotoxin, but after typing in this laboratory they were classified as type A. This phenomenon can of course be explained by the fact that mixing of cultures may have taken place, but the possibility still exists that the cultures have lost their ability to produce beta- and

epsilon-toxins during storage in laboratory media. Antigenic variation in *C. perfringens* type C and type D has been reported previously by Wilson-Taylor (30) and L. S. Smith (personal communication). The mechanism(s) for these variations has yet to be explained since the genetics behind the production of the different toxins are unknown.

It has been demonstrated that a direct relationship exists between sporulation and enterotoxin synthesis (10). Complete sporulation is not necessary for enterotoxin formation because the toxin is synthesized during the early stages of sporulation. Enterotoxin-like spore protein could be extracted from the spores of all the enterotoxin-positive strains tested here except for strain 690. The spore extracts of most of the enterotoxin-negative strains did not show serological or biological activity. This is in contrast to the data presented by Frieben and Duncan (12) in which enterotoxin could be detected in extracts of spore coats of strains in which toxin could not be detected in the cell extract.

Spore coat proteins are known to possess the ability to aggregate into a range of sizes, and this particular problem is discussed in a separate publication (Frieben and Duncan, in press). In that investigation, biologically active enterotoxin-like spore proteins with molecular weights of 36,500, 23,000, and 14,500 were reported, and only one type of enterotoxin-like spore protein was detected in a particular strain. In this study, one strain, 5388, has been shown to have two serologically active proteins in both the spore extract and cell extract that differ in size, but we do not know if both are biologically active.

The molecular weight of the 0.53-Rm enterotoxin from type C was estimated to be 37,000, which is in the same range as reported for enterotoxin from *C. perfringens* type A. The data from this investigation indicate that enterotoxins from type A and type C are very similar. Results of studies on purification and further characterization of the enterotoxin from *C. perfringens* type C will be reported in a separate communication.

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