APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1986, p. 407–412
0099-2240/86/090407-06$02.00/0

Vol. 52, No. 3

Enterotoxin Synthesis by Nonsporulating Cultures of *Clostridium perfringens*†

STEVEN B. GOLDNER,‡ MYRON SOLBERG,* SUSAN JONES,§ AND LAURIE S. POST

Department of Food Science, Cook College, New Jersey Agricultural Experiment Station, Rutgers, The State University, New Brunswick, New Jersey 08903

Received 6 February 1986/Accepted 3 June 1986

Chemostat-cultured *Clostridium perfringens* ATCC 3624 and NCTC 10240, and a nonsporulating mutant strain, 8-5, produced enterotoxin in the absence of sporulation when cultured in a chemically defined medium at a 0.084-h^{-1} dilution rate at 37°C. The enterotoxin was detected by serological and biological assays. Examination of the chemostat cultures by electron microscopy did not reveal sporulation at any stage. The culture maintained enterotoxigenicity throughout cultivation in a continuous system. The enterotoxin was detected in batch cultures of each strain cultivated in fluid thioglycolate medium and a chemically defined medium. No heat-resistant or light-refractile spores were detected in batch cultures during the exponential growth.

Clostridium perfringens food poisoning usually develops after the consumption of food that is contaminated with large numbers of vegetative cells (6, 16). The enterotoxin responsible for the illness is generally thought to be synthesized when the ingested cells sporulate in the intestine and is released from the sporangium after lysis (10). *C. perfringens* enterotoxin has been described as a sporulation-specific gene product (10) or a structural component of the spore coat (11, 12). In contradiction, Walker et al. (40), after examining the ultrastructure of sporulating cells of *C. perfringens* with an antienterotoxin stain, reported that the enterotoxin protein is confined to the cytoplasm and is not associated with spore membranes or the spore coat. Roper et al. (32), using similar techniques, also found no relationship between enterotoxin and the spore coat.

Several studies have shown that *C. perfringens* strains implicated in food poisoning outbreaks did not produce detectable enterotoxin in Duncan-Strong (DS) sporulation medium (5, 33, 34, 38). The lack of detectable enterotoxin was ascribed to poor sporulation frequencies (5, 33, 34). However, strains with a low sporulation frequency produced enterotoxin in DS medium (5, 33), and enterotoxin-negative strains produced approximately the same number of spores per milliliter as did high-level enterotoxin producers (38). Enterotoxin synthesis by vegetative cells of *C. perfringens* was detected by Niilo (28) by using a fluorescent-antibody technique. Smith and McDonel (35) found that enterotoxin was synthesized in a cell-free system by using polysomes from vegetative cells of *C. perfringens* cultured in fluid thioglycolate medium (FTM). Granum et al. (15) detected enterotoxin by using an enzyme-linked immunosorbent assay in three strains of nonsporulating *C. perfringens* grown in FTM. Sporulation and the genes that control sporulation may not be the key to enterotoxin production.

Enzymes that appear during sporulation may not be required, nor are they necessarily specific, for the formation of an endospore (45). The appearance of a protein during sporulation could be the result of some type of derepression of a catabolite gene and not the expression of a sporulation-specific gene (30). Proteins that appear during sporulation in a given medium may be present in vegetative cells growing under some other set of conditions (36). Synthesis of enzymes or protein toxins in late exponential-phase growth or early stationary-phase growth has been attributed to growth-induced changes in the nutritional composition of the medium (24, 30, 41, 44). The effect of medium composition has been further demonstrated by synthesis of the same proteins during the exponential-growth phase in a chemostat at a low dilution rate (25, 42, 44).

Numerous studies attribute the control or regulation of protein synthesis to a specific nutrient (1, 3, 17, 26). *C. perfringens* enterotoxin production may also be regulated by nutritional factors. The present study was undertaken to determine whether *C. perfringens* enterotoxin could be produced under defined nutritional conditions that have been proven not to initiate sporulation, thus separating enterotoxin synthesis from sporulation.

MATERIALS AND METHODS

Test culture maintenance. *C. perfringens* ATCC 3624 was obtained from the Food Science Department, Rutgers University. *C. perfringens* NCTC 10240 and a nonsporulating mutant strain, 8-5, were obtained from the laboratory of R. G. Labbe, University of Massachusetts, Amherst. Stock cultures were maintained in cooked-meat medium (Difco Laboratories, Detroit, Mich.) at 4°C. The stock cultures were subcultured every 4 to 5 weeks through two cycles in FTM (Difco) for 24 h at 37°C, inoculated into fresh cooked-meat medium, and incubated for 24 h at 37°C before storage.

Chemostat construction and operation. The chemostat design (Bioflow model C-30 continuous fermentor; New Brunswick Scientific Co., Inc., Edison, N.J.) was adapted

* Corresponding author.

† Publication no. D-10102-1-86 of the New Jersey Agricultural Experiment Station.

‡ Present address: Microbiological Services Consumer Products, Warner Lambert Co., Morris Plains, NJ 07950.

§ Present address: United States Department of Agriculture, Eastern Regional Research Laboratories, Philadelphia, PA 19118.

from the original model of Novick and Szilard (29) by incorporating the internal sterilization system described by Goldner and Solberg (13) to maintain long-duration, contamination-free operation. The chemostat was operated as previously described by Goldner and Solberg (13).

Monitoring of the continuous culture. Culture purity and stability during continuous culture were assured by following the methods of Goldner et al. (14).

Culture medium. The glucose-limited reformulated defined media described by Goldner et al. (14) were used to culture strains 3624 and 10240. A reformulated defined medium was prepared for the culture of strain 8-5 by addition of lysine (100 mg/liter) to the medium formulated for strain 10240. R & S medium, a chemically defined medium, was prepared by the procedure of Riha and Solberg (31).

Culture inoculum. Inocula were prepared as described by Goldner et al. (14).

Preparation of cell extracts and culture filtrates. Samples were harvested from cultures grown under specific culture parameters and were centrifuged at 12,000 × *g* for 20 min (4°C). The supernatant (100 ml) was decanted and passed through a membrane filter (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). The culture filtrate was dialyzed to dryness against polyethylene glycol (20 M; Union Carbide Corp., New York, N.Y.) for 24 to 30 h at 4°C, reconstituted in 1.0 ml of 0.2 M phosphate buffer (pH 6.8) (concentrated 100-fold), and then stored at −20°C until assayed for enterotoxin. The cell pellet was suspended with 6 ml of cold distilled water and placed in an ice-water bath. The cells were disrupted by sonication (Sonifier Cell Disruptor model W185; Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.; 35 W for 30 min at 30-s intervals) and then centrifuged at 12,000 ×*g* for 20 min (4°C). The cell extract was concentrated by dialysis against polyethylene glycol for 18 to 24 h at 4°C, reconstituted with 1.0 ml of 0.2 M phosphate buffer, and stored at −20°C until assayed for enterotoxin.

Enterotoxin assays. The enterotoxin was assayed in concentrated cell extracts and concentrated culture filtrates both serologically (by counterimmunoelectrophoresis [CIEP] as described by Naik and Duncan [27]) and biologically (by the guinea pig erythemal assay [guinea pig weight, 450 to 500 g, Hartley strain; Charles River Breeding Laboratories, Inc., Wilmington, Mass.] as described by Hauschild [16]). Purified enterotoxin was obtained from C. Duncan and M. W. Pariza, University of Wisconsin. Antienterotoxin serum was obtained from R. Skjelkvale, Norwegian Food Research Institute, Oslo, Norway.

Sporulation characteristics. The sporulation characteristics of *C. perfringens* 10240, 3624, and 8-5 were examined in modified DS sporulation medium with raffinose as the carbohydrate source (21). Modified DS medium (180 ml) was inoculated with an FTM batch culture (16 to 18 h; 10% inoculum) or a 20-ml sample of chemostat-cultured cells. Chemostat cultures were grown in the reformulated chemically defined medium appropriate for each strain. Strains 3624 and 10240 were incubated for 24 h at 37°C, and strain 8-5 was incubated for 48 h at 37°C. Samples of each strain were taken at 0, 4, 8, 10, and 24 h (and also at 48 h for strain 8-5). Counts of heat-resistant spores were determined by heating 5 ml of culture for 20 min at 75°C. Plate counts were made on TSC agar (Difco) without cycloserine and incubated anaerobically for 48 h at 37°C.

Samples from the 24- and 48-h (strain 8-5) cultures were examined for light-refractile spores by phase-contrast microscopy as described by Labbe and Duncan (19). Concentrated culture extracts and concentrated culture filtrates (100 ml) were prepared from 24-h cultures and stored at −20°C until assayed for enterotoxin.

Enterotoxin production by chemostat cultures. *C. perfringens* 3624, 10240, and 8-5 were cultured in the appropriate glucose-limited reformulated medium (37°C) at a dilution rate of 0.084 h^{-1} (generation time, 8.3 h). Steady-state growth was established in the chemostat and was monitored spectrophotometrically (A_{600}) for at least eight culture generations. Samples (106 ml) were harvested from each chemostat and examined for viable cell density, heat-resistant spores, and light-refractile spores and by electron microscopy. Concentrated cell extracts and concentrated culture filtrates were prepared and stored until assayed for enterotoxin. The volume in the culture vessel was then restored to 150 ml, and the cultures were allowed to reestablish steady-state growth.

Enterotoxin production by batch-cultured cells. Tubes of R & S medium (20 ml) were inoculated with 0.5 ml of cooked-meat medium cultures and incubated for 9 to 13 h at 37°C. Transfers were made to fresh R & S medium, and the batch cultures were incubated at 46°C (strains 3624 and 8-5) or 43°C (strain 10240). The cells were harvested at an A_{600} of 0.6 to 0.7 and washed, and the resuspended cells were adjusted to an A_{600} of 1.5. R & S and FTM media (five 25-ml portions per medium) were inoculated with 0.6 ml of the cell suspension and incubated at 37°C. Growth was monitored at 600 nm with a Coleman Junior spectrophotometer (Coleman Systems, Irvine, Calif.) to an A_{600} of 0.3 to 0.4. Tubes of like medium were pooled and examined for viable cell density, heat-resistant spores, and light-refractile spores. Concentrated culture filtrates and cell extracts were prepared from each batch culture (100 ml) and examined for enterotoxin as previously described.

Polyacrylamide gel electrophoresis. Purified *C. perfringens* enterotoxin, concentrated culture filtrates from chemostat cultures of *C. perfringens* 3624, 10240 and 8-5, and a concentrated culture filtrate from strain 8-5 grown in DS sporulation medium with raffinose were analyzed by polyacrylamide gel (7.5%) electrophoresis. Buffers and gels were formulated as described by Laemmli (22). Samples (100 μl) of the culture filtrates were mixed with 40 μl of distilled water or 40 μl of antienterotoxin serum, incubated for 1 h at 37°C, and centrifuged at 12,000 × *g* for 10 min. The purified enterotoxin (40 μl) was mixed with 10 μl of either distilled water or antienterotoxin, incubated for 1 h at 37°C, and centrifuged. A 50% glycerol solution (50 μl) was added to each sample combination. The samples were applied to the stacking gel, a tracking dye was added, and current was applied at 30 mA per gel. The gels were silver stained by the procedure of Wray et al. (43). Protein concentration can be detected at a minimum concentration of several nanograms by using silver staining.

Electron microscopy. A 20-ml sample was harvested from each chemostat and mixed for 30 min with 20 ml of 2% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) for primary fixation. The mixture was centrifuged for 5 min at 10,000 × *g* (4°C). The supernatant was decanted, and the cell pellet was suspended in 5 ml of a phosphate buffer and then stored at 4°C until post-fixed.

Cells were post-fixed in 1% osmium tetroxide in Veronal acetate buffer (Winthrop Laboratories, New York, N.Y.) overnight at room temperature, followed by 90 min in 0.5% uranyl acetate in the same buffer. Cells were embedded in agar, dehydrated with increasing concentrations of ethanol and three changes of propylene oxide, and embedded in Epon epoxy resin. Sections (60 nm) were post-stained with

uranyl acetate and lead citrate and observed in an electron microscope (model 108; Carl Zeiss, Inc., New York, N.Y.) at 60 kV.

RESULTS

Sporulation characteristics and enterotoxin production in a sporulation medium. Heat-resistant spores were detected in 24-h cultures of strains 3624 and 10240 grown in modified DS medium inoculated from FTM batch cultures or chemostat-cultured cells. Spore counts are indicated in Table 1. No heat-resistant or light-refractile spores were detected in cultures of strain 8-5 at 24 or 48 h in modified DS medium inoculated from either source (Table 1).

Enterotoxin was detected by CIEP in concentrated cell extracts and concentrated culture filtrates prepared from the 24-h cultures (Table 1). Strain 10240 produced more enterotoxin than did strain 3624. The experimentally determined limit of detection was 0.9 μg of enterotoxin per ml. An erythemal response was observed with selected test samples (Table 1). Treatment of each sample with antienterotoxin serum resulted in a loss of biological activity.

A concentrated culture filtrate from strain 8-5 was examined by polyacrylamide gel (7.5%) electrophoresis. Two bands were identified that corresponded to two bands characteristic of a purified enterotoxin standard. The two bands were diminished in intensity when the concentrated filtrate was mixed with antienterotoxin serum.

Sporulation characteristics and enterotoxin production by chemostat-cultured cells in a chemically defined medium. No heat-resistant or light-refractile spores were detected in glucose-limited chemostat cultures of strains 10240, 3624, and 8-5 grown in the appropriate reformulated chemically defined medium at a dilution rate of 0.084 h^{-1} (Table 2). No sporulation was observed at any stage when the cultures were evaluated by phase-contrast microscopy or when thin sections of hundreds of cells were examined by electron microscopy. Figure 1 depicts a typical electron micrograph of strain 10240 and is representative of electron micrographs for strains 3624 and 8-5. The cells appear as intact rods with no evidence of transverse spore septum formation or invagination of the cell membrane. The enterotoxin was detected by CIEP in the concentrated cell extract and concentrated culture filtrate from each culture (Table 2). An erythemal response was observed with all samples (Table 2) and was eliminated by treatment of the samples with antienterotoxin.

TABLE 1. Sporulation and enterotoxin production by *C. perfringens* ATCC 3624, NCTC 10240, and 8-5, batch cultured in DS sporulation medium

Inoculum source and strain	Spore count[a] (CFU/ml)	Enterotoxin concn (μg/ml) in[b]:		Erythemal response (diam, cm)[c] in:	
		Cell extract	Culture filtrate	Cell extract	Culture filtrate
FTM[d]					
3624	2.7×10^6	7.2	7.2	0.8	ND[e]
10240	1.9×10^5	28.8	28.8	ND	1.5
8-5	0	3.6	7.2	ND	1.0
Chemostat[f]					
3624	3.8×10^6	7.2	7.2	0.9	ND
10240	1.5×10^6	14.4	28.8	ND	1.4
8-5	0	3.6	7.2	ND	0.9

[a] Heat-resistant spore count.
[b] Enterotoxin assayed by CIEP.
[c] Positive response indicated by a diameter of >0.5 cm.
[d] Inoculum was batch cultured in FTM.
[e] ND, Not done.
[f] Chemostat cultures were grown in the reformulated chemically defined medium appropriate for each strain.

TABLE 2. Sporulation and enterotoxin production by glucose-limited chemostat cultures of *C. perfringens* ATCC 3624, NCTC 10240, and 8-5 cultured in the appropriate chemically defined medium

Strain	Spore count[a] (CFU/ml)	Enterotoxin concn (μg/ml) in[b]:		Erythemal response (diam, cm)[c] in:	
		Cell extract	Culture filtrate	Cell extract	Culture filtrate
3624	0	7.2	14.4	0.8	1.1
10240	0	3.6	14.4	0.6	1.1
8-5	0	3.6	7.2	0.7	1.0

[a] Heat-resistant spore count.
[b] Enterotoxin assayed by CIEP.
[c] Positive response indicated by a diameter of >0.5 cm.

Concentrated culture filtrates from strains 8-5, 3624, and 10240 were subjected to polyacrylamide gel electrophoresis. For strain 8-5, two bands were identified which corresponded to the two identifying bands of purified enterotoxin standard. Complete elimination of one band and diminution of the other band were evident when concentrated culture filtrate and antienterotoxin serum were combined. For strain 3624, one band was identified which corresponded to one of the two identifying bands of purified enterotoxin standard. The band of major interest was absent when purified enterotoxin was mixed with antienterotoxin serum. A substantial diminution of intensity for the band of major interest was evident when concentrated culture filtrate was reacted with antienterotoxin serum. For strain 10240, one very intense band was identified which corresponded to one of the two identifying bands of purified enterotoxin standard. An apparent shifting and a marked decrease in intensity of the band occurred when concentrated culture filtrate and antienterotoxin serum were mixed.

Enterotoxin production by batch-cultured cells in growth media. *C. perfringens* 10240, 3624, and 8-5 were batch cultured in R & S medium, and FTM. Enterotoxin was detected in mid-exponential-phase concentrated culture filtrates and concentrated cell extracts in the absence of light-refractile or heat-resistant spores in both media (Table 3).

DISCUSSION

Sporulation and enterotoxin production by *C. perfringens* are coincidental events which can occur under similar environmental conditions. Enterotoxin was detected without evidence of sporulation in batch R & S cultures, in chemostat cultures in a reformulated chemically defined medium, and in each batch-cultured test strain in FTM. These findings are supported by Labbe (18), who reported enterotoxin production by strain 10240 in a defined medium D, which is similar in composition to R & S medium, and by Granum et al. (15), who detected enterotoxin production by FTM-cultured cells in the absence of sporulation. Smith and McDonel (35) demonstrate the production of enterotoxin by polysomes both from an FTM culture and from a sporulating culture of strain 8798. Enterotoxin production by *C. perfringens* has generally not been reported in a growth-type medium such as FTM, which is believed to suppress sporulation (9, 37). The literature commonly associates enterotoxin

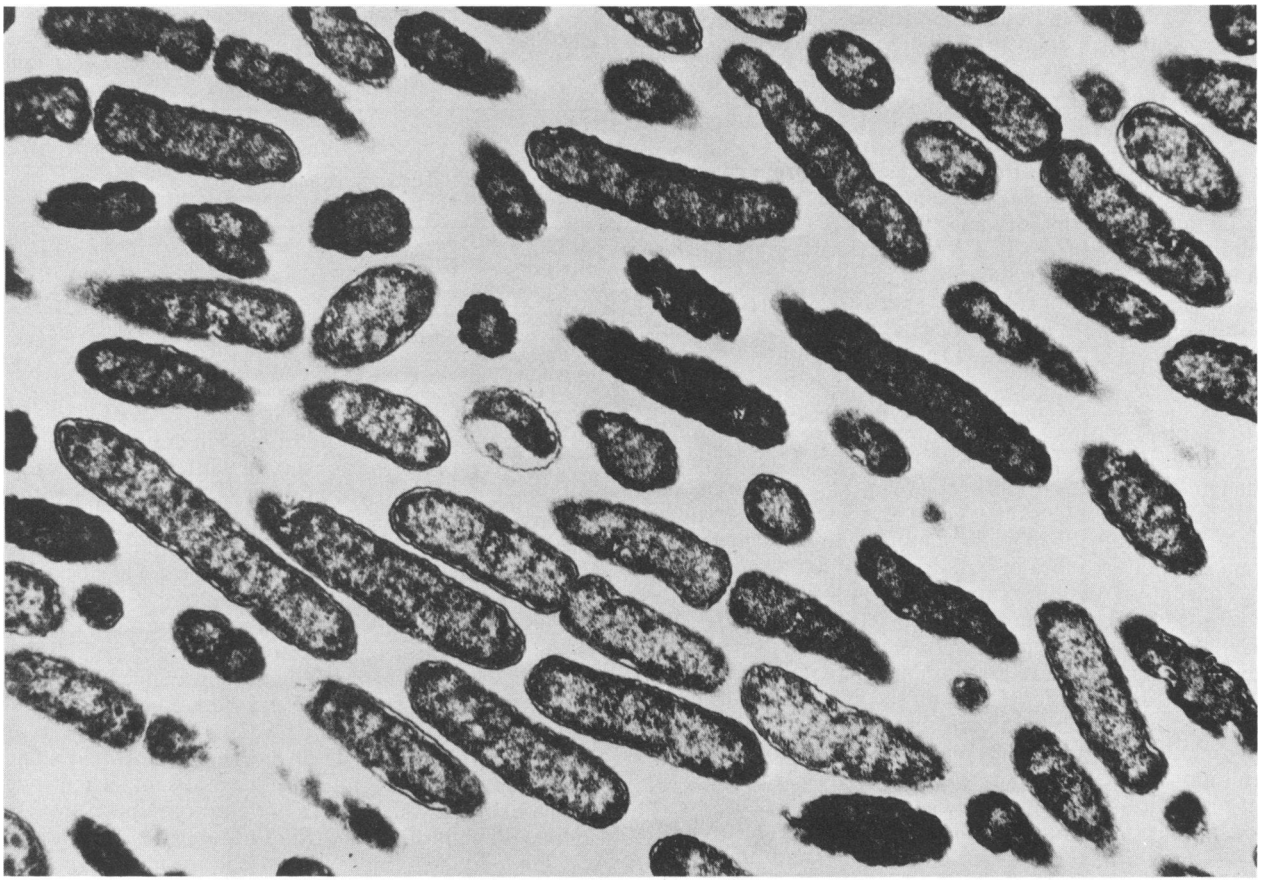

FIG. 1. Electron micrograph of *C. perfringens* 10240 cultured in a glucose-limited chemostat by using a reformulated chemically defined medium at a dilution rate of 0.084 h^{-1}. Magnification, ×22,960.

synthesis with sporulation, on the basis of such findings as those of Duncan and Strong (9), who cultured strain 10240 in FTM and reported a negative response for culture filtrates by the ligated ileal loop assay. The levels of enterotoxin produced by FTM cultures in the present study were below the detection limits of methods used in earlier studies. The use of less sensitive methods may account for the observation that sporulation is a prerequisite for enterotoxin synthesis.

Additional work with strain 8-5, generally considered a nonsporulating, nontoxigenic mutant (10, 23), showed enterotoxin production in DS medium in the absence of sporulation. Duncan et al. (10) did not detect toxin by the ligated ileal loop assay or the guinea pig erythemal assay in concentrated filtrates (33×) or cell extracts of strain 8-5 cultured in DS medium. Failure to concentrate sufficiently and low-level sensitivity of the assay procedures may account for this contradiction. Mutants have been isolated that are defective in their ability to sporulate and synthesize enterotoxin in DS sporulation medium (10). Mutants (Spo^-) which reverted to Spo^+ also regained the ability to produce enterotoxin, and a single gene mutation was presumed to be responsible for both characteristics. The findings with strain 8-5 indicate that enterotoxin is not a sporulation-specific gene product but can be produced by *C. perfringens* under conditions that support sporulation.

The detection of enterotoxin in cell extracts and culture filtrates in the absence of sporulation and the observed enterotoxin production by a nonsporulating mutant refute two accepted lines of evidence linking sporulation with enterotoxin production. A third line holds that proteins from spore coats of *C. perfringens* are serologically identical to the enterotoxin protein (11, 12). In contradiction, Aronson and Fitz-James (2) stated that the specific activity of the extracted spore coat fraction is less than 10% of the specific activity of the purified enterotoxin and might have been only a tightly bound contaminant. Walker et al. (40) examined the ultrastructure of sporulating cells of *C. perfringens* with an antienterotoxin stain and found that the enterotoxin protein was confined to the cytoplasm and was not associated with spore membranes or the spore coat. Roper et al. (32), using similar techniques, could not find a relationship between enterotoxin and the spore coat.

If the enterotoxin is a spore coat protein, an increase in the intracellular level of enterotoxin should be expected to precede the increase in the number of heat-resistant spores (10). However, a study of the time taken for enterotoxin formation revealed that the increase in the intracellular concentration of enterotoxin followed the increase in the number of heat-resistant spores by 2.5 to 5.0 h (7). Labbe and Duncan (20) reported that spore coat proteins of *C. perfringens* 8798, cultured in DS sporulation medium, were detected as early as 2.5 h after sporulation began. Enterotoxin was not detected intracellularly until 3.5 h after the inoculation of the vegetative cells into the sporulation medium. Proteins were extracted from the spore coat of *C. perfringens* 8798, separated by gel electrophoresis, and subjected to immunodiffusion with antienterotoxin. Labbe and Duncan (20) were unable to equate spore coat proteins

of enterotoxigenic strain 8798 with the enterotoxin, as reported by Frieben and Duncan (11, 12).

C. perfringens 10240 was confirmed to be sporogenic and toxigenic in DS medium with raffinose (4, 5, 21). Strains of *C. perfringens* definitely associated with food poisoning outbreaks do not produce detectable levels of enterotoxin in DS sporulation medium (5, 33, 34, 38). Skjelkvale and Duncan (33) concluded that poor sporulation frequencies may have been responsible for the failure to detect enterotoxin. High levels of sporulation have been observed with enterotoxin-negative strains (5, 21, 34, 39). Conversely, there were strains which produced enterotoxin in DS medium while maintaining a low sporulation frequency (5, 34). Stelma et al. (38) reported that only 4 of 14 strains implicated in food poisoning produce enterotoxin in DS medium. The enterotoxin-negative strains produced approximately the same number of spores per milliliter as the high-level enterotoxin producers. Stelma et al. concluded that inability to sporulate was not the cause of failure to produce enterotoxin. No clear connection between sporulation and enterotoxin production seems to exist. Craven et al. (5) suggested that the failure to detect enterotoxin in these cultures is caused, in part, by the loss of enterotoxigenicity from repeated subculturing. In the present study, *C. perfringens* 3624, 10240, and 8-5 were continuously cultivated in a chemostat, with no observable loss in enterotoxigenicity. Selection for nonsporulating cultures did not occur in the chemostat since inoculation of chemostat-cultured cells into sporulation medium resulted in sporulation comparable to that of the original stock culture batch cultured in DS medium.

C. perfringens 3624 sporulated and produced enterotoxin in the DS medium with raffinose. This strain is generally considered to be sporogenic and nonenterotoxigenic (9, 21, 37). Labbe and Rey (21) reported sporulation in DS medium with raffinose at a 93% level, but enterotoxin was not detected in the cell extracts by gel immunoelectrophoresis. Duncan and Strong (9) concentrated (33-fold) a culture filtrate of strain 3624 grown in DS medium and reported good sporulation in the absence of enterotoxin production in the ligated ileal loop assay. Stark and Duncan (37) concentrated (8.25-fold) a 24-h culture filtrate from DS medium. Although sporulation was evident, enterotoxin was not detected by electroimmunodiffusion. In support of the current findings, Uemura et al. (39) reported that *C. perfringens* 3624 sporulates in DS medium and produces enterotoxin (0.6 to 10 μg/ml). Enterotoxin was detected by using reversed passive hemagglutination but was not detected in the culture supernatant by using gel immunodiffusion. Failure to detect enterotoxin in other studies may have been due to insufficiently sensitive assays with high minimum levels of detection or to insufficient sample concentration (8, 15), or both. Culture filtrates were concentrated 100-fold in the present study.

Toxin was present in both cell extracts and culture filtrates. Toxin levels in the culture filtrates were consistently higher than those in the cell extracts. The substance being measured was confirmed to be toxin, and no evidence of cell lysis was found. Two forms of *C. perfringens* enterotoxin may exist, one that is secreted and one that is not. Support for this proposal comes from the characteristic double bands which identify the enterotoxin on polyacrylamide gels.

Enterotoxin production is not a function of sporulation or of physical manipulation of the cells. Nutritional composition of the culture-medium becomes a factor which may influence enterotoxigenesis, since the quantity of enterotoxin produced varied with culture medium. Nutrient balance may prevent sporulation while encouraging the production and secretion of enterotoxin.

TABLE 3. Sporulation and enterotoxin production by mid-exponential-phase *C. perfringens* ATCC 3624, NCTC 10240, and 8-5 batch cultures[a] in FTM and RS growth medium

Strain	Spore count[b] (CFU/ml)	Enterotoxin concn (μg/ml)[c] in:			
		FTM		RS medium	
		Cell extract	Culture filtrate	Cell extract	Cultrue filtrate
3624	0	0.9	3.6	3.6	28.8
10240	0	1.8	3.6	7.2	28.8
8-5	0	0.9	3.6	3.6	14.4

[a] Inoculum source was RS medium inoculated with cooked-meat medium stock cultures.
[b] Heat-resistant spore count.
[c] Enterotoxin assayed by CIEP.

ACKNOWLEDGMENTS

This work was supported by state funds and by U.S. Hatch Act funds.

We thank Rose Ullrich for preparation of the manuscript.

LITERATURE CITED

1. **Adams, M. H., B. D. Hendee, and A. M. Pappenheimer, Jr.** 1947. Factors involved in production of *Clostridium welchii* alpha toxin. J. Exp. Med. **85**:701–713.
2. **Aronson, A. I., and P. Fitz-James.** 1976. Structure and morphogenesis of the bacterial spore coat. Bacteriol. Rev. **40**:360–402.
3. **Boyd, M. J., M. A. Logan, and A. A. Tytell.** 1948. The growth requirements of *Clostridium perfringens* BP6K. J. Biol. Chem. **174**:1013–1025.
4. **Craven, S. E., and L. C. Blankenship.** 1982. Effect of purine derivatives, papaverine hydrochloride, and imidazole on enterotoxin by *Clostridium perfringens* type A. Can. J. Microbiol. **28**:851–859.
5. **Craven, S. E., L. C. Blankenship, and J. L. McDonel.** 1981. Relationship of sporulation, enterotoxin formation, and spoilage during growth of *Clostridium perfringens* type A in cooked chicken. Appl. Environ. Microbiol. **41**:1184–1191.
6. **Dische, F. E., and S. D. Elek.** 1957. Experimental food poisoning by *Clostridium welchii*. Lancet **ii**:71–74.
7. **Duncan, C. L.** 1973. Time of enterotoxin formation and release during sporulation of *Clostridium perfringens* type A. J. Bacteriol. **113**:932–936.
8. **Duncan, C. L., and E. B. Somers.** 1972. Quantitation of *Clostridium perfringens* type A enterotoxin by electroimmunodiffusion. Appl. Microbiol. **24**:801–804.
9. **Duncan, C. L., and D. H. Strong.** 1969. Ileal loop fluid accumulation and production of diarrhea in rabbits by cell-free products of *Clostridium perfringens*. J. Bacteriol. **100**:86–94.
10. **Duncan, C. L., D. H. Strong, and M. Sebald.** 1972. Sporulation and enterotoxin production by mutants of *Clostridium perfringens*. J. Bacteriol. **110**:378–391.
11. **Frieben, W. R., and C. L. Duncan.** 1973. Homology between enterotoxin protein and spore structural protein in *Clostridium perfringens*. Eur. J. Biochem. **39**:393–401.
12. **Frieben, W. R., and C. L. Duncan.** 1975. Heterogeneity of enterotoxin-like protein extract from spores of *Clostridium perfringens* type A. Eur. J. Biochem. **55**:455–463.
13. **Goldner, S., and M. Solberg.** 1981. Comparison of growth response by chemostat cultured and batch cultured *Clostridium perfringens* cells in various food substrates. J. Food Sci. **46**:138–142.
14. **Goldner, S. B., M. Solberg, and L. S. Post.** 1985. Development of a minimal medium for *Clostridium perfringens* by using an

anaerobic chemostat. Appl. Environ. Microbiol. **50**:202–206.
15. **Granum, P. E., W. J. Telle, O. Olsvik, and A. Stavin.** 1984. Enterotoxin formation by *Clostridium perfringens* during sporulation and vegetative growth. Int. J. Food Microbiol. **1**:43–49.
16. **Hauschild, A. W. H.** 1970. Erythemal activity of the cellular enteropathogenic factor of *Clostridium perfringens* type A. Can. J. Microbiol. **16**:651–654.
17. **Jayko, L. G., and H. C. Lichstein.** 1959. Nutritional factors concerned with growth and lecithinase production by *Clostridium perfringens*. J. Infect. Dis. **104**:142–151.
18. **Labbe, R. G.** 1981. Enterotoxin formation by *Clostridium perfringens* type A in a defined medium. Appl. Environ. Microbiol. **41**:315–317.
19. **Labbe, R. G., and C. L. Duncan.** 1974. Sporulation and enterotoxin production by *Clostridium perfringens* under conditions of controlled pH and temperature. Can. J. Microbiol. **20**:1493–1501.
20. **Labbe, R. B., and C. L. Duncan.** 1977. Spore coat protein and enterotoxin synthesis in *Clostridium perfringens*. J. Bacteriol. **131**:713–715.
21. **Labbe, R. G., and D. K. Rey.** 1979. Raffinose increases sporulation and enterotoxin production by *Clostridium perfringens* type A. Appl. Environ. Microbiol. **37**:1196–1200.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
23. **Loeffler, A., and R. G. Labbe.** 1983. Intracellular proteases during sporulation and enterotoxin formation by *Clostridium perfringens* type A. Curr. Microbiol. **8**:187–190.
24. **Markus, Z., and G. J. Silverman.** 1969. Enterotoxin B synthesis by replicating and nonreplicating cells of *Staphylococcus aureus*. J. Bacteriol. **97**:506–512.
25. **Meers, J. L.** 1972. The regulation of amylase production in *Bacillus licheniformis*. Antonie van Leeuwenhoek J. Microbiol. **38**:585–590.
26. **Murata, R., A. Yamanota, S. Soda, and A. Ito.** 1965. Nutritional requirements of *Clostridium perfringens* BP6K for alpha toxin production. Jpn. J. Med. Sci. Biol. **18**:189–202.
27. **Naik, H. S., and C. L. Duncan.** 1977. Rapid detection and quantitation of *Clostridium perfringens* enterotoxin by counterimmunoelectrophoresis. Appl. Environ. Microbiol. **34**:125–128.
28. **Niilo, L.** 1977. Enterotoxin formation by *Clostridium perfringens* type A studied by use of fluorescent antibody. Can. J. Microbiol. **23**:908–915.
29. **Novick, A., and L. Szilard.** 1950. Description of the chemostat. Science **112**:715–716.
30. **Priest, F. G.** 1977. Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol. Rev. **41**:711–753.
31. **Riha, W. E., Jr., and M. Solberg.** 1971. Chemically defined medium for the growth of *Clostridium perfringens*. Appl. Microbiol. **22**:738–739.
32. **Roper, G., J. A. Short, and P. D. Walker.** 1976. The ultrastructure of *Clostridium perfringens* spores, p. 279–296. *In* A. M. Baker, J. Wold, D. J. Ellar, G. H. Dring, and G. W. Gould (ed.), Spore research, 1976. Academic Press, Inc. (London), Ltd., London.
33. **Skjelkvale, R., and C. L. Duncan.** 1975. Enterotoxin formation by different toxigenic types of *Clostridium perfringens*. Infect. Immun. **11**:563–575.
34. **Skjelkvale, R., M. F. Stringer, and J. L. Smart.** 1979. Enterotoxin production by lecithinase positive and lecithinase negative *Clostridium perfringens* isolated from food poisoning outbreaks. J. Appl. Bacteriol. **47**:329–339.
35. **Smith, W. P., and J. L. McDonel.** 1980. *Clostridium perfringens* type A: in vitro system for sporulation and enterotoxin synthesis. J. Bacteriol. **144**:306–311.
36. **Sonenshein, A. L., and K. M. Campbell.** 1978. Control of gene expression during sporulation, p. 179–192. *In* G. Chambliss and J. C. Vary (ed.), Spores VII. American Society for Microbiology, Washington, D.C.
37. **Stark, R. L., and C. L. Duncan.** 1971. Biological characteristics of *Clostridium perfringens* type A enterotoxin. Infect. Immun. **4**:89–96.
38. **Stelma, G. N., Jr., J. C. Wimsatt, P. E. Kauffman, and D. B. Shah.** 1984. Radioimmunoassay for *Clostridium perfringens* enterotoxin and its use in screening isolates implicated in food poisoning outbreaks. J. Food Prot. **46**:1069–1073.
39. **Uemura, T., G. Sakaguchi, and H. P. Riemann.** 1973. In vitro production of *Clostridium perfringens* enterotoxin and its detection by reversed passive hemagglutination. Appl. Microbiol. **26**:381–385.
40. **Walker, P. D., J. A. Short, and G. Roper.** 1975. Location of antigens on ultrathin sections of sporeforming bacteria, p. 572–579. *In* P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), Spores VI. American Society for Microbiology, Washington, D.C.
41. **Whooley, M. A., J. A. O'Callaghan, and A. J. Mcloughlin.** 1982. Effect of substrate on the regulation of exoprotease production by *Pseudomonas aeruginosa* ATCC 10145. J. Gen. Microbiol. **129**:981–988.
42. **Wiersma, M., and W. Harder.** 1978. A continuous culture study of the regulation of extracellular protease production in *Vibrio* SA1. Antonie van Leeuwenhoek J. Microbiol. **44**:141–155.
43. **Wray, W., T. Boulikas, V. P. Wray, and R. Hancock.** 1981. Silver staining of proteins in polyacrylamide gels. Anal. Biochem. **118**:197–203.
44. **Wu, C.-H., and M. S. Bergdoll.** 1971. Stimulation of enterotoxin B production. II. Synthetic medium for staphylococcal growth and enterotoxin B production. Infect. Immun. **3**:784–792.
45. **Young, M., and J. Mandelstam.** 1979. Early events during bacterial endospore formation. Adv. Microb. Physiol. **20**: 104–154.