JOURNAL OF BACTERIOLOGY, Apr. 1972, p. 378-391

Vol. 110, No. 1
Printed in U.S.A.

Sporulation and Enterotoxin Production by Mutants of *Clostridium perfringens*

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Received for publication 17 December 1971

The ability of *Clostridium perfringens* type A to produce an enterotoxin active in human food poisoning has been shown to be directly related to the ability of the organism to sporulate. Enterotoxin was produced only in a sporulation medium and not in a growth medium in which sporulation was repressed. Mutants with an altered ability to sporulate were isolated from an sp^+ ent^+ strain either as spontaneous mutants or after mutagenesis with acridine orange or nitrosoguanidine. All sp_0^- mutants were ent^-. Except for one isolate, these mutants were not disturbed in other toxic functions characteristic of the wild type and unrelated to sporulation. A total of four of seven osp_0 mutants retained the ability to produce detectable levels of enterotoxin. None of the ent^- mutants produced gene products serologically homologous to enterotoxin. A total of three sp^- mutants, blocked at intermediate stages of sporulation, produced enterotoxin. Of these mutants, one was blocked at stage III, one probably at late stage IV, and one probably at stage V. A total of three sp^+ revertants isolated from an sp^- ent^- mutant regained not only the ability to sporulate but also the ability to produce enterotoxin. The enterotoxin appears to be a sporulation-specific gene product; however, the function of the enterotoxin in sporulation is unknown.

The morphological changes accompanying bacterial sporulation may be divided into sequential stages starting with the vegetative cell (stage 0) and ending with liberation of the mature spore (stage VII). These stages are determined by the appearance of cell thin sections in an electron microscope (26) and are generally adopted reference points by which comparisons can be made between different species. Enzymes and other gene products which are known either to appear first or to increase significantly during sporulation have been described in recent reviews (12, 20, 27). Only a few of these gene products, such as dipicolinic acid, sporal antigens, proteins of the spore coat, and some of the enzymes involved in synthesis of the cortical peptidoglycan, are known definitely to be associated with the spore or sporulating cell (12). Other products that are not necessarily sporulation specific may be the result of sporulation-related biochemical events that are essential for the morphological changes occurring during spore development.

Most studies of the biochemical events that accompany sporulation have been conducted using *Bacillus* species. The few studies that have been made using anaerobic spore-forming clostridia have dealt with the relationship between toxin-producing ability and the ability to form spores (27). Many protein toxins are known to be synthesized by members of the genus *Clostridium*. Their synthesis in many instances may be restricted to the sporulation period. However, the role of these toxic proteins in the physiology of the cells producing them generally is unknown (35). The production of at least one toxin, the lethal toxin of *C. histolyticum*, has been shown to be directly related to the ability of sporulating cells to reach stage II of sporulation (29). When the production of lethal toxin was compared in a series of asporogenous (sp^-) mutants of *C. histolyticum*, the strains blocked at stage II of sporulation or later produced toxin (tox^+) in normal amounts. However, three sp^- tox^- mutants were blocked at stage 0. One oligosporogenous (osp) revertant recovered from one of the sp^- tox^- mutants had recovered the ability to produce some toxin. This direct relationship between sporulation and toxigenicity was not observed when asporogenous mutants of *C.*

perfringens, induced by treatment with acridine orange, were tested for their ability to produce the lethal alpha toxin (28). This was not surprising, since it is known that *C. perfringens* alpha toxin is produced during the logarithmic phase of growth and that strains generally sporulate best in media where little alpha toxin is produced.

C. perfringens is known to be a major causative organism in human food poisoning (6). Although ingestion of viable cells is usually considered to be necessary for production of this type of food poisoning, it has been established that an enterotoxin is produced by certain strains of *C. perfringens* and that this toxin is responsible for the food-poisoning symptoms (9, 10, 15, 16, 33, 34). The enterotoxin can be detected in vitro only when cells are sporulating and not when they are grown in a growth medium in which sporulation does not occur. This is consistent with the fact that the organism is known to sporulate readily in the intestine and under such conditions would synthesize and release the biologically active toxin. Production of the enterotoxin only in a sporulation medium and not in a growth medium raised the question of the relationship of this biochemical event to sporulation in this organism. In this communication, evidence is presented which indicates that a direct relationship exists between enterotoxin production and spore formation in *C. perfringens* and that the enterotoxin is a sporulation-specific gene product.

MATERIALS AND METHODS

Bacterial strain. *C. perfringens* type A, strain NCTC 8798 (Hobbs' serological type 9), was the wild-type strain. The enteropathogenic activity and enterotoxin-producing ability of this strain have been reported (10, 33, 34). The sporulation frequency of this strain varied in different culture preparations and ranged from about 5.0×10^{-2} to 1.2×10^{-1}. The spores produced are heat resistant (34).

Isolation of mutants and revertants. The wild-type colonies of *C. perfringens* are usually opaque. However, some sp^- mutants form transparent colonies. Clones of this phenotype also may appear spontaneously as sectors in the wild-type opaque colonies. Mutants with an altered ability to sporulate were isolated from such transparent sectors or colonies either as spontaneous mutants or after mutagenesis with a noninhibitory concentration of acridine orange or nitrosoguanidine, as described (28). Spontaneous sp^+ revertants of sp^- mutants were isolated as opaque colonies after heating for 10 min at 80 C and plating of about 10^{10} spores.

Stock cultures were stored lyophilized in skim milk (Difco) at −20 C. Working stock cultures were maintained in cooked meat medium (Difco) at room temperature.

Growth and sporulation conditions. An active culture was obtained by overnight (16 to 20 hr) growth of each strain in 10 ml of fluid thioglycolate (FTG) medium (BBL). This culture was then inoculated into 100 ml of DS sporulation medium (7) and incubated for 3 hr. The entire culture was then inoculated into 1,000 ml of DS sporulation medium and incubated for 24 hr (procedure A). The sporulation frequency was then determined. Alternatively, 10 ml of the active FTG culture was inoculated into 1,000 ml of DS sporulation medium followed by incubation for 24 hr (procedure B). All incubations were at 37 C. When desired, sterile culture filtrates were obtained by filtration of culture supernatant fluids through Seitz filters.

Determination of sporulation frequency. The total colony-forming units (V) in a culture was determined by diluting the culture in 0.1% peptone water and plating in tryptone-sulfite-neomycin (TSN) agar (21) without added antibiotics. The total viable heat-resistant spore population (S) was determined similarly after heating of a portion of the culture at 75 C for 20 min. Incubation was at 37 C under a 90% N_2-10% CO_2 mixture. The frequency of spores per viable cell was measured as the ratio S/V. The spore recovery was also tested systematically on media supplemented with egg lysozyme (2 μg/ml) to characterize mutants with a lysozyme-dependent germination (5).

All the mutants were checked also for sporulation on Ellner's medium and SEC medium (28). In no case was sporulation better in these media than in DS medium.

In vitro enterotoxin production and determination. Enterotoxin production was tested both in culture supernatant fluids and in cell extracts. Culture supernatant fluids were obtained from cultures grown for 24 hr at 37 C in DS sporulation medium by procedure A. The fluids were passed through a Seitz filter to remove residual organisms and were then concentrated by dialysis against Carbowax 20,000 (polyethylene glycol, Union Carbide Corp.) at 4 C. The filtrates were reconstituted with 0.9% saline to a concentration 33 times that of the original supernatant fluid. Concentrated filtrates were stored at −20 C.

Cell extracts were obtained from cells grown for 8 hr at 37 C in DS sporulation medium by procedure B. In some instances, FTG was used instead of DS medium to obtain nonsporulating cells for cell extracts. Cells were suspended in 10 ml of cold 0.9% saline, and extracts were obtained with a Branson Sonifier. The extraction chamber was cooled in an ethyl alcohol, dry-ice bath. The degree of cell disruption was monitored by phase-contrast microscopy. Vegetative cell or sporangial disruption was achieved after 15 to 20 min; spores, when present, were not ruptured in this time interval. The extract was centrifuged at 4 C and 27,000 $\times$ *g* for 10 min. The supernatant fluid was removed and stored at −20 C.

Concentrated culture filtrates were tested qualitatively for the presence of enterotoxin by their ability to induce fluid accumulation in ligated ileal loops of rabbit intestine. Cell extracts and culture filtrates were tested quantitatively for the presence of entero-

toxin by their ability to produce erythema when injected intradermally into depilated guinea pigs as previously described (33). The activity of samples was always compared to a standard injected into the same guinea pig. The standard was a partially purified enterotoxin preparation containing 1,000 erythemal units per ml. The standard was stored at −20 C.

Animal assay for enteropathogenicity. Viable cells were tested for enteropathogenicity by their ability to induce fluid accumulation in ligated ileal loops of rabbit intestine or overt diarrhea after injection into the normal, nonligated rabbit intestine. The length and fluid volume of positive ileal loops were measured to calculate the loop volume (milliliters)/length (centimeters) ratio (V/L ratio), which was used as a measure of the response obtained. The operative procedures and techniques used have been described (8, 9). For ileal loop challenge, 1 ml of a 16-hr FTG culture was inoculated into 10 ml of skim milk and incubated for 4 hr at 37 C. Loops were injected with 2 ml of this culture. After sacrifice of the animals at 20 to 24 hr, the sporulation frequency of *C. perfringens* in both the challenged loops and in control, nonchallenged loops was determined as described previously (11). In testing for diarrhea-producing ability, cultures were prepared by using the inoculation sequence described in procedure A. However, the cells were harvested from the final DS medium after only 4 hr of incubation. The cells were suspended in 25 ml of skim milk, and 10 ml was used for each intraluminal challenge (8).

Antisera. Antiserum against the enterotoxin was obtained by immunization of New Zealand White rabbits with enterotoxin found in the cell extract of the wild-type strain NCTC 8798. The enterotoxin had been partially purified by Sephadex G-200 column chromatography (33). Specific antiserum was obtained by absorption of the antiserum against crude cell extract of sp^- mutant 8-1.

Clostridium diagnostic serum, *C. perfringens* (*welchii*) type A, was obtained from Wellcome Research Laboratories.

Neutralization of biological activity. DS medium culture filtrates and cell extracts, at various concentrations in 0.9% NaCl, were mixed 1:1 with immune serum prepared against the enterotoxin or with *C. perfringens* type A diagnostic serum that had been diluted previously 1:4 with saline. All antigen-antitoxin mixtures were incubated at 37 C for 30 min before assay for erythemal activity in guinea pig skin.

Immunodiffusion. Immunodiffusion studies were performed on glass slides (3 by 1 inches) with an agar layer consisting of 1% Noble agar (Difco), 1% NaCl, and 1:10,000 merthiolate. Gelman immunodiffusion equipment was used for holding slides and cutting of wells (Gelman Instrument Co.). Slides were incubated for 24 hr at room temperature in a humidity chamber and photographed using a Polaroid MP-3 camera (Polaroid Corp.).

Theta toxin production. Theta toxin, present in culture filtrates adjusted to *p*H 6.8 with phosphate buffer, was determined by using sheep blood-agar (28).

Hyaluronidase (mu toxin) production. Hydrolysis of sodium hyaluronidate in the presence of bovine albumin fraction-V was measured by the plate test of Smith and Willett (30). Both sterile culture filtrates of strains grown in DS medium by procedure A for 6, 24, and 48 hr and viable cells were tested. Viable cells were from cultures grown for 16 to 20 hr in cooked meat medium.

Lecithinase (alpha toxin) production. Sterile culture filtrates of strains grown in DS medium for 6, 24, and 48 hr and viable cells were tested for activity on plates of McClung-Toabe egg yolk-agar (22). Plates spotted with cooked meat medium cultures were incubated anaerobically for 48 hr at 37 C. Plates spotted with culture filtrates were incubated for 12 hr at 37 C. In addition, sterile culture filtrates were tested for activity by the lecithovitellin test (38).

Collagenase (kappa toxin) production. The test for collagenase activity, using bovine achilles tendon collagen (Worthington Biochemical Corp.) as the substrate, was that described in the Worthington enzymes manual (Worthington Biochemical Corp., Freehold, N.J.). Filtrates from 6-, 24-, and 48-hr DS cultures were tested.

Neuraminidase production. Neuraminidase hydrolysis of mucin was carried out by the procedure described in the Worthington enzymes manual (Worthington Biochemical Corp.) except that digestion was for 60 instead of 30 min. The release of *N*-acetylneuraminic acid was tested for by using the thiobarbituric acid method of Aminoff (1). Filtrates from 6-, 24-, and 48-hr DS medium cultures were tested.

Protease production. Mutants were tested for both gelatinase and caseinase activity. For gelatinase activity, filtrates from 6-, 24-, and 48-hr DS cultures were tested by spotting on plates of 0.4% gelatin (Difco) plus 1% agar. The plates were incubated for 18 hr at 37 C and flooded with 15% $HgCl_2$ in 20% HCl. Clear zones surrounding the spots indicated gelatinase activity. Tests for caseinase activity were conducted by using plates inoculated with viable cells of 16- to 20-hr cooked meat medium cultures. Two different plate media used for the caseinase test were Trypticase soy agar (BBL) plus 3.3% skim milk and DS sporulation medium plus 3.3% skim milk and 1.5% agar. The plates were incubated anaerobically at 37 C and examined after 24, 48, and 144 hr of incubation for caseinase activity.

Protein determination. Protein determinations were made by the method of Lowry et al. (19).

Dipicolinic acid determination. For the colorimetric assay of dipicolinic acid (DPA) in spores, a total of 100 ml of sporulating cell culture grown for 16 hr in DS sporulation medium was centrifuged, and the pellet was suspended in 5 ml of distilled water. This suspension was autoclaved for 15 min to release the DPA. The culture supernatant fluid of the sporulating cells was also tested for the presence of DPA, since the possibility existed that mutants might be synthesizing DPA but losing it to the surrounding medium. A total of 100 ml of supernatant fluid was acidified with concentrated HCl and extracted three times with 1-butanol by the procedure

of Waites et al. (36). The presence of DPA was determined by the procedure of Janssen et al. (18) using the modified reagent of Rotman and Fields (24).

Electron and phase-contrast microscopy. Mutants grown in DS sporulation medium were initially screened by examination under negative contrast with an electron microscope to determine the stage at which sporulation was blocked. In addition, thin sections of preparations of certain mutants and the wild-type strain NCTC 8798 grown for 8 hr in DS medium by procedure B were examined by electron microscopy. Thin sections of the wild type grown for 16 hr in FTG growth medium were also examined. An osmium tetroxide-glutaraldehyde mixture fixation was used. Cells from 30 ml of culture were centrifuged for 5 min at 3,000 × *g*. The pellet, while chilled on cracked ice, was fixed for 2 hr in a mixture of 1 ml of 0.2 M cacodylate buffer (*p*H 7.3), 0.5 ml of 5% oxmium tetroxide, and 1.5 ml of 2.5% glutaraldehyde. The pllet was washed once with 0.066 M cacodylate buffer (*p*H 7.3), and mixed with molten agar on a glass slide. The agar was cut into small blocks and dehydrated by a 10-min exposure in each of a graded series of 35, 70, 95, and 100% ethanol, followed by two exposures of 20 min each in propylene oxide. The agar blocks were then embedded in Spurr's resin (32). Thin sections, obtained with a Porter-Blum Sorvall MT-2 microtome and a diamond knife, were mounted on 200-mesh copper grids which had been coated with carbon and parlodion. Sections were stained for 10 min with uranyl acetate and lead citrate and examined with a Zeiss EM 9A electron microscope.

For phase-contrast photomicrography, cells were immobilized on thin, 1% agar layers on a glass slide and covered with a glass cover slip. The preparations were examined and photographed with a Zeiss phase-contrast microscope mounted with a Polaroid MP-3 camera.

RESULTS

Initial classification of mutants and determination of sporulation frequency. The frequency of sporulation varied somewhat in different culture preparations of the same strain. However, the mutants could be divided into the following three classes based on their sporulation frquencies at 24 hr in DS sporulation medium: sp^+, which had a frequency of at least 10^{-2}; sp^-, in which no heat-resistant spores were formed; and osp, which usually had a frequency of 10^{-4} or less (Table 1). The sporulation frequency of the mutants in vivo in the rabbit ileal loop is also shown in Table 1. It was of interest to know if discrepancies existed in the ability to sporulate in vivo versus in vitro, since the in vivo rabbit ileal loop test was used as a measure of pathogenicity of the viable cells. *C. perfringens* spore counts in control ileal loops injected only with DS ranged from 0 to 3.1×10^2 spores per loop. The spore counts in ileal loops injected with sp^- strains ranged from 0 to 5.0×10^2. Therefore, the in vitro and in vivo responses of these strains were essentially identical. Only two of the osp strains (8-8 and 8-21) seemed to sporulate higher in vivo than in vitro. However, it is probable that the higher spore counts were due to a higher background of indigeneous spores.

About half of the transparent colony mutants isolated from *C. perfringens* are sp^+. Mutant 8-2 is an example of such a transparent sp^+ strain. The spores of mutants 8-4 and 8-6 are dependent on exogenous lysozyme for germination. In the presence of 2 μg of lysozyme/ml of plating medium, the heated sporulated cultures appear as sp^+. In the absence of lysozyme, they appear as osp. Such lysozyme dependence has been previously described (5) and is related to aberrant coat formation in these two mutants; fragments of coats are formed but do not assemble around the cortex. Mutant R-3 is a revertant isolated from mutant 8-6 that is no longer lysozyme dependent and that produces normal spore coats (M. Cassier and A. Ryter, *in press*).

Mutant 8-15 was unstable and tended to revert to the wild type. However, in testing for in vitro toxin production, the mutant was osp.

Enterotoxin production and enteropathogenic activity of the mutant classes. The enterotoxic activities of the wild-type and the sp^+ mutants are shown in Table 2. All sp^+ strains were capable of producing fluid accumulation in the rabbit ileal loop (a positive response) when either viable cells or concentrated DS medium culture filtrates of the sporulating cultures were tested. In addition, enterotoxin as measured by its ability to produce erythema in guinea pig skin was detected both extracellularly in 24-hr DS culture filtrates and intracellularly in sporulating cells grown for 8 hr in DS sporulation medium. Therefore, these strains were considered enterotoxin positive (ent^+). The enterotoxin concentration ranged from 6.2 to 34.7 erythemal units/ml of culture filtrate and from 7.4 to 100.8 erythemal units/mg of protein in cell extracts of 8-hr cultures. Cultures grown for 8 hr in DS medium had heat-resistant spores, but the spores were not released from the sporangia until about 10 to 12 hr. The erythemal activity in the culture filtrates and cell extracts could be neutralized with absorbed antiserum prepared against the enterotoxin but not with *C. perfringens* type A diagnostic serum.

When FTG growth medium was used instead of DS medium, concentrated culture filtrates were negative in rabbit ileal loops, and

TABLE 1. *Origin and sporulation frequencies of the Sp⁺, Osp, and Sp⁻ classes of mutants*

Strain	Class	Origin	24-Hr spore counts/ml of DS sporulation medium[a] (frequency S/V)[b]	Spore counts/ileal loop injected with 4-hr milk culture (frequency S/V)[c]
NCTC 8798	Sp⁺	Wild type	1.2×10^{7} / 9.2×10^{-2}	6.1×10^{6} / 2.8×10^{-1}
8-2	Sp⁺	AO[d]	1.1×10^{6} / 1.4×10^{-2}	2.2×10^{6} / 1.3×10^{-2}
8-4	Sp⁺; Lzd[e]	Spontaneous	2.4×10^{7} / 6.8×10^{-2}	8.5×10^{2} / 3.0×10^{-3}
8-6	Sp⁺; Lzd	Spontaneous	2.5×10^{6} / 8.9×10^{-2}	2.4×10^{4} / 1.0×10^{-5}
R-3	Sp⁺	Lzd⁻ revertant of 8-6	1.1×10^{7} / 9.0×10^{-2}	Not tested
8-3	Osp	AO	$1.2 \times 10^{1} - 1.9 \times 10^{5}$ / $3.3 \times 10^{-6} - 5.5 \times 10^{-6}$	6.9×10^{5} / 5.9×10^{-3}
8-8	Osp	Spontaneous	$3.7 \times 10^{1} - 8.4 \times 10^{2}$ / $5.6 \times 10^{-6} - 1.0 \times 10^{-5}$	4.0×10^{5} / 3.0×10^{-2}
8-14	Osp	AO	$1.2 \times 10^{2} - 4.2 \times 10^{4}$ / $8.0 \times 10^{-4} - 2.8 \times 10^{-3}$	4.0×10^{4} / 2.8×10^{-3}
8-15	Osp	AO	$3.3 \times 10^{2} - 2.6 \times 10^{4}$ / $3.5 \times 10^{-4} - 8.4 \times 10^{-3}$	6.8×10^{5} / 7.2×10^{-3}
8-21	Osp	AO	$5.3 \times 10^{2} - 9.9 \times 10^{2}$ / $1.2 \times 10^{-5} - 3.0 \times 10^{-5}$	3.3×10^{6} / 1.8×10^{-2}
8-22	Osp	AO	$1.2 \times 10^{2} - 1.1 \times 10^{3}$ / $6.3 \times 10^{-5} - 1.1 \times 10^{-4}$	5.8×10^{4} / 1.2×10^{-3}
8-23	Osp	AO	$1.8 \times 10^{1} - 8.0 \times 10^{3}$ / $4.1 \times 10^{-5} - 5.0 \times 10^{-4}$	5.5×10^{2} / 2.3×10^{-5}
8-1	Sp⁻	AO	0	6.1×10^{2} / 1.0×10^{-5}
8-5	Sp⁻	Spontaneous	0	0.4×10^{1} / 1.5×10^{-7}
8-7	Sp⁻	Spontaneous	0	3.2×10^{1} / 3.4×10^{-7}
8-16	Sp⁻	AO	0	4.5×10^{2} / 1.3×10^{-6}

[a] In some instances, the counts and frequencies given are the ranges of values obtained in different culture preparations.

[b] S/V: total viable, heat-resistant spores/total colony-forming units.

[c] Spore range of the Sp⁻ class-control loops: 0 to 3.1×10^{2}; avg 4.0×10^{1}.

[d] AO: acridine orange.

[e] Sp⁺: when 2 μg of lysozyme per ml is added to the plating medium for recovery of spores in cultures heated 10 min at 80 C. If lysozyme is not present, the heated cultures appear as Osp.

[f] NTG: nitrosoguanidine.

TABLE 1—*Continued*

Strain	Class	Origin	24-Hr spore counts/ml of DS sporulation medium[a] (frequency S/V)[b]	Spore counts/ileal loop injected with 4-hr milk culture (frequency S/V)[c]
8-17	Sp^-	AO	0	$\frac{2.5 \times 10^1}{7.8 \times 10^{-8}}$
8-20	Sp^-	AO	0	$\frac{3.1 \times 10^1}{3.4 \times 10^{-7}}$
8-46	Sp^-	NTG[f]	0	$\frac{5.0 \times 10^2}{2.6 \times 10^{-5}}$
8-47	Sp^-	NTG	0	$\frac{0.7 \times 10^1}{9.3 \times 10^{-5}}$
8-61	Sp^-	NTG	0	0
8-62	Sp^-	NTG	0	$\frac{2.2 \times 10^1}{5.4 \times 10^{-6}}$
8-65	Sp^-	NTG	0	$\frac{2.3 \times 10^2}{4.9 \times 10^{-5}}$

TABLE 2. *Enterotoxic activity of the Sp^+, Osp, and Sp^- classes of mutants*

Strain	Class	Activity in rabbit ileal loops				Erythemal activity[b]	
		4-Hr milk cultures		33 × DS culture filtrate			
		Vol/length ratio[a]	No. of loops +/no. tested	Vol/length ratio	No. of loops +/no. tested	Units/ml 24-hr culture filtrate	Units/mg of protein in 8-hr cell extract
NCTC 8798	Sp^+	0.9	4/4	1.8	4/4	34.7	51.2
8-2	Sp^+	0.5	5/8	0.8	6/7	6.2	100.8
8-4	Sp^+	0.9	4/4	1.2	3/3	15.8	13.4
8-6	Sp^+	0.8	4/4	1.6	4/4	17.8	7.4
R-3	Sp^+	0.9	2/2	1.7	2/2	+	98.0
8-3	Osp	0.4	5/9	–	0/5	–[d]	–
8-8	Osp	–[c]	0/7	–	0/5	–	0.64; –
8-14	Osp	–	0/3	–	0/3	–	–
8-15	Osp	–	0/3	–	0/3	–	–
8-21	Osp	–	0/9	–	0/8	4.5; –	–
8-22	Osp	–	0/6	–	0/4	1.3; –	–
8-23	Osp	–	0/4	–	0/6	2.8; –	3.8; –
8-1	Sp^-	–	0/8	–	0/9	–	–
8-5	Sp^-	–	0/6	–	0/5	–	–
8-7	Sp^-	0.1	2/12	1.1	10/14	3.2	0.9
8-16	Sp^-	0.4	3/8	0.1	2/9	–[d]	–
8-17	Sp^-	0.9	10/10	2.0	6/6	2.3	2.1
8-20	Sp^-	0.6	6/8	2.0	5/6	7.3	96.2
8-46	Sp^-	0.2	3/13	–	0/4	–[d]	–
8-47	Sp^-	–	0/3	–	0/2	–	–
8-61	Sp^-	–	0/3	–	0/2	–	–
8-62	Sp^-	–	0/3	–	0/2	–	–
8-65	Sp^-	–	0/9	–	0/2	–[d]	–

[a] Ileal loop volume (milliliters)/length (centimeters) ratio as measured 20 to 24 hr postchallenge.

[b] In some instances, both a negative and positive result are indicated, showing the variability in different culture preparations.

[c] Negative sign indicates no response.

[d] Erythemal activity neutralized by *C. perfringens* type A diagnostic antiserum.

enterotoxin, as measured by its erythemal activity, could not be detected in either culture filtrates or cell extracts. Heat-resistant spores are not present in 8-hr FTG cultures. At 24 hr, very low levels of spores may occasionally be detected; however, the sporulation frequency is usually 10^{-7} to 10^{-6}.

The enterotoxic activities of the osp mutants are presented also in Table 2. Only 1 mutant, 8-3, produced fluid accumulation in the rabbit ileal loop when viable cells were tested. However, a low V/L ratio was obtained. Although mutant 8-3 produced an erythemal reaction in guinea pig skin, it was neutralized with *C. perfringens* type A diagnostic serum. The reaction was thus due to some other *C. perfringens* toxin. Mutant 8-3 is atypical in that it is sensitive to a virulent phage (ϕb) to which the wild-type strain and all other mutants are resistant. Nevertheless, as are the wild-type strain and most of the mutants, 8-3 is inducible lysogenic for the same phage (ϕx) (*unpublished data*). Thus, the possibility that mutant 8-3 is a contaminant can be eliminated. None of the concentrated culture filtrates of the osp mutants were positive in rabbit ileal loop. When 24-hr culture filtrates were tested for erythemal activity, three of the mutants, 8-21, 8-22, and 8-23, had activity. The production of enterotoxin was not consistent in different culture preparations and often could not be detected. Repeated testing of the culture filtrates of the remaining osp mutants did not reveal enterotoxin production. Enterotoxin activity was detected in the cell extracts of mutants 8-8 and 8-23, but again variations were obtained between different cell preparations. The erythemal activity of the various preparations was neutralized with antiserum against the enterotoxin but not type A diagnostic serum. The variation in enterotoxin production in different culture preparations of the four mutants possessing erythemal activity was apparently dependent on the frequency of sporulation which also varied in different culture preparations. The sensitivity of the skin test has been shown to be about 1,000 times greater than the ileal loop test (13). This allowed detection of low concentrations of enterotoxin by the skin test which could not be detected by the ileal loop test with the mutants. Repeated testing of culture filtrates and cell extracts of mutant 8-3 failed to show any erythemal activity due to enterotoxin. Therefore this mutant was considered ent$^-$. Of the seven mutants tested, 8-8, 8-21, 8-22, and 8-23 were considered ent$^+$. These mutants were blocked at stage zero of sporulation (osp$_0$) as determined by negative-contrast electron microscopy, but in the cultures of 8-21 and 8-23 many lysed bodies were noticed that might be abnormal spores. A minor number of cells that had reached stage III were observed in 8-21.

The enterotoxic activities of the sp$^-$ class of mutants are shown in Table 2. Three of the mutants, 8-7, 8-17, and 8-20, were considered ent$^+$ since they produced positive responses in the ileal loop test and possessed erythemal activity in both culture filtrates and cell extracts.

Mutants 8-16 and 8-46 were occasionally positive in the ileal loop test. However, these were apparently nonspecific responses, since enterotoxin could not be detected by the skin test. These two mutants and mutant 8-65 possessed erythemal activity, but the activity was neutralized by *C. perfringens* type A diagnostic serum and not by enterotoxin antiserum. Thus, 3 of the 11 sp$^-$ mutants tested were ent$^+$.

Serological testing for homologous enterotoxin protein. In order to exclude the possibility that biologically inactive enterotoxin protein was produced by the ent$^-$ mutants, all mutants were tested for the presence of serologically homologous enterotoxin protein. Cell extracts of 8-hr cultures grown in DS medium were used as the crude protein material. The extracts were tested both unconcentrated and concentrated 10 times. A cell extract of the wild-type strain grown for 8 hr in FTG growth medium was also used. The extracts were tested for precipitin line of identity by the microslide double-diffusion technique. The specific antiserum was against the wild-type enterotoxin. Only the mutants that possessed biological activity produced a precipitin line of identity with the wild-type cell extract. A precipitin line was not obtained with cell extract of the wild type grown in FTG. Figure 1 shows a typical immunodiffusion pattern with sp$^-$ ent$^-$ mutant (8-1), an osp ent$^-$ mutant (8-3), the wild-type NCTC 8798 grown in DS medium, and the wild type grown in FTG.

Electron microscopy of thin sections of sp$^-$ ent$^+$ mutants. All of the sp$^-$ mutants grown by procedure A were originally examined by negative-contrast electron microscopy; they were all blocked at stage zero of sporulation except 8-20 which had many lysed bodies looking like abnormal spores. The three sp$^-$ ent$^+$ mutants were reexamined by observation of 8-hr DS sporulation medium cultures, grown by procedure B, under phase-contrast microscopy (Fig. 2). A partial reversion for the ability to sporulate apparently appeared between both examinations by electron microscopy, for the results surprisingly revealed that the mutants

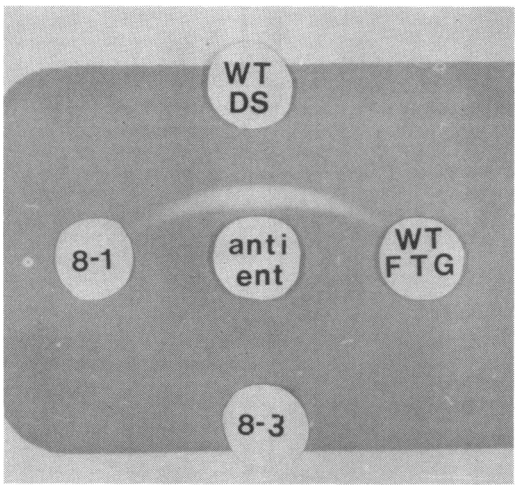

FIG. 1. *Immunodiffusion pattern in agar gel. The center well contained antiserum against partially purified enterotoxin. WT DS, cell extract of the wild type grown in DS sporulation medium; WT FTG, cell extract of the wild type grown in FTG growth medium; 8-3, cell extract of osp ent⁻ mutant 8-3 grown in DS sporulation medium; 8-1, cell extract of sp⁻ ent⁻ mutant 8-1 grown in DS sporulation medium.*

really were blocked at an intermediate stage of sporulation. Phase-dark forespores did not become refractile with continued incubation. The forespores of mutant 8-7 seemed abnormal in that they tended to be smaller and located at the extreme tip of the cell. The typical refractile appearance of the heat-resistant wild-type strain is also shown in Fig. 2.

Thin sections of 8-hr sporulating cultures of the three mutants and the wild type were prepared and examined by electron microscopy. The morphology of the normal heat-resistant spore of the wild type (Fig. 3a) is similar to that of other anaerobic and aerobic spores (17, 37). The spore core, cortex, and inner and outer spore coats are easily distinguishable. Knoblike extensions of the spore coats occur at intervals around the spore. The abnormal appearance of the forespores of mutant 8-7 as observed under phase contrast was confirmed in thin sections of this strain (Fig. 3b). The forespore remained at the extreme end of the sporulating cell and did not become completely engulfed by the sporangium. In a rare number of cells, fragments of coat material could be seen deposited in the cytoplasm out-

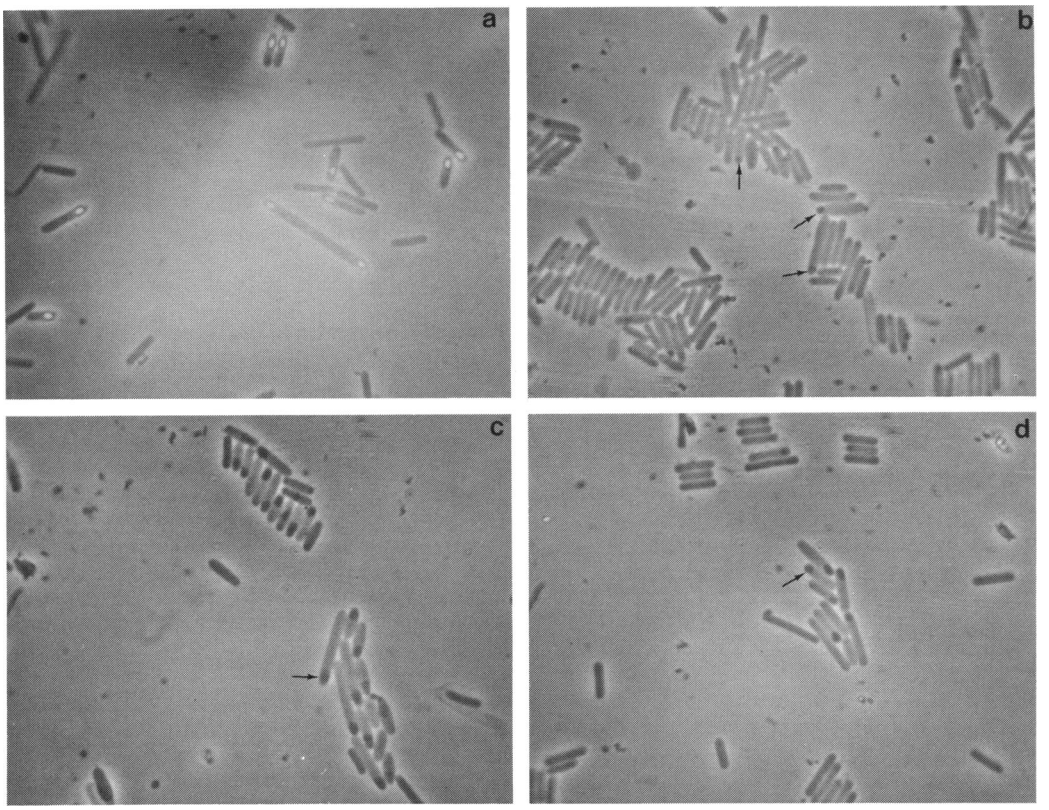

FIG. 2. *Phase-contrast photomicrographs of 8-hr sporulating cells of the wild type (a), mutant 8-7 (b), mutant 8-17 (c), and mutant 8-20 (d).*

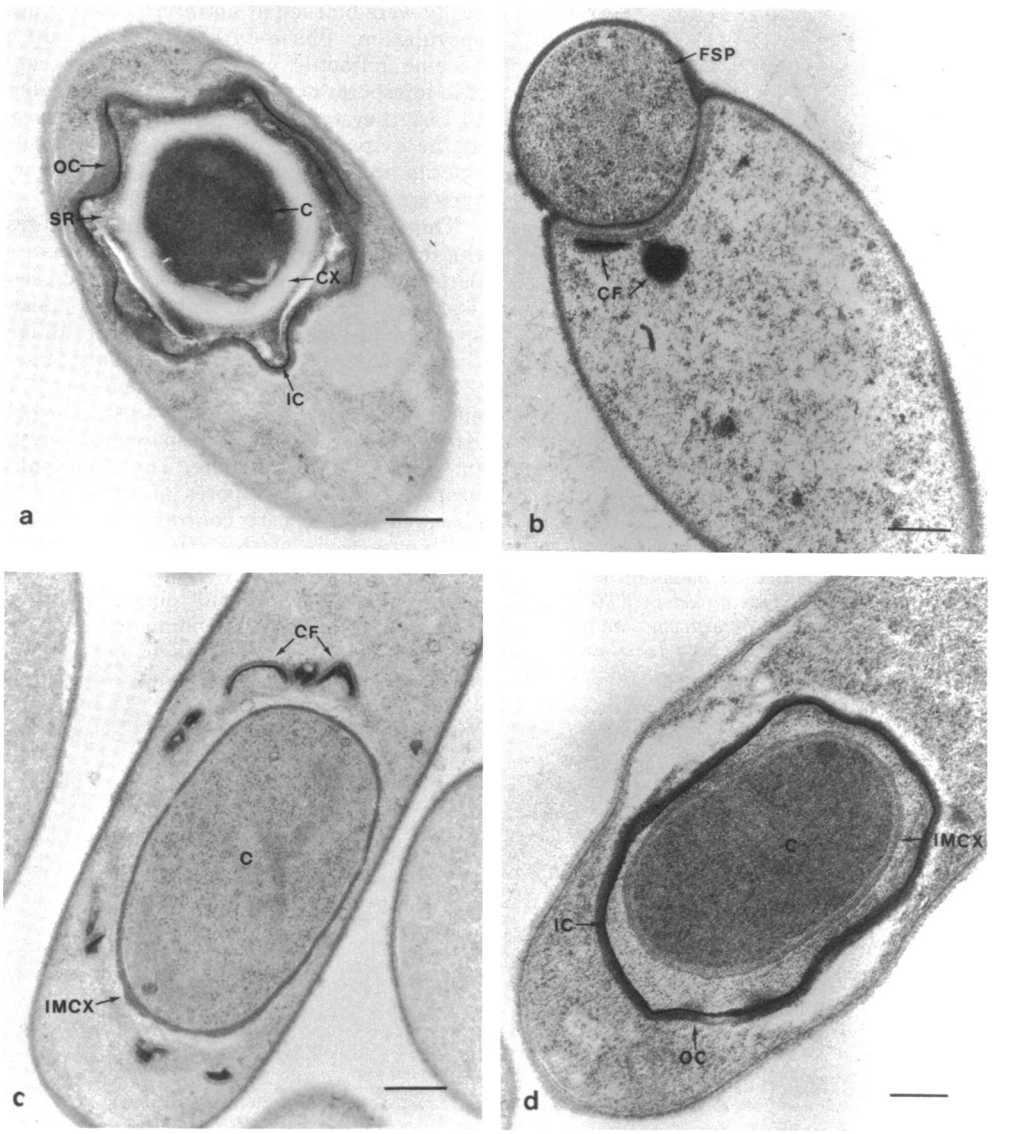

FIG. 3. *Electron micrographs of thin sections of 8-hr sporulating cells of Clostridium perfringens ent+ wild type and mutants. a, Wild type; b, mutant 8-7; c, mutant 8-20; d, mutant 8-17. Sporulation did not progress beyond the stages shown for mutants 8-7, 8-20 and 8-17. Symbols: C, core; CX, cortex; IC, inner coat; OC, outer coat; SR, subcoat region; FSP, forespore; CF, coat fragment; and IMCX, immature cortex. Bar represents 0.2 nm.*

side the forespore. Mutant 8-7 is apparently blocked at stage III of sporulation.

Mutants 8-20 and 8-17 were blocked at a later stage of sporulation than was mutant 8-7 (Fig. 3c and 3d). Mutant 8-20 had spore coat material deposited discontinuously in the cytoplasm around the forespore. Although a mature cortex was not present, a narrow, dense cortical layer was present external to the inner forespore membrane. A continuous double layer of inner and outer spore coat material was present around most of the forespores of mutant 8-17, indicating a block somewhat later than that seen with mutant 8-20. Primordial cortical material could be detected around some of the prespores. DPA was present in spores of the wild type but could not be detected in sporulating cells or the culture filtrates of mutants 8-7, 8-17, and 8-20.

It is difficult to determine the stage at which

mutants 8-17 and 8-20 are blocked. Normal cortical development and spore coat deposition may occur simultaneously in *C. perfringens* (17). Such simultaneous development in the wild type is shown in Fig. 4. Both an inner, dense cortical layer and a less dense outer cortical layer can be seen. The inner, dense cortical layer apparently corresponds to that seen in mutant 8-20. Both the inner and outer coat layers are deposited simultaneously in a discontinuous pattern. The sporulation stages described for *Bacillus* species generally include cortex synthesis in stage IV and coat deposition in stage V, although coat deposition may begin at the end of stage IV (3). This sequence of events may vary somewhat, especially with *Clostridium* species (25). Fragments of coat material may be deposited in stage IV. Although mutants 8-17 and 8-20 do not produce appreciable amounts of cortical material, some can be detected in thin sections under an electron microscope. Based on this observation and the extent of spore coat development, mutant 8-20 is provisionally classed as being blocked at late stage IV and mutant 8-17 at stage V.

Production of exoenzymes and toxins other than enterotoxin. Mutants and the wild type were characterized as to their ability to produce known toxins of *C. perfringens* type A, other than the enterotoxin, and extracellular proteolytic enzymes. The excretion of extracellular proteases is an early event in the sporulation of some *Bacillus* species. However, their role in sporulation is uncertain (12). It was of interest to know not only if asporogeny in *C. perfringens* affected protease production but also if such mutants were disturbed in other toxic functions presumably unrelated to sporulation.

Collagenase (kappa toxin) could not be detected in either the wild type or mutant strains.

Hyaluronidase (mu toxin) was produced by all strains except mutants 8-3 and 8-16. Mutants 8-14 and 8-15 failed to show hyaluronidase activity when viable cells were tested on spot plates; however, when DS sporulation medium culture filtrates were tested, activity was present.

Lecithinase (alpha toxin) was produced by all strains when plate tests were conducted with viable cells. In contrast to hyaluronidase production, when DS medium culture filtrates were tested, lecithinase activity could be detected with only six of the mutants. Only two of these, mutants 8-3 and 8-16, were strongly positive. Thus, with most of the mutants and the wild type, lecithinase production was not compatable with growth in sporulation medium.

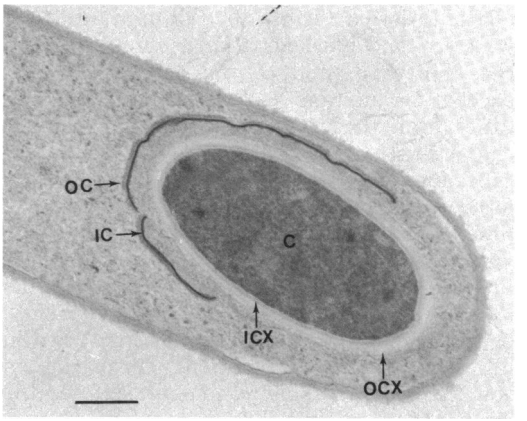

FIG. 4. *Electron micrograph of a thin section of the wild-type strain at an intermediate stage of sporulation. C, Core; IC, inner coat; OC, outer coat; ICX, inner cortical layer; OCX, outer cortical layer. Bar represents 0.2 nm.*

The production of neuraminidase and theta toxin was not consistent in either the wild type or in the mutants. When present, activity of the two toxins was considered to be positive/negative.

Extracellular protease was produced by viable cells of all strains when skim milk incorporated into Trypticase soy agar base was used as the substrate. When DS medium was used as the agar base, protease production was variable, with extended incubation of the test plates being necessary for detection of activity. Proteolytic activity on skim milk could not be detected by using 6-, 24-, or 48-hr DS medium culture filtrates of the mutants or wild type. However, when these same filtrates were tested with gelatin used as the substrate, proteolytic activity was detected in some strains. No correlation existed between asporogeny and the presence or absence of gelatinolytic activity. Obviously, more than one extracellular protease may be produced by *C. perfringens*. The detection of a given protease is dependent on the sensitivity of the test. Low levels of proteolytic activity may not have been detected by the plate procedures used here. The level of protease activity also may vary as a reflection of the nutritional conditions of growth. Since all strains possessed proteolytic activity when viable cells were tested, they are considered to be protease positive.

Sporulation and biochemical characterization of the wild type and mutants. The mutants were separated into groups on the basis of their sporulation and biochemical

characteristics (Table 3). All mutants that were sp$^-$ and blocked at stage zero (sp$_0^-$) lost the ability to produce the enterotoxin and, except for mutant 8-16, did not otherwise differ from the wild type in the biochemical characteristics tested. Mutant 8-16 and the osp$_0$ mutant 8-3 were not only ent$^-$ but also differed from the wild type in being unable to produce hyaluronidase. Mutants 8-14 and 8-15 were osp$_0$ ent$^-$ but otherwise toxigenically identical to the wild type. Four osp$_0$ mutants (8-21, 8-22, 8-23, and 8-8) were classed as ent$^+$ or ent$^-$, since enterotoxin production varied in different culture preparations depending on the frequency of sporulation. Except for the frequency of sporulation, these mutants were phenotypically similar to the wild type. Mutants 8-7, blocked at stage III of sporulation (sp$_{III}^-$), 8-20, blocked at stage IV of sporulation (sp$_{IV}^-$), and 8-17, blocked at stage V of sporulation (sp$_V^-$), retained the ability to produce enterotoxin. All sp$^+$ isolates from the wild type were similarily ent$^+$. Thus, in *C. perfringens* a direct relationship exists between the ability to produce enterotoxin and the ability to sporulate.

Characterization of sp$^+$ revertants. Three sp$^+$ revertants were isolated from the sp$_0^-$ ent$^-$ mutant 8-1. The sporulation frequency of the revertants, their ability to produce enterotoxin, and their enteropathogenic activity in rabbits as compared to mutant 8-1 and the wild type are shown in Table 4. All three revertants regained not only the ability to sporulate but also the ability to produce enterotoxin and were enteropathogenic in rabbits. The defect in mutant 8-1 was apparently due to a single mutation. When tested by immunodiffusion, a precipitin line of identity was obtained between the three revertants and the wild type by using specific antiserum against the enterotoxin. The restoration of enterotoxin production in these sp$^+$ revertants is further evidence of the relationship between the production of enterotoxin and sporulation.

DISCUSSION

The enterotoxin produced by certain strains of *C. perfringens* type A is a heat-labile protein that possesses several demonstrable biological characteristics (9, 10, 13, 14, 23, 33, 34).

The results presented in this paper confirm the previously observed close relationship between sporulation and enterotoxin production and indicate that the enterotoxin is a sporulation-specific gene product. The possibility of such a relationship has been indicated previously by S. Nishida (*personal communication*). In the present study, three sp$^+$ mutants isolated from the wild-type sp$^+$ ent$^+$ strain were ent$^+$. Eight sp$_0^-$ mutants isolated from an sp$^+$ ent$^+$ strain were unable to synthesize enterotoxin protein. Except for one isolate (8-16), these mutants were not disturbed in other toxic functions, presumably unrelated to sporulation. Therefore, it seems that the ent$^-$ characteristic is due directly to loss in the ability to sporulate and not to a general metabolic disturbance in the cell. Three sp$^+$ revertants isolated from an sp$^-$ ent$^-$ mutant regained not only the ability to sporulate but also the ability to produce enterotoxin. It appears that a single gene mutation was responsible for both the sp$^-$ and the ent$^-$ traits, thus providing further evidence of the direct relationship between enterotoxin production and sporulation.

The formation of mature, heat-resistant spores is not a prerequisite for enterotoxin production, for three sp$^-$ mutants blocked at intermediate stages of sporulation are still ent$^+$. These three sp$^-$ mutants were first de-

TABLE 3. *Summary of sporulation and biochemical characteristics of wild type and mutant strains*[a]

Strains	Sporulation class	Ent	Hya	Lec	Neur	Theta	Prot
NCTC 8798, 8-2, 8-4, 8-6, R-3	Sp$^+$	+	+	+	±; −	±; −	+
8-7	Sp$_{III}^-$	+	+	+	±	−	+
8-17	Sp$_V^-$	+	+	+	±	−	+
8-20	Sp$_{IV}^-$	+	+	+	±	−	+
8-21, 8-22, 8-23, 8-8	Osp$_0$	+; −	+	+	±	−	+
8-14, 8-15	Osp$_0$	−	+	+	±; −	±; −	+
8-3	Osp$_0$	−	−	+	−	−	+
8-16	Sp$_0^-$	−	−	+	−	±; −	+
8-1, 8-5, 8-46, 8-47, 8-61, 8-62, 8-65	Sp$_0^-$	−	+	+	±; −	−	+

[a] Traits indicated: Ent, Enterotoxin; Hya, hyaluronidase; Lec, lecithinase; Neur, neuraminidase; Theta, theta toxin; Prot, protease.

TABLE 4. *Sporulation and enterotoxic activity of three sp⁺ revertants of sp_0^- ent⁻ mutant 8-1*

Strain	Spores/ml of DS sporulation medium (frequency S/V)[a]		Enterotoxic activity		
	24 hr	8 hr	Erythemal units/mg of protein (8-hr cell extract)	Diarrhea in rabbits	Rabbit ileal loop fluid accumulation
NCTC 8798 (WT)	$\frac{1.2 \times 10^7}{9.2 \times 10^{-2}}$	$\frac{3.0 \times 10^6}{1.2 \times 10^{-1}}$	51.2	+	+
8-1	0	0	0	−	−
R-8	$\frac{6.3 \times 10^6}{7.9 \times 10^{-2}}$	$\frac{5.0 \times 10^6}{5.6 \times 10^{-2}}$	108.3	+	+
R-42	$\frac{1.1 \times 10^6}{7.8 \times 10^{-2}}$	$\frac{3.7 \times 10^5}{3.7 \times 10^{-3}}$	42.2	+	+
R-43	$\frac{1.9 \times 10^6}{6.7 \times 10^{-2}}$	$\frac{4.7 \times 10^5}{4.7 \times 10^{-3}}$	29.6	+	+

[a] See Table 1.

tected on the basis of their ability to produce enterotoxin, even though it was known that the mutants did not produce heat-resistant spores and were thus sp⁻. Since mutants blocked at stage II have not been isolated, it is impossible to know whether a block at this stage would permit enterotoxin production. However, mutants 8-17 (blocked at stage V) and 8-20 (blocked at stage IV) routinely produce higher levels of intracellular enterotoxin than does mutant 8-7 (blocked at stage III), and this characteristic may be associated with the block at a later stage. It often is difficult to accurately relate the time of production of a sporulation-specific gene product with a given morphological stage of sporulation. It has been suggested that the gene products resulting from biochemical events occurring prior to stage III of sporulation may be essential to sporulation, but not unique to the sporulating cell, and that the events occurring subsequent to stage III are sporulation-specific (12). However, it has been shown that coat protein synthesis begins early in sporulation even though morphologically distinct coats normally are not present until late stage IV or V (2, 27). A coat structure has also been shown in some mutants blocked at stage II (27) and in some cortexless mutants (3). In the present study, ent⁺ mutant 8-7, blocked at stage III, produced morphologically distinct fragments of coat material, ent⁺ mutant 8-17 produced normal coats but an immature cortex, and ent⁺ mutant 8-20 produced incomplete coats and an immature cortex. All three mutants failed to produce DPA, a gene product associated with a late sporulation stage, and did not become refractile. Thus, it is difficult at this time to assign enterotoxin synthesis to a given stage of sporulation. The time-course of intracellular enterotoxin accumulation indicates that it is a late-stage sporulation product (*unpublished data*), which would be consistent with the observed enterotoxin production by mutants blocked at stage III, IV, or V.

The function of *C. perfringens* enterotoxin in sporulation of this organism is unknown. It is also not known whether the enterotoxin possesses any enzymatic activity that might permit assignment of a physiological role in sporulation. The failure to detect gene products serologically homologous to enterotoxin in sp_0^- ent⁻ mutants would indicate that transcription of relevant sporulation genes is essential for synthesis of enterotoxin protein. However, it is not clear whether enterotoxin synthesis is essential for sporulation. We have established that only certain strains of *C. perfringens* synthesize biologically active and serologically detectable enterotoxin (33, 34). Among nonenterotoxin-producing strains are those that sporulate as well as enterotoxin-producing strains. The possibility exists that enterotoxin is a protein normally associated with or comprising some spore structural component. Such a relationship may exist between the parasporal crystalline protein inclusion, known to be toxic to certain insect larvae, and the spore of *Bacillus thuringiensis* (31). Intracellular accumulation of *C. perfringens* enterotoxin in only certain strains may result from loosely regulated synthesis of the toxin pro-

tein, whereas in the non-enterotoxin-producing strains, tight regulation of toxin synthesis may prevent enterotoxin detection. Some sporulation-specific events are not necessarily detected in all strains of a given species. The synthesis of sulfolactic acid has been shown to be a sporulation-specific event occurring at a late stage of sporulation in *B. subtilis* Marburg (39). However, some other strains of *B. subtilis* or other *Bacillus* species do not appear to produce sulfolactic acid (4).

In an attempt to determine the requirement of preformed enterotoxin for sporulation, cross-feeding studies were conducted by adding exogenous enterotoxin at various time intervals to DS medium cultures of the sp_0^- ent^- mutant 8-1 (*unpublished data*). Under no circumstance did sporulation occur. However, this was not surprising, since the enterotoxin protein is not excreted from the sporulating cell but is released from the sporangium with its lysis, concomitantly with the mature spore release (*unpublished data*); thus the enterotoxin would not be readily available to the intact cell.

These results indicate that a single gene mutation is responsible for both the sp^- and ent^- traits. Nevertheless, phage research was also made with these mutant strains, and some mutants were found to differ from the wild-type strain by restriction of a virulent phage, or by a defective lysogenic state (*unpublished data*), or both. There is no evident relationship between these traits and both properties studied in this communication. These traits show only that some of the mutants are pleiotropic mutants.

ACKNOWLEDGMENTS

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, by Public Health Service grants 1-RO1-FD-00203-01, 5-RO1-FD-00203-02, and 5-RO1-FD-00068-10, and by contributions to the Food Research Institute by member industries.

The assistance of A. Ryter in the examination of our cultures in the electron microscope and Robert R. Reich in the preparation and examination of cell thin sections by electron microscopy is gratefully acknowledged.

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