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Sporulation-Promoting Ability of *Clostridium perfringens* Culture Fluids

NENG-JEN SHIH AND RONALD G. LABBÉ*

Food Microbiology Laboratory, Department of Food Science, University of Massachusetts, Amherst, Massachusetts 01003

Received 6 October 1995/Accepted 24 January 1996

The culture supernatant fluids (CSFs) of 12 strains of *Clostridium perfringens* types A, B, C, and D stimulated sporulation of test strains NCTC 8238 and NCTC 8449 of this organism. The sporulation-promoting ability was present in vegetative and sporulating CSFs of both enterotoxin-positive (Ent^+) and Ent^- strains. The sporulation factor possessed a molecular weight between 1,000 and 5,000 and was heat and acid stable. This study suggests a potential role for Ent^- strains in food-borne disease outbreaks caused by Ent^+ strains of *C. perfringens* type A.

Clostridium perfringens type A produces an enterotoxin whose concentration increases dramatically during sporulation (10). Contrary to early reports (5), its synthesis is apparently not closely related to spore coat synthesis (18) and its true function remains unknown. The enterotoxin is responsible for food-borne illness following the consumption of large numbers of vegetative cells, which sporulate in the small intestine (14).

Most studies of the sporulation process have focused on *Bacillus* species. Details of the sporulation process in members of this genus have been described previously (4, 9). In the case of *C. perfringens* several methylxanthines, such as caffeine, theophylline, and isobutylmethylxanthine, have been reported to enhance sporulation (12, 19). Human bile salts also affect sporulation and enterotoxin production in some *C. perfringens* strains (8). These substances may result in a decrease in the guanosine triphosphate pool, which triggers sporulation. Several enzyme systems that are associated with sporulation (proteases, peptidoglycan hydrolase, and amylase) in *C. perfringens* have been studied (11, 13, 21, 22). Here we report a sporulation-inducing factor produced by enterotoxin-positive (Ent^+) and Ent^- strains of *C. perfringens.*

Two drops of stock cultures of *C. perfringens* from cooked-meat medium (Difco, Detroit, Mich.) were added to 10 ml of fluid thioglycolate medium (Difco) and heated for 10 min at 75°C. The heat-activated cultures were incubated for 16 to 18 h at 37°C. The cultures were transferred (2% inoculum) into a defined (D) medium (200 ml) containing no glucose and incubated for 8 h at 37°C (21). Cells were removed by centrifugation (10,000 × *g*, 20 min) and the supernatant fluid was ultrafiltered by using successive membranes (Amicon) of decreasing mol weight cut-off, frozen, and lyophilized. The concentrated culture supernatant fluid (CSF) was then redissolved in water (5 ml), collected by centrifugation (13,000 × *g*, 5 min), and filter sterilized (0.45-μm-pore-size filters). The CSF (50 μl) was mixed with 10 ml of Duncan Strong (DS) sporulation medium (2). Because *C. perfringens* NCTC 8238 and NCTC 8449 sporulate poorly in DS medium, they were selected as the test strains for stimulation of sporulation. Overnight cultures of *C. perfringens* NCTC 8239 or NCTC 8449 in fluid thioglycolate medium were added (1% inoculum) to DS medium and incubated for 7 h at 37°C. The levels of heat-resistant spores were determined as previously described (21).

The CSFs of 12 strains of *C. perfringens* were assayed; all CSFs promoted sporulation of strain NCTC 8238, and 11 of the 12 promoted sporulation of strain NCTC 8449. The sporulation factor was produced by all type strains examined and by both sporulating and vegetative cells (Table 1). CSFs from NCTC 8238 and PS 51 were least effective with NCTC 8449 as the test strain. This may due to the specificity of the fluid concentrate. Waldburger et al. (24) reported that CSF from *Bacillus subtilis* 168 stimulated sporulation of *B. subtilis* BR 151 but that the reverse was not true. The sporulation-promoting substances released by bacterial cells have been reported for *Bacillus cereus* (termed sporogen [23]) and *B. subtilis* (termed sporulation factor [24] or extracellular differentiation factor [7]), but the substances have resisted efforts at isolation and detailed characterization. This is the first report of a similar factor produced by an anaerobe.

The sporulation-promoting ability of *C. perfringens* CSF was concentration dependent (Fig. 1). Using CSF from NCTC 8238, the heat-resistant-spore levels (HRSL) of test strain NCTC 8238 increased as the volume of CSF added was increased. Observation of 7-h cell cultures of NCTC 8238 by phase-contrast microscopy revealed mature, refractile spores, with 85 to 90% sporulation. However, the CSF (from strains NCTC 8679 and 748) did not induce sporulation of strain 8-62, a Spo^- mutant of *C. perfringens* blocked at stage 0 of sporulation (3) (data not shown). This is not surprising since the enterotoxin is transcribed only in subsequent stages of sporulation (15, 16).

An interesting question is whether these sporulation-promoting factors in CSF function naturally as signals between cells. Waldburger et al. (24) have studied the production of the sporulation-promoting substances in *B. subtilis spo* mutants and found that all *spo* mutants tested were able to produce a functional sporulation-promoting substance in spite of their inability to sporulate themselves. Our results also indicate that production of this factor is not sporulation specific. For example, we have found that addition of a small amount of glucose (8 mM) in D medium results in vegetative growth by strain NCTC 8679 whereas the absence of glucose results in significant sporulation ($>10^7$ spores per ml) (21). Yet the CSFs produced by vegetative and sporulating cells of this strain ex-

* Corresponding author. Phone: (413) 545-1021. Fax: (413) 545-1262.

TABLE 1. Sporulation-promoting abilities of CSFs of homologous and heterologous strains of *C. perfringens*

Type	Producer strain	Physiological status in D medium[a]	HRSL of test strain[b]	
			NCTC 8238	NCTC 8449
	Control[c]		<100	<100
A, Ent$^+$	NCTC 8238	S	$(2.0 \pm 0.3) \times 10^6$	$(6.3 \pm 3.9) \times 10^3$
	NCTC 8449	S	$(2.6 \pm 0.5) \times 10^7$	$(1.9 \pm 0.6) \times 10^6$
	NCTC 8679	S	$(3.2 \pm 0.7) \times 10^7$	$(1.7 \pm 0.1) \times 10^6$
		V[d]	$(3.6 \pm 0.6) \times 10^7$	$(2.4 \pm 1.0) \times 10^6$
A, Ent$^-$	ATCC 3624	S	$(1.1 \pm 0.1) \times 10^7$	$(7.4 \pm 3.6) \times 10^5$
	FD 800	V	$(5.6 \pm 1.7) \times 10^6$	$(6.5 \pm 1.6) \times 10^5$
B	3627	V	$(4.3 \pm 2.3) \times 10^6$	$(3.8 \pm 2.5) \times 10^5$
	PS 49	V	$(2.0 \pm 0.7) \times 10^7$	$(1.2 \pm 0.5) \times 10^6$
C	197	M	$(3.2 \pm 0.6) \times 10^7$	$(3.1 \pm 1.8) \times 10^6$
	243	M	$(1.5 \pm 0.6) \times 10^7$	$(4.1 \pm 2.1) \times 10^5$
	PS 51	S	$(6.9 \pm 3.0) \times 10^6$	<100
D	748	S	$(3.2 \pm 1.1) \times 10^6$	$(9.9 \pm 7.4) \times 10^5$
	PS 52	S	$(8.7 \pm 1.6) \times 10^6$	$(5.5 \pm 3.4) \times 10^5$

[a] S, sporulating (HRSL, $>10^5$ CFU/ml); V, vegetative (HRSL, <30 CFU/ml); M, mixed (HRSL, $\sim 10^3$ CFU/ml).
[b] HRSL in DS medium.
[c] Concentrated, uninoculated D medium.
[d] In the presence of 8 mM glucose.

hibited the same ability in promoting sporulation of the test strains (Table 1).

C. perfringens NCTC 8679 is able to sporulate in the presence of glucose (8 mM) in the presence of 30 mM organic acids (acetic, lactic, or butyric acid) (20). To determine the possible role of such acids as inducers of sporulation, their presence in the CSF of vegetative cultures of strain NCTC 8679 was examined by high-pressure liquid chromatography with an Aminex PHX-87H hydrogen column (Bio-Rad, Hercules, Calif.). A sample of 50 μl was loaded on the column and eluted with 0.012 N H_2SO_4 at a flow rate of 0.8 ml/min (6). Only one organic acid, acetic acid, at a concentration of 11.7 mM, was detected. This is equivalent to an acetic acid concentration of 0.06 mM in the DS sporulation medium. However, the addition of 0.06 mM acetic acid alone or with sterile, concentrated D medium (50 μl) to DS medium was not effective in stimulating sporulation of the test strains (data not shown). Thus, acetic acid is not likely the responsible agent in the CSF.

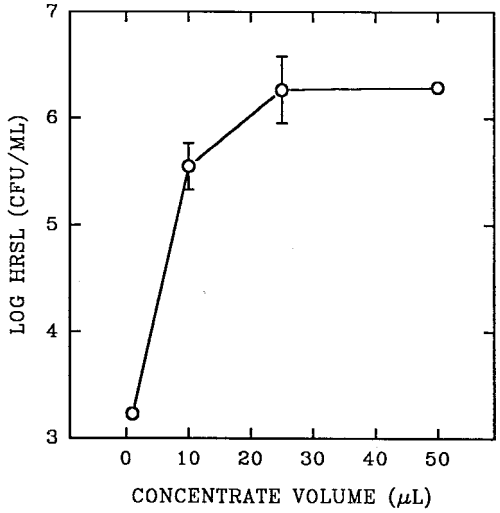

FIG. 1. Effect of volume of CSF from *C. perfringens* NCTC 8238 on sporulation of NCTC 8238 in DS medium. The first datum point represents the addition of 1 μl.

Addition of exogenous purified enterotoxin (640 μg/ml) has been reported to hasten the sporulation process of *C. perfringens* (1). It was possible that enterotoxin in the CSF from Ent$^+$ strains due to cell lysis could be the sporulation-promoting substance. However, antienterotoxin serum did not cross-react with 7-h-concentrated CSF from NCTC 8238 (an Ent$^+$ strain) by counterimmunoelectrophoresis (17) (data not shown). Since the CSF from Ent$^-$ strains also stimulated sporulation in *C. perfringens* NCTC 8238, it is unlikely that enterotoxin is involved in this case.

Heat (121°C, 15 min) only partially inactivated the sporulation-promoting activity of strains NCTC 8238, NCTC 8679, and ATCC 3624, resulting in an approximately 1-log decrease in sporulation of test strain NCTC 8238 (data not shown). Examination of the molecular mass by ultrafiltration of the CSF containing the sporulation-promoting substance(s) through a YM5 membrane indicated a molecular size of less than 5,000. Thus, the sporulation-promoting substance(s) in CSF is heat stable and of relatively small size. By comparison, the sporulation factor reported in the case of *B. subtilis* has an estimated molecular size between 1,000 and 5,000 and is also heat stable (7, 24).

In food poisoning outbreaks caused by *C. perfringens*, vegetative cells survive stomach passage, and it has been proposed that the acidic environment may serve to stimulate sporulation of this organism (25). To examine the acid stability of the sporulation factor, CSF (0.5 ml) from strain ATCC 3624 was mixed with 1 N HCl to a pH between 1.8 and 2.0. The acidic CSF was incubated at 37°C for 2 h and then neutralized with 1 N NaOH to its original pH (7.2). The acid-treated CSF was adjusted to a final volume of 5 ml, filter sterilized, and added (0.5 ml) to 10 ml of DS sporulation medium. Test strain NCTC 8238 sporulated well in the presence of the acid-treated CSF from ATCC 3624, FD-800, or NCTC 8449 (data not shown). Thus, the sporulation-promoting factor is also acid stable.

In spite of the etiological role of Ent$^+$ strains in food-borne illness, Ent$^-$ strains of *C. perfringens* are more widespread in foods and can be found in the soil and intestinal contents of humans and animals. Thus, many food items, in particular raw meat and poultry, can be contaminated with Ent$^+$ strains, Ent$^-$ strains, or both (10). Our studies of the sporulation-promoting abilities of Ent$^-$ strains of *C. perfringens* suggests a possible role for the sporulation factor in food-borne outbreaks caused by Ent$^+$ *C. perfringens* type A. Previous or concomitant growth of Ent$^-$ strains could result in sporulation-promoting substances which could enhance sporulation and enterotoxin production of Ent$^+$ *C. perfringens* strains in the small intestine. Since the sporulation-promoting ability is heat stable, raw materials contaminated with Ent$^-$ strains could cause food safety problems in either of two ways: if food is contaminated with Ent$^+$ strains after processing, allowing vegetative cell growth to occur, and by the traditional scenario, in which spores of Ent$^+$ strains alone may survive a heat-processing step, germinate, and resume vegetative cell growth.

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