

## Inhibition of *Clostridium botulinum* Toxin Formation by *C. sporogenes* in Culture Media and in a Meat System

C. N. HUHTANEN

### ABSTRACT

Vegetative cells and spores of types A and B *Clostridium botulinum* and *C. sporogenes* were coinoculated into culture media and into mechanically deboned chicken meat. Botulin toxin formation was inhibited; the degree of inhibition depended on the relative concentrations of the two microorganisms. Inhibition of toxin formation by *C. sporogenes* also occurred in the meat system, but not all strains of *C. botulinum* were affected.

Early reports (2,5) indicated that *Clostridium sporogenes* inhibits toxin formation by *C. botulinum* under some conditions. Sommer and Glunz (9) found no inhibition in meat, but toxin inhibition occurred in spinach or asparagus; inhibition was dependent on spore ratios of the two organisms. Kautter et al. (6) described *C. botulinum*-like bacteria capable of inhibiting *C. botulinum* type E. Smith (8) analyzed 31 soil samples and found eight with strains of *C. sporogenes* that inhibited *C. botulinum* toxin formation. In most of these studies, the *C. botulinum* strains could not be related to those now in use.

The purpose of the present study was to verify the results of early workers using well characterized strains of *C. botulinum*.

### MATERIALS AND METHODS

The cultures of *C. botulinum* were proteolytic and were obtained from the Food and Drug Administration, Washington, DC [62(A) and 426(A)]; Centers for Disease Control, Atlanta, GA [20PL(A)]; Animal and Plant Health Inspection Service, Beltsville, MD [770(B)]; U.S. Army Natick Laboratories, Natick, MA [53(B)]; and American Type Culture Collection, Silver Spring, MD [7949(B)]. The *C. sporogenes* cultures were B1219 from the Northern Regional Research Center, USDA, Peoria, IL and a strain isolated from the intestinal tract of *Apis mellifera* (4). The culture medium was NTT, a mixture of 0.4% nutrient broth (Difco, Detroit, MI), 1.5% trypticase soy broth with glucose (BBL, Cockeysville, MD), and 0.05% Na thioglycollate. Incubation was at 35°C in anaerobic jars (BBL) with a gas mixture of 10% H<sub>2</sub>, 10% CO<sub>2</sub> and 80% N<sub>2</sub>, and a palladinized asbestos catalyst.

Cultures were grown at 35°C for 2 weeks in NTT and were then centrifuged at 3000 g for 15 min at 2°C. The sediments were

resuspended in water and recentrifuged. Water was used for the final suspensions that were heated at 80°C for 10 min. Spore enumeration was in NTT supplemented with 1.5% agar, incubation was 3 d at 35°C in anaerobic jars.

Mechanically deboned chicken meat (MDCM) was locally obtained from a manufacturer of poultry products. The meat was placed in aluminum tab-top cans (208 x 107) which were sealed under nitrogen. Some of the cans were irradiation sterilized at 42 kGy (at a rate of 125 Gy/min) at 0-2°C. The cans were stored at -12°C and were thawed overnight at room temperature as needed.

Freshly prepared NTT medium was placed into culture tubes (16 x 125 mm), 8 ml per tube, and 0.1 ml of appropriate culture or spore suspension was added. Sterile NTT was used for controls. The cultures were incubated in anaerobic jars for 2 weeks at 35°C.

The tests in MDCM were done by adding 10 g meat to petri dishes in a biohazard hood using aseptic precautions. Spore suspensions, 0.1 ml each, were mixed into the meat with sterile wooden applicator sticks and the petri dishes were incubated in anaerobic jars for 2 weeks at 35°C. The meat cultures were tested for aerobic contamination by streaking onto plate count agar (Difco) and incubating aerobically 2 d at 35°C.

The NTT cultures were diluted with two parts gelatin phosphate buffer (0.2% gelatin, 0.36% KH<sub>2</sub>PO<sub>4</sub> and 0.15% Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O) followed by serial 10-fold dilutions in the same buffer. The meat samples were diluted with 20 ml gelatin phosphate buffer in 40-ml centrifuge tubes and were centrifuged at 3000 g for 15 min at 2°C. The supernatant fluids were diluted as needed with the gelatin phosphate buffer.

Toxin tests were done by intraperitoneal injection of pairs of mice (15-20 g) with 0.5 ml of sample and observing them for 3 d. Positive toxin results were those where the mice died with typical symptoms of respiratory muscle paralysis. Confirmation was by injecting boiled extracts or in cases of doubt by injecting antiserum-neutralized samples (1 IU of antiserum per 0.5 ml sample incubated 1 h at 35°C).

### RESULTS AND DISCUSSION

The effect of rapidly growing (5 h at 35°C) vegetative cells of *C. sporogenes* B1219 on toxin production by *C. botulinum* was investigated by adding each in concentrations of approximately 10<sup>5</sup> or 10<sup>7</sup> per ml NTT medium. In the presence of the lower level of *C. sporogenes* B1219, a small amount of toxin (positive in the 1/30 dilution) was

produced by the higher levels of strains 770(B) and 426(A), but no toxin was produced when B1219 was added at the higher concentration. Toxin production by both levels of 62(A) and 20PL(A) was inhibited (no toxin in 1:2 dilution) by both concentrations of the sporogenes culture.

Stationary phase (22 h cultures) of *C. sporogenes* B1219 also inhibited toxin production in cultures of *C. botulinum* 62(A), 770(B), and 20PL(A). Toxin was not produced when both cultures were added at the same level (the concentrations used were  $10^3$  and  $10^5$ /ml) or when *C. sporogenes* was added at 100 times the level of *C. botulinum*, but if the inoculum of *C. botulinum* cells was 100 times that of *C. sporogenes*, toxin was present in the cultures.

When spores of *C. botulinum* and B1219 were coinoculated at a level of 1000/g each into NTT medium (Table 1), toxin was not detected in cultures of ATCC 7949(B), 62(A), or 20PL(A) although mice injected with the lowest dilution (1:2) of ATCC 7949(B) or 62(A) showed symptoms of neuromuscular intoxication (pinched abdomen).

TABLE 1. Effect of *C. sporogenes* B1219 spores on toxin formation by *C. botulinum* in culture medium.<sup>a</sup>

<i>C. botulinum</i> Strain	Dilution tested	No spores (toxin occurrence)	Spores
770 (B)	1:30000	+ <sup>b</sup>	+
770 (B)	1:3000	+	+
770 (B)	1:300	+	+
770 (B)	1:30	nd	nd
ATCC 7949 (B)	1:30000	+	-
ATCC 7949 (B)	1:3000	+	-
ATCC 7949 (B)	1:300	+	-
ATCC 7949 (B)	1:30	nd	s
62 (A)	1:30000	+	-
62 (A)	1:3000	+	-
62 (A)	1:300	+	-
62 (A)	1:30	nd	s
20PL (A)	1:30000	+	-
20PL (A)	1:3000	+	-
20PL (A)	1:300	+	-
20PL (A)	1:30	nd	-

<sup>a</sup>Medium was NTT (0.4% nutrient broth, 0.75% trypticase soy broth and 0.05% sodium thioglycollate). Spores of *C. botulinum* and *C. sporogenes* were added at levels of 1000/ml each.

<sup>b</sup>Explanation of symbols: (+) toxin, (-) no toxin, (s) symptoms of muscle paralysis (pinched abdomen) without death, (nd) test not done.

Toxin formation by strain 770(B) was not affected by *C. sporogenes* under these conditions.

Sommer and Glunz (9) used mice and guinea pigs in studies of the effect of various ratios of spores of *C. botulinum* and *C. sporogenes* on toxin formation in media prepared from asparagus, spinach and meat. The highest level of inhibition was in asparagus medium which also gave the lowest titer of botulinal toxin in the absence of *C. sporogenes*. Toxin was produced in the meat (beef heart) medium in contrast to the results of Jordan and Dack (5) who showed that toxin formation by their strain of *C. botulinum* was inhibited by *C. sporogenes* in this medium. The results of Sommer and Glunz (9) also showed that the inhibition was more pronounced with increasing levels of *C. sporogenes* spores. However, they studied only a single strain of *C. botulinum* (strain 97 from home canned peas).

The results in Table 2 provide a possible explanation for the differences in results of the earlier workers on the inhibition of *C. botulinum* by *C. sporogenes*. In this experiment the MDCM was sterilized by gamma radiation to eliminate indigenous microorganisms that might interfere with the growth of or toxin production by *C. botulinum*. The MDCM was inoculated with 10 or 1000 spores/g of the two organisms. The log MLD/ml (minimal lethal dose/ml) was 3.8 when only spores of *C. botulinum* were added at levels of either 10 or 1000/g. No toxin was detected in MDCM inoculated with spores of 62(A) when spores of it and *C. sporogenes* were added to meat in ratios of 10:10, 10:1000, 1000:10, or 1000:1000/g. Toxin formation in meat inoculated with spores of ATCC 7949(B) was inhibited when the concentrations of spores of the two organisms were equal, but detectable toxin was found when the spore ratio was 1000:10. *C. sporogenes* spores coinoculated with three of the strains [770(B), 426(A), and 53(B)] did not inhibit toxin formation. Toxin production by strain 20PL(A) was decreased when the spore ratios were 10:1000 or 1000:1000, but there was no decrease in the amount of toxin when the ratios were 10:10 or 1000:10.

These results confirm early work on the effect of *C. sporogenes* on toxin production by *C. botulinum*, but they show that the inhibition is not a general phenomenon but rather depends on the particular strain of *C. botulinum* present in the culture being studied.

The inhibition of botulinal toxin formation by *C.*

TABLE 2. Effect of *C. sporogenes* B1219 spores on toxin production by *C. botulinum* in mechanically deboned chicken meat.

Culture	Inoculum (spores <i>C. botulinum</i> : <i>C. sporogenes</i> )/g meat <sup>a</sup>					
	10:0	10:10	10:1000	1000:0	1000:10	1000:1000
	(log MLD/ml)					
62(A)	3.8 <sup>b</sup>	0	0	3.8	0	0
770(B)	3.8	3.8	3.8	3.8	3.8	3.8
426(A)	3.8	3.8	3.8	3.8	3.8	3.8
53(B)	3.8	3.8	3.8	3.8	3.8	3.8
20PL(A)	3.8	3.8	1.8	3.8	3.8	1.8
ATCC 9749(B)	3.8	0	0	3.8	1.8	0

<sup>a</sup>Meat was radiation sterilized (42 kGy). Duplicate portions of 10 g were placed in petri dishes and 0.1 ml of the appropriate spore suspensions was added; incubation was in anaerobic jars at 25°C for 2 weeks. For assay 5 parts gelatin phosphate buffer was added to 1 part sample; the samples were mixed, kept overnight at 4°C, and centrifuged 15 min at 3000 g.

<sup>b</sup>The results are averages of two replicates (both values were the same). A "0" means no deaths at the lowest dilution tested; dilutions (1/6, 1/30, 1/3000) were made in gelatin phosphate buffer. For details of the mouse test see text.

*sporogenes* might be of great significance in the epidemiology of botulism. The establishment of *C. botulinum* in the intestinal tract of infants, for example, may well be influenced or even controlled by inhibitory strains of bacteria such as *C. sporogenes* [or of *C. perfringens* whose inhibitory effect was reported by Dack (2) and Smith (8)]. Support for this hypothesis was the work of Moberg and Sugiyama (7) who found that normally susceptible gnotobiotes became resistant to *C. botulinum* colonization when exposed to conventional mice. Burr and Sugiyama (1) reported that normal mice fed erythromycin and kanamycin sulfate became susceptible to colonization by *C. botulinum* but no toxic symptoms developed. This suggested that toxin may have been formed but was inactivated by the intestinal flora. The effect of several defined bacterial floras (including one with an unidentified clostridium) was investigated by Wells et al. (10) for their effect on colonization and toxin production by *C. botulinum* 62(A). Colonization was not influenced, but the death rate from botulism was significantly reduced by the introduced flora.

*C. sporogenes* inhibition may affect isolation procedures for *C. botulinum* since the two organisms cannot be distinguished biochemically. Studies of the incidence of *C. botulinum* in honey (4), for example, showed that many samples contained *C. sporogenes* but very few had *C. botulinum*. The true incidence of *C. botulinum* in nature may thus be obscured by inhibitory strains of *C. sporogenes*.

The nature of the inhibitory factor has not been determined. Dack (2) indicated that filtered supernatant fluids of *C. botulinum* gradually lost toxicity in the presence of *C. sporogenes*; he also reported that *C. botulinum* growth and toxin production was inhibited if the culture was grown in a sterile supernatant of *C. sporogenes*. Growth was in-

creased if the supernatants were heated suggesting that enzymes may have been involved. The inhibitory activity may be associated with bacteriocins as reported by Kautter et al. (6) for type E *C. botulinum* or with bacteriophage which has been reported to be involved in the interconversion of *C. botulinum* and *C. novyiii* (3).

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