JOURNAL OF BACTERIOLOGY, Apr., 1967, p. 1461-1462
Vol. 93, No. 4

Outgrowth and Toxin Production of Nonproteolytic Type B *Clostridium botulinum* at 3.3 to 5.6 C

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Received for publication 12 December 1966

Although the growth and toxin production of *Clostridium botulinum* type E within the temperature range of 3.3 to 10 C has been documented (C. E. Dolman et al., Can. J. Public Health **41**:215, 1950; D. F. Ohye and W. J. Scott, Australian J. Biol. Sci. **10**:85, 1957; C. F. Schmidt, R. V. Lechowich, and J. F. Folinazzo, Food Sci. **26**:626, 1961; J. T. Graikoski and L. L. Kempe, Bacteriol. Proc., p. 8, 1963), the ability of other nonproteolytic types of *C. botulinum* to grow and produce toxin in this same temperature range has not been reported.

The first reported isolation of a nonproteolytic *C. botulinum* type B strain on the North American continent was from salmon eggs implicated in an outbreak of human botulism in Canada (C. E. Dolman et al., J. Infect. Diseases **106**:5, 1960). These authors reported that their type B isolate would grow and produce gas after 6 weeks of incubation at 6 C. However, the production of toxin was not determined, nor was a study made of the outgrowth at temperatures lower than 6 C.

The purpose of this investigation was to determine the growth and toxin production of a nonproteolytic *C. botulinum* type B strain in the range of 2.2 to 5.6 C (36 to 42 F). The strain used in these studies was isolated at our laboratory from marine sediments during a study of the incidence of *C. botulinum* on the Pacific Coast of the United States.

Spore suspensions were prepared from 5-day cooked-meat (CM) medium (glucose, Trypticase, peptone, yeast extract, beef infusion, and ground-meat particles) cultures incubated at 30 C. After the incubation period, the broth portion of the CM medium was decanted and centrifuged. The spores were washed three times in sterile, distilled water and resuspended in physiological saline. Viable counts of the spore stock were prepared by use of TPG agar containing 1% yeast extract.

For low-temperature outgrowth studies, CM medium was sterilized in screw-capped tubes (16 by 150 mm). All tubes were prechilled for 2 hr in an ice-water bath and were inoculated with either 2×10^5 or 2×10^6 spores. The inoculated medium was overlaid with a layer of sterile Vaspar, and the tubes were held for an additional 2 hr in an ice-water bath prior to incubation at the corresponding temperatures. The tubes were incubated in a water bath in biochemical-oxygen-demand low-temperature incubators. Extreme precautions were taken to ensure against any rise in temperature during the incubation period or during observation of the tubes for growth.

Outgrowth was ascertained by the observation of gas bubbles beneath the Vaspar seal. Confirmation of growth was established by assaying the supernatant fluid of the culture for type B toxin. In the absence of gas production, the production of toxin was the only criterion used for outgrowth.

In previous studies on the characteristics of our nonproteolytic type B isolate, maximal toxin titers were obtained after 3 days of incubation at 30 C. These cultures contained 356 LD_{50} per ml of medium prior to trypsin activation and 112,000 LD_{50}/ml after activation with trypsin. Therefore, in the low-temperature outgrowth experiments, toxin assays were made with trypsinized cultures (J. T. Duff, G. G. Wright, and A. Yarinsky, J. Bacteriol. **72**:455, 1956). Trypsinized samples were diluted in half-log intervals in gelatin-phosphate diluent, and 0.5 ml of each dilution was injected intraperitoneally into white mice.

The LD_{50} dose was calculated by the method of H. Tint and A. Gillen (J. Appl. Bacteriol. **24**:83, 1961). The lethal toxin was identified as type B *C. Botulinum* toxin by use of the mouse-protection test, in which monovalent type B antiserum was used for serological neutralization.

Immediately after inoculation, the contents of three tubes of each inoculum level were assayed for the presence of *C. botulinum* toxin in both the trypsinized and untrypsinized state to determine whether the inoculum contained any residual toxin. A second set of inoculated tubes was incubated at 2.2 C and assayed for toxin after different incubation periods. All cultures assayed for toxin immediately after inoculation and after 120 days of incubation at 2.2 C were nontoxic (sensitivity of assay, 4 minimal lethal doses per ml of culture).

The incubation time for the production of gas and toxin at the various temperatures is summarized in Table 1. When the inoculum level was 2×10^6 spores per tube, the earliest time in which visible amounts of gas were observed was 17 days

TABLE 1. *Production of gas and toxin in CM medium inoculated with spores of Clostridium botulinum type B (nonproteolytic) and incubated at 3.3 to 5.6 C*

Spore inoculum per tube	Tube no.	Incubation time and toxin titer at								
		5.6 C			4.4 C			3.3 C		
		Gas production[a]	Toxin assay[b]	Toxin titer[c]	Gas production	Toxin assay	Toxin titer	Gas production	Toxin assay	Toxin titer
		days	*days*	*LD_{50}/ml*	*days*	*days*	*LD_{50}/ml*	*days*	*days*	*LD_{50}/ml*
2×10^6	1	17	18	35	24	27	20	>85	85	>10
	2	17	21	113	25	29	356	>85	85	>10
	3	18	21	200	25	29	113	>90	90	36
	4	18	21	356	25	29	356	>102	102	36
	5	18	21	200	28	32	200	>102	102	0
	6	18	21	100	26	32	100	>109	102	36
2×10^5	1	21	26	356	33	36	356	>109	109	0
	2	22	27	356	33	36	356	>109	109	0
	3	22	27	356	33	39	2000	>109	109	0
	4	22	27	834	35	39	1126	>109	109	0
	5	22	27	356	36	39	356	>109	109	0
	6	22	38	356	37	39	356	>109	109	0

[a] Incubation time before visible gas production.
[b] Incubation time before toxin assay.
[c] Mouse intraperitoneal LD_{50} per milliliter of culture.

at 5.6 C, 24 days at 4.4 C, and greater than 109 days at 3.3 C. If the inoculum level was reduced to 2×10^5 spores per tube, the earliest outgrowth time as determined by gas production was 21 days at 5.6 C, 33 days at 4.4 C, and greater than 109 days at 3.3 C. Toxin was detected shortly after gas was observed in the tubes incubated at 4.4 and 5.6 C. The toxin titers varied from tube to tube, but, in general, they increased with increased incubation time. The maximal toxin titer that could be obtained during prolonged incubation in the temperature range of 4.4 to 5.6 C was not determined.

At 3.3 C, gas was not observed in any of the tubes during the 109-day incubation period; toxin was detected, however, in tubes inoculated with 2×10^6 spores and incubated for 85 days. None of the tubes inoculated with 2×10^5 spores contained detectable amounts of toxin after 109 days of incubation. However, in another experiment (data not shown in Table 1), toxin was detected in tubes inoculated with 2×10^5 spores after 129 days of incubation; the toxin titer ranged from 36 to over 200 LD_{50} per ml of culture.

When the nonproteolytic type B strain was grown at 3.3 to 5.6 C, over 90% of the potential toxin (data not shown) was in the form of the protoxin. The protoxin was activated by treatment with trypsin.

The outgrowth and toxin production of our nonproteolytic type B culture is comparable with that reported for type E at 5.6 C. Based upon these preliminary data, the outgrowth time of Type B in CM medium at 3.3 C is considerably longer, however, than that reported for type E strains.

Studies are currently in progress to determine the minimal temperature for outgrowth of nonproteolytic type B spores in various substrates. These data will be reported later.

This investigation was supported by the U.S. Atomic Energy Commission Contract no. AT(49-7)-2442.