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Heat Resistance of Spores of Marine and Terrestrial Strains of *Clostridium botulinum* Type C

W. P. SEGNER AND C. F. SCHMIDT
Metal Division Research and Engineering, Continental Can Co., Inc., Chicago, Illinois 60620

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Resistance to heat of spores of marine and terrestrial strains of *Clostridium botulinum* type C in 0.067 M phosphate buffer (*p*H 7.0) was determined. The marine strains were 6812, 6813, 6814, and 6816; the terrestrial strains were 468 and 571. The inoculum level equaled 10^6 spores/tube with 10 replicate tubes for each time-temperature variable. Heating times were run at three or more temperatures to permit survival of some fraction of the inoculum. Survivors were recovered at 85 F (30 C) in beef infusion broth containing 1% glucose, 0.10% L-cysteine hydrochloride, and 0.14% sodium bicarbonate. *D* values were calculated for each fractional survivor end point after 6 months of incubation. Thermal resistance curves were constructed from the *D* value data. D_{220} (104 C) values for spores of 468 and 571 equaled 0.90 and 0.40 min, respectively. The corresponding values for spores of 6812, 6813, 6814, and 6816 were 0.12, 0.04, 0.02, and 0.08 min. The *z* values for the thermal resistance curves ranged from 9.0 to 11.5 F (5.0 to 6.2 C).

The classical paper of Bengston (2) is the only comprehensive report concerning the heat resistance of spores of *Clostridium botulinum* type C. Bengston studied spores of seven type C strains. Three strains were from fly larvae (*Lucilia caesar* or *L. sericata*), two from chickens showing symptoms of "limberneck," and one from the stomach contents of a horse reportedly dead from botulism poisoning; one nontoxigenic strain was included which Bengston had isolated by a single-cell technique from the prototype toxigenic culture. Spores of each strain were produced in a cooked-meat medium and heated in the same medium in which they were formed. Tubes were inoculated, flame-sealed, and completely submerged in a "boiling"-water bath. Spores showing the greatest heat resistance (strain from *L. sericata*) at an inoculum reportedly between 10^5 and 10^6 spores per tube survived heating for 60 min, but not 90 min.

The purpose of the present study was to compare the heat resistance of spores of marine and terrestrial strains of *C. botulinum* type C.

MATERIALS AND METHODS

Suspensions. Terrestrial strains 468 and 571 and marine strains 6812, 6813, 6814, and 6816 were used. The sources, method of preparation, and standardization of spore suspensions were as previously presented (5). Aqueous suspensions were standardized to contain 10^7 spores/ml, based on preheating at 160 F (71 C) for 15 min. The standardized suspensions were stored unheated at 34 F (1.1 C).

Heat resistance determinations. Ten-tube replicate sets were inoculated for each time-temperature combination. Sterile screw-cap tubes (16 by 125 mm) were inoculated with 0.1 ml of suspension (10^6) viable spores, and then 0.9 ml of heat-sterilized Sørensen's buffer (0.067 M, *p*H 7.0) was pipetted into each tube. Each set of tubes was heated simultaneously. For temperatures of 205 F (96 C) and below, a thermostatically controlled water bath with auxillary stirrer and insulated cover was used. Prior to heating, the screw caps were firmly tightened and sealed with a waterproof, heat-resistant tape. The tubes were placed in a metal holder, weighted down, and completely submerged in the bath.

For temperatures of 215 F (101 C) and above, thermal death time retorts were used (4). The correction factor for the come-up time, taken from previous heat resistance studies, was considered to be 1.2 min.

Various time-temperature combinations were run to obtain survivors in some fraction of the replicates. Survivors were recovered with beef infusion broth containing 1% glucose, 0.10% L-cysteine hydrochloride, and 0.14% sodium bicarbonate. The medium was prepared as previously described (5), dispensed in 200-ml quantities, and autoclaved at 250 F (121 C) for 15 min. The L-cysteine hydrochloride solution (20%) was heat-sterilized, and the bicarbonate (10%) was membrane-sterilized. Each was added aseptically to the medium immediately before use; then heat-sterilized 1 N sodium hydroxide was added to adjust the medium to *p*H 7.0 to 7.2. About 10 ml of the re-

TABLE 1. *Heat resistance of spores of terrestrial strains 468 and 571*

Temp	Strain 468			Strain 571		
	Heating time[a]	Positive tubes[b]	*D* value	Heating time[a]	Positive tubes[b]	*D* value
	min		*min*	*min*		*min*
215 F (101 C)	14.0	10/10	—	6.5	8/10	1.07
	15.0	3/10	2.31	7.0	4/10	1.09
	16.0	6/10	2.58	7.5	2/10	1.12
	17.0	1/10	2.43	8.0	1/10	1.14
				8.5	2/10	1.27
				9.0	0/10	—
220 F (104 C)	5.0	8/10	0.82	2.0	10/10	—
	5.5	9/10	0.92	2.5	6/10	0.40
	6.0	1/10	0.86	3.0	0/10	—
	6.5	3/10	1.00			
	7.0	0/10	—			
225 F (107 C)	1.4	10/10	—	0.60	7/10	0.10
	1.6	4/10	0.25	0.80	5/10	0.13
	1.8	3/10	0.29	1.00	0/10	—
	2.0	2/10	0.30			
	2.5	0/10	—			
230 F (110 C)	0.4	10/10	—			
	0.6	5/10	0.10			
	0.8	0/10	—			
	1.0	1/10	0.14			

[a] Corrected for the lethality of the come-up time.
[b] Fraction of tubes showing growth after heating.

TABLE 2. *Heat resistance of spores of marine strains 6812 and 6813*[a]

Temp	Strain 6812			Strain 6813		
	Heating time	Positive tubes	*D* value	Heating time	Positive tubes	*D* value
	min		*min*	*min*		*min*
200 F (93 C)				14.0	7/10	2.26
				15.0	3/10	2.31
				16.0	1/10	2.39
				17.0	0/10	—
205 F (96 C)	12.0	10/10	—	5.5	9/10	0.92
	14.0	8/10	2.29	6.0	7/10	0.97
	16.0	7/10	2.58	6.5	5/10	1.03
	18.0	1/10	2.77	7.0	4/10	1.09
	20.0	1/10	2.85	7.5	2/10	1.12
				8.0	3/10	1.23
				8.5	1/10	1.20
				9.0	0/10	—
				9.5	1/10	1.36
215 F (102 C)	1.2	9/10	0.20	0.4	2/10	0.06
	1.4	4/10	0.22	0.6	1/10	0.09
	1.6	4/10	0.25	0.8	1/10	0.11
	1.8	1/10	0.26			
	2.0	2/10	0.28			
220 F (104 C)	0.6	5/10	0.10			
	0.8	5/10	0.13			
	1.0	1/10	0.14			
	1.2	0/10	—			

[a] See footnotes to Table 1.

TABLE 3. *Heat resistance of spores of marine strains 6814 and 6816*[a]

Temp	Strain 6814			Strain 6816		
	Heating time	Positive tubes	*D* value	Heating time	Positive tubes	*D* value
	min		*min*	*min*		*min*
200 F	18.0	3/10	2.77			
(93 C)	20.0	5/10	3.17			
	22.0	1/10	3.14			
	24.0	0/10	—			
205 F	5.0	10/10	—	14.0	6/10	2.26
(96 C)	5.5	7/10	0.89	16.0	4/10	2.50
	6.0	4/10	0.94	18.0	1/10	2.57
	6.5	1/10	0.93	20.0	1/10	2.86
	7.0	0/10	—	22.0	0/10	—
215 F	0.2	10/10	—	1.5	5/10	0.24
(102 C)	0.4	9/10	0.07	2.0	1/10	0.29
	0.6	0/10	—	2.5	0/10	—
220 F				0.4	7/10	0.07
(104 C)				0.6	3/10	0.09
				0.8	0/10	—

[a] See footnotes to Table 1.

TABLE 4. *Thermal resistance values of spores of marine and terrestrial strains of C. botulinum type C heated in neutral phosphate buffer*

Strain	Avg *D* value (min)[a]						*z* values (F)
	200 F (93 C)	205 F (96 C)	215 F (101 C)	220 F (104 C)	225 F (107 C)	230 F (110 C)	
468			2.44	0.90	0.28	0.12	11.5
571			1.14	0.40	0.12		10.0
6812		2.62	0.24	0.12			10.8
6813	2.32	1.11	0.09	0.04[b]			10.7
6814	3.02	0.91	0.07	0.02[b]			9.5
6816		2.55	0.27	0.08			10.0

[a] *D* values are based on arithmetic averages of the data in the preceding tables.
[b] Value from extrapolation of thermal resistance curve based on data obtained at lower temperatures.

covery medium was poured into each heated tube, and then stratified with melted Vaspar. The tubes were incubated at 85 F (30 C) and examined for turbidity and gas at periods up to 6 months.

D values (time in minutes at a specified temperature to cause a 10-fold reduction in count) were calculated for each fractional survivor end point. The formula of Stumbo (6) was used:

$$D = \frac{t}{\log A - \log S}$$

where t is corrected heating time (minutes), A is the population per tube times the number of replicates, and S is the number of positive tubes, presuming that growth originated from one surviving spore in each positive tube. Thermal resistance curves for each strain were constructed by plotting the average *D* values on the logarithmic axis versus temperature on the linear scale on semilogarithmic graph paper.

RESULTS AND DISCUSSION

Determinations of thermal processes for foods involve calculations in degrees Fahrenheit; hence, the actual heating temperatures used are shown in degrees F with conversion to the nearest degree C. Tables 1 through 3 show the survivor data and *D* values of spores of the six type C strains studied.

Where heat-resistant survivors were recovered, growth was usually detectable within 2 weeks after incubation. Occasionally, there was a marked lag, sometimes ranging up to 1 month, before turbidity and gas were seen in some tubes. Such lags were commonly associated with the longer heating times or higher temperatures used and apparently reflect the slow recovery of severely heat-damaged spores.

The presence of type C toxin was confirmed

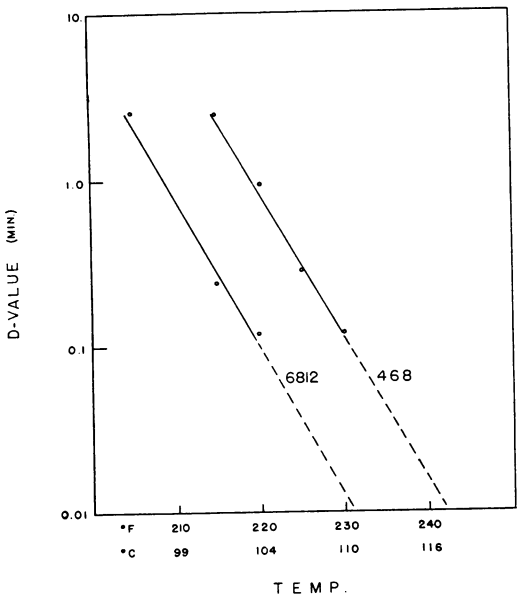

FIG. 1. *Comparative heat resistance of spores of terrestrial strain 468 and marine strain 6812.*

at most fractional end points by the intraperitoneal injection of white mice, unprotected and protected with type-specific botulinum antitoxin. No evidence of type C growth without toxin production was encountered.

Table 4 compares the average *D* values of spores of the terrestrial and marine strains and summarizes the *z* values from a plot of the thermal resistance data. Figure 1 contrasts the heat resistance of spores of the most resistant terrestrial strain (468) to that of spores of the most resistant marine strain (6812). Based on the limited number of cultures studied, spores of marine strains appear to be much more heat-sensitive than spores of most terrestrial strains.

In general, the data presented suggest that spores of both marine and terrestrial type C strains possess a resistance to heat which is intermediate between that shown by spores of *C. botulinum* type A or proteolytic type B (3) and that shown by spores of type E (1).

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