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Characteristics of *Clostridium botulinum* Type F Isolated from the Pacific Coast of the United States

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Some of the physiological and biochemical characteristics of a type F strain recently isolated from the United States were studied and compared with those of the prototype Langeland type F strain. The recent isolates were nonproteolytic, fermented sucrose and ribose, produced spores of low thermal resistance, produced a protoxin activated by trypsin, and grew and produced toxin at 38 F (3.3 C) from a spore inoculum. The prototype Langeland strain was proteolytic, did not ferment sucrose or ribose, and produced spores of relatively high thermal resistance, and the toxin of 3-day-old cultures was not activated by trypsin. Approximately two to three times the minimal lethal dose (MLD) of type F toxin from either Langeland or nonproteolytic strains was cross-neutralized by 1,000 anti-MLD of type E antitoxin. Antitoxin serums prepared by immunizing rabbits with the toxoid of the nonproteolytic type F isolate neutralized the toxin of the Langeland strain, but did not show cross-neutralization with the toxins of other types of *Clostridium botulinum*.

The first known strain of *Clostridium botulinum* type F was isolated by Moller and Scheibel (11) from a home-prepared liver paste implicated as the source of human botulism on the Danish Island, Langeland. This culture was studied more extensively by Dolman and Murakami (4) and designated as *C. botulinum* type F. The Langeland strain resembled type A and proteolytic type B strains of *C. botulinum* in its proteolytic and saccharolytic properties, but the toxin was antigenically distinct and differed from that of previous known types of *C. botulinum*.

In 1965, *C. botulinum* type F was demonstrated in marine sediments collected from the Pacific Coast of the United States (6) and isolated from fish taken from the Columbia River (1). Preliminary data on the characteristics of a pure culture of type F have been reported (Eklund, Poysky, and Wieler, Proc. Botulism Symp. Moscow, 1966, *in press*). The organism has also been reported to be present in mud samples collected from a stream in the interior of the United States (16).

In October 1966, the first outbreak of human botulism from *C. botulinum* type F in the United States occurred in California from venison jerky. Of the 20 people who ate the jerky, 3 developed symptoms of botulism, 2 developed mild transient gastroenteritis, and 15 remained asymptomatic (P. K. Conduit and H. A. Renteln, Morbidity and Mortality Weekly Report, **15**:349, 1966).

The purposes of this investigation were (i) to determine the physiological and biochemical characteristics of the *C. botulinum* type F strain recently isolated from the Pacific Coast of the United States, and (ii) to determine whether the characteristics of the isolate differ from those of the prototype Langeland strain.

MATERIALS AND METHODS

Cultures. *C. botulinum* type D and the Langeland strain of type F were obtained from L. V. Holdeman (Virginia Polytechnic Institute, Blacksburg, Va.).

The nonproteolytic type F isolates used in these studies were isolated at our laboratory from marine sediments collected from the Pacific Coast of the United States. From one of the enrichment cultures of marine sediments, 10 different type F isolates were separated into pure cultures. Of these 10 isolates, 1 (culture 202F) was selected as the representative for this study. The other nine isolates, however, were used in some of the different experiments. To avoid confusion in the discussion of the characteristics of the Langeland strain and the nonproteolytic isolates from the Pacific Coast of the United States, the latter isolates will henceforth be referred to by number.

Cultures of *C. botulinum* type A and nonproteolytic strains of type B were isolated at this laboratory from marine sediments. The Beluga strain of type E was received from C. E. Dolman (University of British

Columbia) and the type C strain was received from A. W. Anderson (Oregon State University).

Cooked meat (CM) medium. A modification of the medium of Dolman (3) was used. In the preparation of this medium, 500 g of freshly ground beef was heated in 1 liter of water, cooled, and infused overnight at 5 C. The fat layer was removed, and the infusion was adjusted to *p*H 8. The infusion was then heated, cooled, and again adjusted to *p*H 8.0. This procedure was repeated for a third time and the infusion was finally adjusted to *p*H 7.5. This procedure was found essential to stabilize the *p*H of the medium. The meat particles were separated from the liquid, and to the liquid portion were added 0.5% NaCl, 0.08% Na_2HPO_4, 0.5% peptone, 1.0% Trypticase (BBL), 0.4% glucose, 0.1% soluble starch, and 1% yeast extract. The concentration of glucose was reduced to 0.2% when the medium was used for the production of spores. Approximately 5 g of meat particles and 20 ml of liquid were added to each screw-capped culture tube and sterilized. The final *p*H of this medium was 7.2. Before inoculation, the tubes containing the CM medium were heated to expel dissolved oxygen, then cooled.

Propagation of culture. CM medium was used for the propagation of the Langeland and nonproteolytic type F isolates. Spore suspensions were prepared from 5-day cultures incubated at 30 C. Spores were centrifuged, washed five times in sterile distilled water, and resuspended in physiological saline. The spore suspensions were heat-shocked for 30 min at 60 C before use. Viable spore counts were made by use of (i) TPG agar (14) containing 1% yeast extract and 0.1% sodium thioglycolate, and (ii) 5% peptone agar (C. F. Schmidt *personal communication*) containing 1% yeast extract and 0.1% sodium thioglycolate.

Thermal resistance of spores. In the test for thermal resistance, spore suspensions were diluted in physiological saline so that 1 ml contained 100,000 spores. Of spore suspension, 3 ml was dispensed into each of a number of sterile 5-ml ampoules and the openings of the ampoules were sealed with a flame. The ampoules were then completely immersed in a water bath at the appropriate temperature. After predetermined heating periods, three ampoules were removed from the bath and were immediately immersed into ice water. The ampoules were then washed with filter-sterilized ethyl alcohol, flamed, and opened. Of the spore suspension 1 ml was removed and inoculated into CM medium The survival of spores was determined by growth and by gas production in CM medium during a 35-day incubation period at 25 C.

Antitoxin. Supernatant fluids containing toxin of isolates 202F were activated by trypsin (5) and fractionated according to the procedure of Gordon et al. (8). Detoxification was carried out at *p*H 5.5 in the presence of 0.3% Formalin at 37 C. After 7 days, the Formalinized toxin was nontoxic to mice in intraperitoneal doses of 0.5 ml. The fluid toxoid was dialyzed against 0.2 M succinate buffer and was absorbed on aluminum phosphate following the procedure of Gordon et al. (8).

Antiserum was produced in New Zealand white rabbits. The rabbits were injected with $AlPO_4$ toxoid (starting with doses of 1 ml and increasing to 2 ml) by use of a combination of intraperitoneal and subcutaneous routes. The injections were repeated every 6 days for a total of five injections. Whenever trial bleedings indicated a satisfactory level of antitoxin, the rabbits were bled by cardiac puncture. The serum was separated and Merthiolate (1:10,000) was added as a preservative.

Antitoxins of *C. botulinum* types A, B, C, D, E, and F (prepared from Langeland toxoid) were obtained from the National Communicable Disease Center (NCDC), Atlanta, Ga. Type E antitoxin was also obtained from C. E. Dolman.

Toxin assays and neutralization. Toxin determinations were made with centrifuged culture supernatant fluids diluted at half-log intervals in phosphate-gelatin buffer. Toxin assays of the nonproteolytic type F isolates were made by use of trypsin-treated and nontreated toxins. For trypsin activation, the procedures of Duff et al. (5) were followed using a final concentration of 0.5% Trypsin 1:250; Difco) at *p*H 6.0. White mice weighing 18 to 22 g were injected intraperitoneally with 0.5 ml of the appropriate dilution of culture supernatant fluid. Groups of two to three mice were used per dilution, and the minimal lethal dose (MLD) was calculated as the highest dilution which was lethal to mice within 72 hr.

Neutralization tests were made by injecting pairs of mice with 0.5 ml of a mixture composed of 0.4 ml of toxin supernatant fluid or dilution thereof and 0.1 ml (approximately 1 International Unit) of a monovalent antitoxin of *C. botulinum* types A, B, C, D, E, or F.

Specificity of 202F toxin and antitoxin. The cross-neutralization of 202F toxin and antitoxin of *C. botulinum* types A, B, C, D, and E was determined by mixing equal volumes of toxin and antitoxin. The toxin was tested at levels of 2 to 18 MLD/0.1 ml, and the antitoxin was tested by use of approximately 10,000 anti-MLD/0.1 ml for types A, B, C, and D. Type E antitoxin was tested at the 1,000 to 6,000 anti-MLD/0.1 ml level. The mixtures were incubated at 25 C for 1 hr, and mice were injected intraperitoneally with 0.4 ml of the toxin-antitoxin mixtures.

The cross-neutralization of 202F type F antitoxin with the toxins of types A, B, C, D, and E were determined in the same manner described above. The toxins were tested at levels of 2 to 10 MLD/0.1 ml, and the 202F type F antitoxin was used at a concentration of 2,000 anti-MLD/0.1 ml.

The potency of the 202F type F antitoxin was determined by mixing varying dilutions of the antitoxin with approximately 100 MLD of the Langeland and 202F (trypsin-activated) toxins. The mixtures were incubated for 2 hr at 25 C, and 0.5 ml of each mixture was injected intraperitoneally into each of four mice.

Morphological and biochemical characteristics of strains. Colonial morphology and characteristics were compared on Brain Heart Infusion Agar (Difco), blood agar (Brain Heart Infusion Agar to which 5% citrated human blood was added), and beef infusion egg-yolk agar [broth of CM medium without glucose containing 5% egg yolk and neutral red (18) as an indicator]. Anaerobic conditions were obtained by incubating the agar plates in Brewer anaerobic jars. Ability to ferment different carbohydrates was tested

in a basal medium containing 1% Trypticase, 0.5% peptone, and 0.5% yeast extract. Concentrated, filter-sterilized carbohydrates and sodium thioglycolate were added to the basal medium just prior to inoculation, giving respective final concentrations of 1 and 0.1%. Bromothymol blue indicator was added after incubation.

Proteolytic activity was determined with substrates of casein, coagulated egg white, cooked meat medium, nutrient gelatin, and gelatin containing peptone, Trypticase, yeast extract, glucose, and sodium thioglycolate. Urease production was tested for in Urea Broth (Difco) modified by the addition of 0.5% yeast extract, 0.1% sodium thioglycolate, and 0.1% glucose.

Other tests were performed according to the procedures outlined in the *Manual of Microbiological Methods* (15).

Outgrowth and toxin production at 38 to 50 F (3.3 to 10 C). For low-temperature outgrowth studies, CM medium (containing 0.5% yeast extract) was sterilized in 16 × 150 mm screw-capped tubes. All tubes were prechilled for 2 hr in an ice-water bath prior to inoculation.

Different concentrations of spores (ranging from 20 to 2×10^6 in log intervals) were inoculated into the tubes containing CM medium, and the medium was overlaid with a layer of sterile Vaspar. The tubes were held for an additional 2 hr in an ice-water bath and then incubated in water baths in biochemical oxygen demand low-temperature incubators. Extreme precautions were taken to ensure against any rise in temperature during the incubation period or during the observation of the tubes for growth.

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

The lethal toxin was identified as *C. botulinum* type F toxin by the mouse-protection test in which monovalent type F antiserum was used for serological neutralization.

The outgrowth time of different concentrations of 202F spores was determined at 10 C. For minimal temperature outgrowth studies, spore levels of 2×10^5 and 2×10^6 per tube were used, and the incubation temperatures ranged from 38 to 42 F (3.3 to 5.6 C).

Immediately after inoculation, the contents of three tubes of each inoculum level were assayed for the presence of botulinum toxin in both the trypsinized and untrypsinized states to determine whether the inoculum contained any residual toxin. All cultures assayed for toxin immediately after inoculation were nontoxic (sensitivity of assay, 4 MLD/ml of cultures).

RESULTS

The demonstration, isolation, and identification of *C. botulinum* type F from marine sediments collected from the coastal areas of Oregon and California have previously been discussed (6; Eklund, Poysky, and Wieler, Proc. Botulism Symp. Moscow, 1966, *in press*).

Two of the demonstrations of type F were from different 5-g samplings of a sediment collected 83 km from the northern coast of California (42° N. Latitude) at a depth of 1,646 meters. A third demonstration was from a sediment sample taken 100 km off the coast of Oregon (43° N. Latitude) at a depth of 1,326 meters, and a fourth from sediments taken 25 km off the southern coast of California (35° N. Latitude) at a depth of 235 meters.

From one of the toxic enrichment cultures of sediments collected at 1,646 meters, a strain of *C. botulinum* type F was separated into pure cultures. Ten of the approximately 300 different colonies picked from beef infusion egg-yolk agar containing 0.1% sodium thioglycolate produced toxin that was neutralized only by type F antitoxin. The characteristics of these isolates were studied and are reported below.

Cultural and morphological characteristics. During the early stages of growth, all of the nonproteolytic type F isolates produced large quantities of gas in CM medium. Within 18 hr of incubation at 20 to 37 C, the production of gas was so vigorous that all of the meat particles were raised to the top of the broth. The Langeland strain also produced gas in this medium at these temperatures but to a far lesser degree.

On blood-agar plates, both the nonproteolytic and the Langeland type F strains showed definite β-hemolysis. The colonies of the nonproteolytic type F were 2 to 3 mm in diameter and translucent with very irregular margins. On Brain Heart Infusion Agar, the colonies were larger and more nearly circular. On both blood-agar and Brain Heart Infusion Agar plates, the colonies of the Langeland strain were generally larger and more opaque and circular than those of the 202F strain.

Both type F strains produced a precipitate in beef infusion egg-yolk agar and a pearly (iridescent) layer surrounding and covering the colonies. Other types of *C. botulinum* give a similar reaction in beef infusion egg-yolk agar medium (10, 17, 18). Microscopically, the vegetative cells of both 202F and Langeland type F strains were quite uniform in size when grown in CM medium. The average size of the cells of the 202F and Langeland strains was 1 by 5 μ and 1 by 4 μ, respectively. Young cultures of 202F strain were gram-positive, but became gram-negative as the culture aged. Hanging drop preparations from young cultures of isolate 202F showed vigorous motility of the organism imparted by the many peritrichous flagella (Fig. 1).

Spores were produced abundantly in CM medium, by both the Langeland and 202F strains. The spores were oval-shaped and were formed

subterminally to terminally, causing a moderate swelling of the bacilli (Fig. 2). TPG medium (14) or CM medium without yeast extract often yielded very few spores, and the vegetative cells were very filamentous.

Biochemical characteristics. The biochemical characteristics of the Langeland and the 202F type F strains are summarized in Table 1 and are generally similar to those reported by Moore et al. (12). Both strains produced acid and gas from glucose, fructose, maltose, trehalose, dextrin, glycerol, and sorbitol. However, the action on the different carbohydrates was greater with the nonproteolytic strain. The main difference in saccharolytic activity between the strains was the ability of the nonproteolytic strain and the inability of the Langeland strain to produce acid and gas from sucrose and ribose.

The most striking difference between the two cultures lay in their ability to attack protein. The Langeland strain attacked casein, cooked meat particles, coagulated egg albumin, and gelatin.

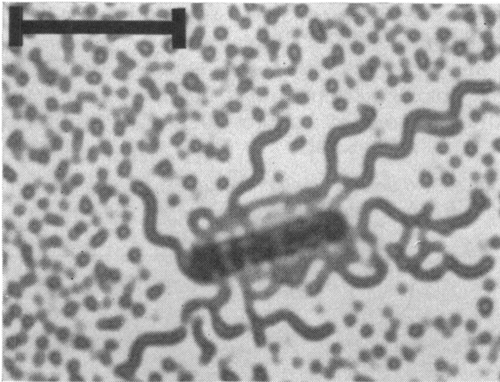

FIG. 1. *Nonproteolytic type F organism with peritrichous flagella. Marker represents 5 μ.*

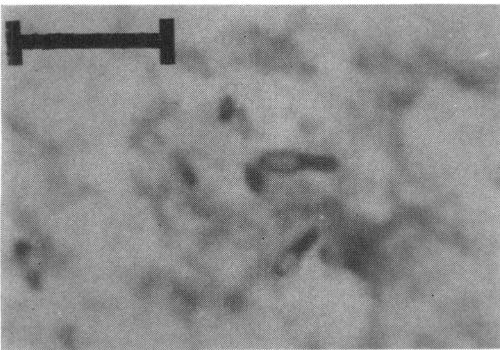

FIG. 2. *Nonproteolytic type F spores from center of 72-hr colony. Marker represents 10 μ.*

TABLE 1. *Biochemical activities of nonproteolytic (isolate 202F) and Langeland strains of Clostridum botulinum type F*

Property	Reaction of	
	Nonprotolytic type F	Langeland type F
Saccharolytic		
Glucose	AG++++[a]	AG+
Fructose	AG++++	AG+
Galactose	A[b]	A
Maltose	AG++	AG+
Sucrose	AG++	–
Lactose	–	–
Dextrin	AG++	AG
Trehalose	AG++	AG
Raffinose	–	–
Melibiose	–	–
Salicin	–	A
Glycerol	AG	AG
Mannitol	–	–
Sorbitol	AG+++	AG
Inositol	–	–
Ribose	AG+++	–
Proteolytic		
Casein	–	+
Cooked meat	–	+
Coagulated egg albumin	–	+
Gelatin	–	+
Other		
Nitrate reduction	–	–
Indole production	–	–
Urease production	–	–
H_2S production	SL[c]	+

[a] AG = acid and gas production.
[b] A = acid only.
[c] Slight amounts of H_2S detected on lead acetate paper strips wedged into the mouth of tubes of cooked meat cultures.

The nonproteolytic isolates, of course, were unable to attack any of the complex proteins, nor could they attack gelatin. The Langeland strain produced large amounts of hydrogen sulfide in CM medium and in Triple Sugar Iron Agar (Difco). In contrast, hydrogen sulfide was not produced by any of the nonproteolytic isolates in Triple Sugar Iron Agar and was only faintly detectable in CM medium as determined by the use of lead acetate paper.

Toxicity of nonproteolytic type F cultures incubated at different temperatures. The toxin titers of type F (isolate 83F) cultures incubated for different periods of time at 20, 25, 30, and 37 C are summarized in Table 2. Maximal toxicity of the cultures was obtained after 72 hr of incubation for cultures incubated at 25 and 30 C and after 136 hr for cultures incubated at 20 C. Tryp-

TABLE 2. *Toxicity of supernatant fluids from nonproteolytic type F (isolate 83F) cultures incubated at different temperatures*

Incubation temp	Incubation time	Toxin titer (mouse MLD/ml of medium) for	
		Untrypsinized	Trypsinized
C	*hr*		
20	65	<10	100
	88	<10	200
	112	20	200
	136	20	>2,000
	160	20	2,000
	234	20	2,000
25	24	<10	20
	48	<10	200
	72	20	2,000
	96	20	2,000
30	24	<10	200
	48	<10	1,000
	72	20	2,000
	96	20	2,000
	144	20	2,000
37	24	<10	<10
	48	<10	10
	72	<10	10
	96	<10	<10

sin activation produced a 100-fold increase of the type F toxin at the time of maximal toxin production. Even though good growth was obtained at 37 C, only 10 MLD of toxin was detected per ml of medium after 48 and 72 hr of incubation. After 96 hr of incubation at 37 C, toxin was no longer detectable in either the untrypsinized or trypsinized state.

Toxicity of different nonproteolytic type F isolates. The toxin titers of 10 different isolates of type F (different toxic colonies isolated from the original toxic enrichment sample) were determined after 3 days of incubation at 30 C in CM medium. The titers of the trypsin-activated cultures fell roughly into four different categories: 2,000, 10,000, 20,000, and >20,000 MLD/ml of cultures. The titers of the nonactivated cultures varied from a low of 20 MLD/ml to a high of 200 MLD/ml of medium. In some cases, trypsin activation potentiated the toxin titers over 100-fold.

In the early phases of this study, we found that one unit of NCDC Langeland type F antitoxin (approximately 5,000 anti-LD_{50}/unit) would only neutralize approximately 100 MLD of 202F type F toxin. However, when trypsin-activated toxin was selected as the challenging toxin, 1 unit of NCDC antitoxin would neutralize over 2,000 MLD of 202F toxin.

Neutralization of type F toxin by other botulinum antitoxins. Neutralization of type F toxin by type E antitoxin has been previously reported (4, 6, 11).

Dolman and Murakami (4) reported that 4,000 type E anti-MLD would neutralize 10 MLD of type F toxin (Langeland strain). Since the characteristics of the nonproteolytic type F isolate differ from those of the Langeland strain, we attempted to determine whether the cross-neutralization patterns were also different.

The results of these studies with type E antitoxin (from the NCDC and from C. E. Dolman) indicated that approximately 1,000 type E anti-MLD would neutralize 2 to 3 MLD of toxin of isolate 202F. The same degree of neutralization by type E antitoxin and type F toxin therefore exists in the Langeland and 202F type F strains.

Neutralization studies were also made by use of types A, B, C, and D antitoxins. None of these antitoxins (at levels up to 10,000 anti-MLD) showed any degree of cross-neutralization with the toxin of the 202F isolate.

Specificity of 202F type F antitoxin. Antitoxin was obtained from rabbits immunized with the $AlPO_4$ toxoid of culture 202F. The antitoxin

TABLE 3. *Thermal resistance of Clostridium botulinum nonproteolytic type F spores (10^6/ml) in physiological saline*

Temperature	Exposure time	Thermal resistance[a] of					
		Isolate 202F in replicate			Isolate 288F in replicate		
		1	2	3	1	2	3
C	*min*						
90	2	+	+	+	+	+	+
	4	−	−	−	−	−	−
	6	−	−	−	−	−	−
	8	−	−	−	−	−	−
80	5	+	+	+	+	+	+
	10	+	+	+	+	+	+
	20	+	+	+	+	+	+
	30	−	−	−	−	−	+
	40	−	−	−	−	−	−
70	30	+	+	+	+	+	+
	60	+	+	+	+	+	+
	90	+	+	+	+	+	+
	120	+	+	+	+	+	+
	150	+	+	+	+	+	+
	180	+	+	+	+	+	+

[a] Symbols: + = survivors within 35 days of incubation in CM medium; − = no survivors within 35 days of incubation in CM medium.

contained approximately 4,000 and 20,000 anti-MLD/ml when the challenging toxin was that of the Langeland and 202F (trypsin-activated) type F cultures, respectively.

When 2,000 anti-MLD of the 202F antitoxin (based upon the 202F toxin) was mixed with the various levels of botulinum toxins of types A, B, C, D, and E and when the mixtures were injected into mice, the animals invariably died. The toxin of the 202F strain is therefore, to all intents, antigenically monospecific.

Thermal resistance of nonproteolytic type F spores. The results of the thermal resistance of two of the nonproteolytic type F isolates (202F and 288F) are summarized in Table 3. Spores were diluted in physiological saline such that 1 ml of suspension contained 100,000 spores. Heating at 90 C for 4 min destroyed all of the spores present. Both cultures survived 20 min at 80 C and one of the tubes contained survivors after 30 min at 80 C. Heating at 70 C for 180 min was not sufficient to destroy the spores of either of the nonproteolytic cultures.

A more detailed study on the heat resistance of spores of both type F strains is planned, but these preliminary data do indicate that the heat resistance of type F isolates 202F and 288F resembles that of type E strains. The Langeland strain, however, has a much greater heat resistance which more closely resembles that of the type A strains (4).

Minimal outgrowth temperature of nonproteolytic type F spores. The Langeland strain of *C. botulinum* type F has been reported to grow and produce toxin at 4 C (N. W. Walls, Proc. Botulism Symp. Moscow, 1966, *in press*). Since nonproteolytic and Langeland type F strains differ in many of their physiological and biochemical characteristics, the minimal outgrowth temperatures of several of the nonproteolytic type F isolates were also determined.

The incubation periods for outgrowth of cultures 202F and 288F are summarized in Tables 4 and 5. The outgrowth time (gas production) for culture 288F was comparable to that for culture 202F at 42 F (5.6 C). However, the incubation time for gas production at the lower temperatures was somewhat longer for culture 288F. Even though culture 288F was slowest in outgrowth based upon gas production, it produced more toxin at the lower temperatures than did culture 202F. Toxin titers of over 200 MLD/ml of medium were obtained at 42 F (5.6 C) for culture 288F; in contrast, with the same incubation period and inoculation level, culture 202F produced only 20 MLD/ml of medium.

Neither of the cultures produced gas or detectable amounts of toxin at 36 F (2.2 C) during the 102-day incubation period.

Based upon these data, the outgrowth time of the nonproteolytic type F strain in the temperature range of 38 to 42 F (3.3 to 5.6 C) is comparable with that of type E reported by Schmidt et al. (13).

TABLE 4. *Time for production of gas and toxin in CM medium inoculated with spores of Clostridium botulinum type F (nonproteolytic), culture 202F, and incubated at 38 to 42 F (3.3 to 5.6 C)*

Spore inoculum per tube	Tube no.	Incubation time and toxin titer at								
		42 F (5.6 C)			40 F (4.4 C)			38 F (3.3 C)		
		Gas production[a]	Toxin assay[b]	Toxin titer[c]	Gas production[a]	Toxin assay[b]	Toxin titer[c]	Gas production[a]	Toxin assay[b]	Toxin titer[c]
		days	*days*	*MLD/ml*	*days*	*days*	*MLD/ml*	*days*	*days*	*MLD/ml*
2×10^6	1	14	15	<4	18	22	<4	39	47	<4
	2	14	18	<4	18	27	<4	39	52	20
	3	15	20	<4	19	29	10	49	61	<4
	4	15	22	20	19	32	20	52	81	20
	5	16	26	100	19	32	10	52	84	20
	6	19	27	20	20	34	20	52	84	20
2×10^5	1	17	26	20	22	34	20	39	47	<4
	2	18	27	20	22	34	20	39	52	<4
	3	18	27	20	22	35	20	40	61	20
	4	18	29	20	23	35	20	42	63	20
	5	18	29	20	23	35	20	45	61	10
	6	18	29	10	23	35	20	45	81	20

[a] Incubation time before visible gas production.
[b] Incubation time before toxin assay.
[c] For mice which received intraperitoneal inoculation, determined in trypsinized state.

TABLE 5. *Time for production of gas and toxin in CM medium inoculated with spores of Clostridium botulinum type F (nonproteolytic), culture 288 F, and incubated at 38 to 42 F (3.3 to 5.6 C)*

Spore inoculum per tube	Tube no.	Incubation time and toxin titer at								
		42 F (5.6 C)			40 F (4.4 C)			38 F (3.3 C)		
		Gas production[a]	Toxin assay[b]	Toxin titer[c]	Gas production[a]	Toxin assay[b]	Toxin titer[c]	Gas production[a]	Toxin assay[b]	Toxin titer[c]
		days	*days*	*MLD/ml*	*days*	*days*	*MLD/ml*	*days*	*days*	*MLD/ml*
2×10^6	1	15	18	<4	23	29	<4	53	61	4
	2	15	20	<4	23	29	<4	53	63	4
	3	18	20	<4	25	43	20	53	63	4
	4	16	26	200	25	35	20	53	81	100
	5	16	27	200	25	35	100	53	84	200
	6	18	27	200	25	35	100	53	84	200
2×10^5	1	19	26	200	25	29	<4	68	81	10
	2	19	27	200	25	29	<4	77	90	10
	3	20	27	200	26	34	10	78	90	<4
	4	20	27	200	28	35	<4	81	102	4
	5	21	29	200	29	35	20	81	102	20
	6	21	29	>200						

[a] Incubation time before visible gas production.
[b] Incubation time before toxin assay.
[c] For mice which received intraperitoneal inoculation, determined in trypsinized state.

TABLE 6. *Outgrowth time and toxin production of different concentrations of 202F type F spores in CM medium incubated at 50 F (10 C)*

Spore inoculum per tube	Gas producion[a]	Toxin assay[b]	Toxin titer[c]
	days	*days*	*MLD/ml*
2×10^6	5	8	20
		22	2,000
2×10^5	5–6	8	20
		22	2,000
2×10^4	6	9	>100
		22	1,000
2×10^3	7	9	>100
		28	2,000
2×10^2	7–8	12	>200
		28	2,000
2×10^1	8	12	>200
		28	2,000

[a] Incubation time before visible gas production.
[b] Incubation time before toxin assay.
[c] For mice which received intraperitoneal inoculation, determined in trypsinized state.

Outgrowth time of different concentrations of 202F type F spores at 50 F (10 C). The effect of spore-inoculum size (culture 202F) on the outgrowth time at 50 F (10 C) is summarized in Table 6. When the inoculum contained 2×10^6 spores, outgrowth occurred after 5 days of incubation. When the inoculum was reduced to 2×10^1 spores, outgrowth occurred in 8 days. Toxin was detected shortly after gas production at all inoculum levels. Maximal toxin titer obtained was 2,000 MLD/ml. Approximately 99% of the toxin was in the protoxin state and was activated by trypsin.

DISCUSSION

The data presented above indicate that the nonproteolytic and Langeland type F strains differ considerably in the following characteristics: (i) saccharolytic and proteolytic activity, (ii) production of hydrogen sulfide, (iii) thermal resistance of spores, and (iv) activation of toxin by trypsin. Holdeman and Smith (9), however, have reported that 90% of the potential toxin of young cultures of the Langeland strain existed in the form of the precursor and that with the old cultures the greater portion of the precursor usually had been converted to toxin.

The biochemical and physiological characteristics of the nonproteolytic strains of types B, E, and F (isolated at this laboratory from samples collected from the Pacific Coast of the United States) are indistinguishable (*unpublished data*). The nonproteolytic strains of these types: (i) produce toxin activated by trypsin, (ii) produce spores of low thermal resistance, (iii) grow and produce toxin at low temperatures (7, 13), and (iv) possess the same biochemical, cultural, and colonial characteristics.

With these recent findings on type F, nonpro-

teolytic and proteolytic strains of types B and F are now known to exist in nature. In a recent study made by this laboratory on the incidence of *C. botulinum* in marine sediments (*unpublished data*), one of the enrichment cultures yielded type A botulinum toxin which was lethal to mice only after trypsin activation. This culture has not been separated into pure culture, but, because of the necessity for trypsin activation to detect toxicity, the possibility exists that it is a strain of nonproteolytic type A.

It is interesting to speculate on the delayed recognition of *C. botulinum* type F in the United States and possibly in other parts of the world. The low thermal resistance of the spores of the nonproteolytic type F undoubtedly played an important role in its not being detected during earlier surveys. However, a factor of equal importance is the probability of misidentification of type F due to the cross-neutralization between type E antitoxin and type F toxin. In studies made in this laboratory on the incidence of *C. botulinum* on the Pacific Coast of the United States, enrichment cultures often have contained only 4 to 10 MLD of botulinum toxin/ml of the culture (*unpublished data*). Therefore, type E antitoxin, if used in large excess, could neutralize type F toxin, and the culture could thus be misidentified as type E. Protection of low levels of type F toxin from crude enrichment cultures and food materials by excessive amounts of type E antitoxin has previously been reported (6, 11; P. K. Conduit and H. A. Renteln, Morbidity and Mortality Weekly Report, **15**:349, 1966).

The necessity of having a trivalent therapeutic antiserum (types A, B, and E) available for therapeutic purposes has been emphasized by Dolman (2). In view of the recent type F findings in the United States (1, 6, 16; P. K. Conduit and H. A. Renteln, Morbidity and Mortality Weekly Report) the necessity of having a tetravalent antiserum (types A, B, E, and F) available needs serious consideration.

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