Minimal Growth Temperature, Sodium Chloride Tolerance, pH Sensitivity, and Toxin Production of Marine and Terrestrial Strains of Clostridium botulinum Type C

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Received for publication 15 June 1971

Minimal growth temperatures of four marine and two terrestrial strains of Clostridium botulinum type C were determined in a laboratory culture medium, fortified egg meat medium (FEM), and in ground haddock. The inoculum equaled $2 \times 10^6$ viable spores per tube with five-tube replicate sets. The spores were preheated in aqueous suspension at 71°C for 15 min prior to inoculation to reduce toxin carry-over. Similar results were obtained in both substrates. Both the marine and the terrestrial strains grew at 15.6°C, but only the terrestrial strains grew at 12.8°C. None of the strains grew at 10°C during prolonged incubation. The sodium chloride tolerance and the pH sensitivity of the marine and the terrestrial strains were determined at 30°C. The basal medium consisted of beef infusion broth. The inoculum level equaled $2 \times 10^6$ unheated spores per replicate. Growth was inhibited at salt concentrations from 2.5 to 3.0%. The terrestrial strains were more pH-sensitive than the marine strains. Whereas the terrestrial strains failed to grow below pH 5.62, three of the marine strains grew at pH 5.10, but not at pH 4.96, during extended incubation. One marine strain grew at pH 5.25, but not below. FEM and proteose peptone-Trypticase-yeast extract-glucose medium permitted the production of high levels of botulinum toxin among four media tested. Toxin produced by the marine and terrestrial strains showed no increase in toxicity after incubation with trypsin.

Michener and Elliott (13), who reviewed the minimal growth temperatures of various microorganisms, found no evidence of growth of Clostridium botulinum type C at temperatures below 10°C. Tanner and Oglesby (20) observed growth from a vegetative-cell inoculum in a laboratory culture medium at 15°C, but not at 10°C. From a spore inoculum, they noted growth and toxin production at 20°C, but not at 15°C. However, Tanner, Beamer, and Rickher (18) obtained growth from a spore inoculum in peas and in asparagus at 10°C, but they were unable to detect growth at 5°C, the next lower temperature tested. Castell (5) obtained type C growth in ground lobster and ground cod fillets at 25°C, although he was unable to demonstrate growth in either substrate at 3°C during 3 months of incubation. Beerens, Sugama, and Tahon-Castel (2) failed to obtain growth of two type C strains in a laboratory culture medium at 6°C.

Although definite proof is lacking, it appears that all C. botulinum type C strains tested for psychrophilic ability have been isolated from terrestrial sources. Because type C exists in marine environments with botulinum types known to possess low-temperature growth ability, and because of cultural similarities to those types, it was considered that marine strains might show psychrophilic growth characteristics (16). Therefore, the principal objective of this research was to compare the minimal growth temperatures of marine and terrestrial strains of C. botulinum type C. In addition, work was conducted to determine their sodium chloride tolerance, pH sensitivity, and toxin-producing ability in various culture media.

MATERIALS AND METHODS

Type C strains. The origin of the terrestrial and the marine strains was previously described (16). The terrestrial strains are numbered 468 and 571; the marine strains are designated 6812, 6813, 6814, and 6816.

Sporulation and standardization of suspensions. Spores of each strain were produced in FEM medium (Difco Egg Meat Medium fortified with additions of 1% yeast extract, ammonium sulfate, and glucose).
As already described (16), aqueous suspensions were prepared and standardized by a deep-tube technique with beef infusion agar (BIA). The recovery medium was supplemented with 0.1% L-cysteine hydrochloride and 0.14% sodium bicarbonate by aseptic additions immediately before use. Colony counts were made after 24 to 48 h of incubation at 30 C.

Low-temperature growth studies. FEM, autoclaved at 121 C for 15 min, was used in 20-g quantities in screw-cap tubes (20 by 150 mm). A fish substrate was prepared from fresh haddock fillets (Melanogrammus aeglefinus). The fillets were finely ground and distributed in 10- to 12-g amounts in screw-cap tubes (16 by 125 mm). Air pockets in the substrate produced during filling were removed by centrifuging at a low speed. Tubes for incubation at 10 C and below were heated in flowing steam for 30 min; those for incubation above 10 C were autoclaved at 121 C for 15 min. After heating and cooling in cold tap water, the tubes were dried and held at 2 C for inoculation the next day. The inoculum level used equaled 0.1 ml/tube or 2 X 10^9 viable spores. Prior to inoculation, the spores were preheated at 71 C for 15 min. Five-tube replicate sets were inoculated per variable. The inoculated tubes and corresponding unoinoculated controls were Vaspar-sealed and incubated at 15.6 C (60 F), 12.8 C (55 F), 10 C (50 F), and 7.8 C (46 F).

Incubated tubes of FEM medium and ground haddock were examined for gas production on a Monday, Wednesday, and Friday time schedule. After approximately 8 months of incubation at the highest temperatures showing no gas production, each replicate set was assayed for type C toxin and examined for growth by phase-contrast microscopy. The Vaspar was removed from each tube with sterilized cotton-tipped swabs. For the haddock substrate, the contents of each tube were blended with 90 ml of sterilized, prechilled sodium acetate buffer (0.5 M, pH 5.4). About 6 ml of the blend was transferred to a sterilized tube and held overnight at about 3 C; toxin was assayed the next day. For the FEM medium, 1 ml of the liquid portion was diluted with 9 ml of gelatin-phosphate buffer and the assays were run immediately. Duplicate white mice each were injected intraperitoneally with 0.5 ml of a sample. The mice were observed for botulinum symptoms up to 4 days before a sample was considered nontoxic.

Sodium chloride and pH studies. The basal medium used in the sodium chloride and pH studies consisted of beef infusion (BI) broth. Disodium phosphate normally added to the medium was deleted. In the salt experiments, various concentrations of sodium chloride were dissolved in the basal medium on a weight basis. The medium was dispensed in 200-ml quantities in screw-cap bottles and autoclaved at 121 C for 15 min. Before use, sterile L-cysteine hydrochloride was added to the medium to a final concentration of 0.1%. Sterile 1 N sodium hydroxide was added to adjust the medium to pH 7.0 to 7.2. The balance of the loss in weight due to autoclaving was restored to the nearest gram by the addition of sterile distilled water. In the pH experiments, the basal medium was adjusted from pH 6.0 to 5.0 at 0.2 pH unit intervals by the addition of dilute HCl. The medium was bottled and autoclaved as described above. The pH values cited in the tables represent determinations made after the incorporation of 0.1% L-cysteine hydrochloride.

Five-tube replicate sets were inoculated per variable with 0.1 ml of unheated spore suspension (2 X 10^6 spores) per tube. About 10 ml of medium was poured aseptically into each inoculated tube. Each set of replicates was sealed with Vaspar and incubated at 30 C. Examinations for growth (gas and turbidity) were made daily for the first 2 weeks of incubation and then at less frequent times. Unoinoculated control tubes, poured at the beginning of the experiment, were used to detect a possible change in pH of the medium during incubation; no pH changes occurred.

Toxin studies. Cardella's medium (4) consisted of 40 g of Proteose Peptone (Difco), 20 g of Trypticase (BBL), and 10 g of glucose per liter; it was adjusted to pH 7.0 before sterilization. Jensen's medium (12) was slightly modified, consisting of 30 g of Lactalysate (BBL), 20 g of yeast extract (Difco), 3.5 g of sodium citrate, and 10 g of glucose. BI broth and FEM medium were described earlier. Jensen's medium, BI broth, and FEM medium were adjusted to pH 7.2 to 7.4, bottled, and autoclaved at 121 C for 15 min along with Cardella's medium. Toxin titrations were made at intervals during 1 month of incubation at 30 C. Serial 10-fold dilutions were prepared in gelatin-phosphate buffer. Duplicate white mice, weighing 18 to 20 g each, were intraperitoneally injected with 0.5-, 0.2-, and 0.1-ml volumes of the dilutions to obtain a minimal lethal dose (MLD) end point.

Trypsin digestion. To determine the effect of trypsin on type C toxin, single tubes of FEM medium were inoculated for each type C strain and incubated at 30 C for 3 days. For trypsin digestion, 1 ml of the culture was mixed with an equal volume of 1.0% trypsin (Difeo, 1:250) and incubated at 37 C for 1 hr. Serial 10-fold dilutions of the trypsinized and an untrypsined culture were prepared and injected into mice as described above.

RESULTS

Tables 1 and 2 summarize the results of the minimal growth temperature studies. FEM and ground haddock media gave similar results. Both the marine and terrestrial strains grew at 15.6 C, but only the terrestrial strains grew at 12.8 C. Type C toxin was verified in all of the tubes showing gas. The marine strains failed to grow at 12.8 C, and the terrestrial strains did not grow at 10 C or below during prolonged incubation. All inoculated tubes of FEM medium and ground haddock at the highest temperature showing no gas formation were examined microscopically for growth and assayed for botulinum toxin by the injection of white mice. None of the tubes showed growth or detectable toxin. The minimal growth temperature of strain 6816 in ground haddock was not determined, because the results in FEM medium showed that it had
TABLE 1. Minimal incubation temperatures permitting growth of *C. botulinum* type C in FEM medium

| Strain | Avg and range for growth (days)* | 15.6°C | 12.8°C | 10.0°C |
|--------|---------------------------------|--------|--------|--------|
| 468    |                                 | 8 (5-12)| 40 (19-47)| >250  |
| 571    |                                 | 6 (5-9) | 31 (28-33)| >250  |
| 6812   |                                 | 15 (7-26)| >250  | >250  |
| 6813   |                                 | 7 (5-9) | >250  | >250  |
| 6814   |                                 | 9 (7-12)| >250  | >250  |
| 6816   |                                 | 18 (13-24)| >250 | >250  |

* Based on an inoculum of *2 × 10^6* spores per replicate and five replicates per variable. Spores were preheated at 71°C for 15 min before inoculation. Range is shown in parentheses.

TABLE 2. Minimal incubation temperatures permitting growth of *C. botulinum* type C in ground haddock

| Strain | Avg and range for growth (days)* | 15.6°C | 12.8°C | 10.0°C |
|--------|---------------------------------|--------|--------|--------|
| 468    |                                 | 36 (7-152)| 23, 25, 25, | >250  |
| 571    |                                 | 7 (5-7) | 19      | >250  |
| 6812   |                                 | 6 (5-7) | >250  | >250  |
| 6813   |                                 | 6 (5-7) | >250  | >250  |
| 6814   |                                 | 5 (5-6) | >250  | >250  |

* See footnote to Table 1. When fewer than five tubes showed growth, the time of growth for each tube is given; where no range is shown, all five replicates showed gas production at the same time.

In general, FEM medium gave the highest levels of toxin, although Cardella's medium was almost as good. During 1 month of incubation, the toxin remained relatively stable in each case.

The pH of each medium at 3 days showed little change during 30 days of incubation. Jensen's and Cardella's media ranged from pH 5.6 to 5.8 at 3 days depending on the strain; BI broth showed pH 5.2 to 5.6, and FEM ranged from pH 5.8 to 6.5. The marine strains commonly gave a somewhat lower final pH than the terrestrial strains.

The effect of trypsin on type C toxin was determined. The results were based on an MLD comparison; hence, a two- to threefold difference between a trypsinized and an untrypsinized strain was not considered significant. There was no conclusive evidence of activation with trypsin; with toxin from 468, an indication of possible inactivation with trypsin was observed (fourfold reduction).

Mention should be made of the rapidity of mouse death after intraperitoneal injection with type C toxin. At high toxin levels, i.e., 100 MLD/ml and higher, deaths invariably occurred in less than 24 hr; at low toxin levels, deaths often occurred after 24 hr and up to 72 hr. No deaths beyond 72 hr were ever recorded.

**DISCUSSION**

Nonproteolytic strains of *C. botulinum* type B, type E, and type F are capable of growth and toxin production down to about 3°C (7, 8, 14). In contrast, it is generally accepted that type A and proteolytic type B are unable to grow at 10°C or below (13). Although type C coexists in a limiting temperature for growth similar to those of the other marine strains tested.

The sodium chloride sensitivity of the marine and terrestrial strains is shown in Table 3. Vegetative-cell growth from a spore inoculum was shown by the development of turbidity and gas. Each strain grew in the presence of 2.0% salt, but only 468 and 6814 grew in 2.5% salt. None of the strains grew in 3.0% salt. Recovery of spores of each strain at the end of incubation from the 3.0% salt medium showed that some spore germination had occurred, although many spores were still refractile by phase-contrast microscopy.

The limiting pH for type C growth is presented in Table 4. The terrestrial strains failed to grow below pH 5.62. In contrast, three of the marine strains grew as low as pH 5.10, but not below. Strain 6812 was somewhat less pH-tolerant than the other marine strains.

The effect of various media formulations on type C toxin production is shown in Table 5.
marine environments with the nonproteolytic botulinum types, neither marine nor terrestrial strains apparently possess psychrophilic growth characteristics. The minimal temperature for growth of *C. botulinum* type C is very near that accepted as limiting for growth of type A and proteolytic type B. The usual pattern that nonproteolytic strains of *C. botulinum* exhibit low-temperature growth characteristics does not appear to be applicable to type C.

It is usually recognized that about 10% sodium chloride (calculated as per cent brine concentration) is necessary to inhibit growth and toxin production of *C. botulinum* type A and proteolytic type B (1, 9, 19). In contrast, type E growth is inhibited by a brine concentration of about 5.0% (15). Type C appears to be even less salt-tolerant than type E, its growth being inhibited by 3.0% salt.

The lowest pH permitting growth and toxin production of *C. botulinum* is 4.7 to 5.0, based on numerous strains, inoculum levels, and culture media (11, 15, 21). Judged by the data presented here, there is no evidence to show that type C can grow at any lower pH than the other botulinum types. Cardella et al. (4) and Skulberg (17) reported that a proteose peptone-Trypticase-yeast extract-glucose medium permitted the production of high levels of botulinum toxin by type C. Work presented here shows that both marine and terrestrial strains produce appreciable quantities of toxin.
in either Cardella's medium or FEM medium. Of course, Cardella's medium has the distinct advantage of being an aparticulate medium, in contrast to FEM.

*C. botulinum* includes both proteolytic and nonproteolytic strains based largely on their ability or inability to digest coagulated egg albumin or meat particles. All available type A strains are reportedly proteolytic, whereas all type C, D, and E strains are nonproteolytic. Both proteolytic and nonproteolytic strains of types B and F are known, but most of the nonproteolytic strains are weakly proteolytic, as shown by the fact that they often hydrolyze gelatin. Until recently, it was generally accepted that only the nonproteolytic strains produced botulinum toxin that could be activated upon treatment with the proteolytic enzyme trypsin. The phenomenon of trypsin activation of botulinum toxin has been studied thoroughly since the first report by Duff, Wright, and Yarinsky (6) on type E toxin. It commonly presumed that type E toxin is elaborated by the organism as a prototoxin (17). Since type E, unlike types A and B, lack strongly active proteolytic enzymes, the toxin is released by the organism principally in the prototoxin form that is commonly activated by trypsin. In contrast, toxin from the proteolytic strains usually does not show activation with trypsin except in very young cultures (3). The organism's cellular proteases presumably cause activation of the toxin in the same manner as trypsin causes activation of toxin of many nonproteolytic strains. It was recently shown that the proteolytic enzymes of some botulinum strains do not always activate the prototoxin; similarly, toxins produced by some nonproteolytic strains do not always show activation with trypsin. Iida (10) observed both situations in his studies. Two type C strains studied by Iida produced toxin that showed no significant indications of activation by trypsin. In this work, toxin produced by the marine and the terrestrial strains also showed no conclusive signs of activation when incubated with trypsin.

**ACKNOWLEDGMENT**

This work was conducted under contract AT (11-1) 1183 with the Division of Biology and Medicine of the U.S. Atomic Energy Commission.

**LITERATURE CITED**

1. Anderton, J. I. 1963. Pathogenic organisms in relation to pasteurized cured meats. Scientific and Technical Surveys No. 40, p. 1-158. The British Food Manufacturing Industries Research Association, Leatherhead, Surrey, England.

2. Beerens, H., S. Sugama, and M. Tahon-Castel. 1965. Psychrotrophic clostridia. J. Appl. Bacteriol. 28:36-48.

3. Bonventre, P. F., and L. L. Kempe. 1959. Toxicity enhancement of *Clostridium botulinum* type A and B culture filtrates by proteolytic enzymes. J. Bacteriol. 78:892-893.

4. Cardella, M. A., J. T. Duff, C. Gottfried, and J. S. Beigel. 1958. Studies on immunity to toxins of *Clostridium botulinum*. IV. Production and purification of type C toxin for conversion to toxoid. J. Bacteriol. 57:360-365.

5. Castell, C. H. 1947. Growth of *Clostridium* in seaweeds and marine fish. J. Fish. Res. Board Can. 7:62-69.

6. Duff, J. T., G. G. Wright, and A. Yarinsky. 1956. Activation of *Clostridium botulinum* type E toxin by trypsin. J. Bacteriol. 72:455-460.

7. Eklund, M. W., F. T. Powsky, and D. I. Wieler. 1967. Characteristics of *Clostridium botulinum* type F isolated from the Pacific Coast of the United States. Appl. Microbiol. 18:1316-1323.

8. Eklund, M. W., D. I. Wieler, and F. T. Powsky. 1967. Outgrowth and toxin production of nonproteolytic type B *Clostridium botulinum* at 3.3 to 5.6 C. J. Bacteriol. 93:1461-1462.

9. Greenberg, R. A., H. J. Siliker, and L. D. Fatta. 1959. The influence of sodium chloride on toxin production and organoleptic breakdown in perishable cured meat inoculated with *Clostridium botulinum*. Food Technol. 13:509-511.

10. Iida, H. 1970. Activation of *Clostridium botulinum* toxin by trypsin, p. 336-340. In M. Herzberg (ed.), Proc. First U.S.-Japan Conf. on Toxic Microorganisms. U.S. Dept. of Interior and UJNR Panels on Toxic Microorganisms. U.S. Govt. Printing Office, Washington, D.C.

11. Ingram, M., and R. H. M. Robinson. 1951. The growth of *Clostridium botulinum* in acid bread media. Proc. Soc. Appl. Bacteriol. 14:62-72.

12. Jensen, W. I., and R. B. Gritman. 1967. An adjuvant effect between *Clostridium botulinum* types C and E toxins in the mallard duck (*Anas platyrhynchos*), p. 407-413. In M. Ingram and T. A. Roberts (ed.), *Botulism* 1966. Chapman and Hall, Ltd., London.

13. Michener, H. D., and R. P. Elliott. 1964. Minimum growth temperature for food poisoning, focal indicator, and psychrophilic microorganisms. Advan. Food Res. 13:349-396.

14. Schmidt, C. F., R. V. Lechowich, and J. F. Folinazoo. 1961. Growth and toxin production by type E *Clostridium botulinum* below 40°C. J. Food Sci. 26:626-630.

15. Segner, W. P., C. F. Schmidt, and J. K. Boltz. 1966. Effect of sodium chloride and pH on the outgrowth of spores of type E *Clostridium botulinum* at optical and suboptimal temperatures. Appl. Microbiol. 14:49-54.

16. Segner, W. P., C. F. Schmidt, and J. K. Boltz. 1971. Enrichment, isolation, and cultural characteristics of marine strains of *Clostridium botulinum* type C. Appl. Microbiol. 22:1017-1024.

17. Skulberg, A. 1964. Studies on the formation of toxin by *Clostridium botulinum*. Inst. Food Hygiene and Microbiology, Veterinary College of Norway, Oslo. A/S Kaare Grypting,在香港

18. Tanner, F. W., P. R. Beamer, and C. J. Rickher. 1940. Further studies on development of *Clostridium botulinum* in refrigerated foods. Food Res. 5:323-333.

19. Tanner, F. W., and F. L. Evans. 1933. Effect of meat curing solutions on anaerobic bacteria. I. Sodium chloride. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. II 88:44-54.

20. Tanner, F. W., and E. W. Oglesby. 1936. Influence of temperature on growth and toxin production by *Clostridium botulinum*. Food Res. 1:481-494.

21. Townsend, C. T., L. Yee, and W. A. Mercer. 1954. Inhibiton of growth of *Clostridium botulinum* by acidification. Food Res. 19:536-542.