Differentiation of Clostridium botulinum Types A, B, and E by Pyrolysis-Gas-Liquid Chromatography

R. D. Cone and R. V. Lechowich
Department of Food Science, Michigan State University, East Lansing, Michigan 48823

Received for publication 18 September 1969

Vegetative cells and spores of 10 strains of Clostridium botulinum representing types A, B, and E were grown in Trypticase-peptone-sucrose-yeast extract (TPSY) medium. Five type E strains were also grown in Multipeptone-sucrose-Nutramino acids (MSN) medium. Lyophilized samples were subjected to pyrolysis-gas-liquid chromatography (PGLC) analysis, and the resulting pyrograms were examined for variations in elution patterns between spores and vegetative cells of types A, B, and E grown in the TPSY medium and spores and vegetative cells of type E grown in the TPSY medium and spores and vegetative cells of type E grown in TPSY and MSN media. Growth and toxin production of all 10 strains of C. botulinum were investigated by using a modified dialysis sac culture technique. The dialysate supernatant fluid (DSF) obtained after centrifugation of the 5-day-old cultures from the dialysate was also subjected to PGLC analysis. Control samples consisting of (i) noninoculated DSF, (ii) noninoculated DSF plus partially purified toxin, and (iii) 1.0 mg of partially purified toxin were also analyzed by PGLC. Differences between pyrograms of cultures were suitable for positive identification at the type level but not at the strain level. Pyrograms permitting differentiation were also obtained between spores and vegetative cells as well as between the same cultures grown in different media. The dialysis sac technique was useful in detecting growth but not toxin production of C. botulinum.

The increase in type E botulism outbreaks throughout the world (2, 3) and in the United States (1) during the last decade has led to a substantial increase in studies related to the microorganism Clostridium botulinum. The study of the food-borne disease of botulism usually involves (i) demonstration that the filtrate of a suspected food sample contains type-specific toxin by use of animal neutralization tests, (ii) similar detection of type-specific toxin in a patient's blood sample, and (iii) cultural procedures to isolate and identify the causative microorganism. Animal toxin assay procedures have definite time, cost, and technique limitations. The recently described detection methods that may include selective growth on egg yolk medium, fluorescent-antibody staining, polycrylamide gel electrophoresis, immunofluorescence, electron microscopy, and immunological techniques appear promising only as supplementary detection methods since their reliability has not been adequately established.

One of the most promising techniques for the identification of bacteria appears to be pyrolysis-gas-liquid chromatography (PGLC). Since several workers have reported the successful use of this technique for rapid identification of other genera and species of bacteria (5-8), it seemed feasible that it might also be used for detection and identification of cultures of C. botulinum. This method would not only detect materials that would provide adequate identification of cultures, but it might also decrease the minimum time of 5 days required for detection and identification by conventional methods. Preparation of the sample for analysis by growth and lyophilization is also relatively simple compared to some other methods.

The purpose of this study was to investigate the uses of PGLC for the detection of growth and toxin production of selected serotypes of pure cultures of C. botulinum in laboratory media. No food systems were used in these preliminary efforts. The effects of the growth medium and of per cent sporulation on the resulting elution patterns after pyrolysis were also studied.
MATERIALS AND METHODS

Microorganisms. The microorganisms studied in this experiment included two strains of *Clostridium botulinum* type A, two proteolytic strains of *C. botulinum* type B, and six strains of *C. botulinum* type E (five toxic strains and one nontoxic variant). The strain numbers and the source of each strain are shown in Table 1.

| Type | Strain | Source |
|------|--------|--------|
| A    | 62     | C. F. Schmidt, Continental Can Co., Chicago, Ill. |
| A    | 78     | C. F. Schmidt, Continental Can Co., Chicago, Ill. |
| B    | 169    | C. F. Schmidt, Continental Can Co., Chicago, Ill. |
| B    | 213    | C. F. Schmidt, Continental Can Co., Chicago, Ill. |
| E    | Vancouver Her-ring (VH) | C. F. Schmidt, Continental Can Co., Chicago, Ill. |
| E    | Kalamazoo (Kal) | C. F. Schmidt, Continental Can Co., Chicago, Ill. |
| E    | Seattle Forks (SF) | J. T. Graikoski, Bureau of Commercial Fisheries, Ann Arbor, Mich. |
| E    | A6247  | Donald A. Kautter, Food and Drug Administration, Washington, D.C. |
| E    | 066B nontoxic (NT) | Haim M. Solomon, Food and Drug Administration, Washington, D.C. |

TABLE 1. Sources of various strains of *Clostridium botulinum* types A, B, and E used in pyrolysis-gas-liquid chromatography experiments

Carry-over of materials from the inoculation medium to the saline solution was prevented by centrifuging the inoculum (15 ml of 16- to 18-hr actively growing culture) for 10 min at 1,000 × g by using a Sorvall RC-2 refrigerated centrifuge. The culture was then resuspended by using 5 ml of sterile distilled water and inoculated into the saline solution. The flasks were incubated at 32°C, and duplicate flask cultures were prepared for each strain.

Harvesting vegetative cells and spores. Cultures incubated at 32°C for 8 to 10 hr in TPSY and MSN media to obtain vegetative cells were immediately cooled to 4°C to prevent sporulation, dispensed into 250-ml centrifuge bottles, and centrifuged for 15 min at 5,000 × g with the centrifuge cooled to 4°C. The cells were then washed by centrifugation as above and resuspended in 200 ml of sterile distilled water until they appeared clean under microscopic examination (required five to eight washings). The cells were suspended in 100 ml of sterile distilled water and stored in sterile glass bottles at 2°C after the final washing.

Spore crops of *C. botulinum* type E were grown in both media and harvested after 24 to 30 hr of incubation when spores were free from their sporangia. Longer incubation (30 to 36 hr) was necessary to obtain adequate sporulation of type A and B strains. It was then necessary to treat these latter cultures with lysozyme (0.75 mg of lysozyme per ml of 0.1 N KCl) for 4 to 6 hr at 32°C to obtain free spores. All washing and storage procedures of vegetative cells and spores were the same.

Microorganisms grown by the use of the dialysis sac technique were separated from the dialysate fluid by centrifugation at 5,000 × g, and the dialysate supernatant fluid (DSF) was retained for subsequent analysis. The DSF was filtered through a membrane filter (0.80 μm pore size) to remove any microorganisms not removed by centrifugation and stored in sterile glass bottles at 2°C.

Direct microscopic counts. Direct microscopic counts were made on all culture samples by using a Petroff-Hauser counting chamber. A 1-ml amount of each culture was added to either 3 or 7 ml of 30% glycerol solution, depending on the concentration of the microorganisms. The direct counts gave an indication of the total count as well as the per cent sporulation. Refractile bodies were considered to be spores.

Toxin assays. Swiss Webster white mice weighing 15 to 20 g were injected intraperitoneally with 0.1, 0.2, and 0.5 ml of serial dilutions of each DSF sample. Before injection, the type E samples were digested for 60 min at 37°C with 0.5 ml of 5% sodium thioglycolate adjusted to pH 7.2. One liter of 0.1% Trypsin (Difco, 1:250) was added to the samples and homogenized, and an equal volume of 0.05 M sodium phosphate buffer (pH 6.0), and dilutions of all toxins were made in 0.05 M sodium phosphate buffer. The highest dilution at which injected mice died was used to calculate the minimal lethal dose (MLD).

Controls. Control samples consisted of (i) noninoculated DSF; (ii) noninoculated DSF plus partially purified toxin of the VH strain of *C. botulinum* type E, obtained from Alexander Emodi, Department of
Food Science, Michigan State University, to give a final toxin titer of $10^6$ MLD per ml; and (iii) a 1.0-mg sample of the partially purified toxin ($5.0 \times 10^6$ MLD per mg of N).

**Lyophilization of samples.** All cultures as well as the DSF and control samples were lyophilized by using a VirTis Freeze Mobile. Approximately 5 ml of each culture or sample was added to a 10-ml freeze-drying ampoule. The contents were then shell-frozen by using a dry ice and alcohol bath and lyophilized for 8 to 10 hr under a vacuum of 0.25 mm of Hg.

**PGLC analysis.** A Hewlett-Packard pyrolysis unit (model 80; F and M Scientific Division, Avondale, Pa.) was used to pyrolyze the samples. This instrument utilized an automatic pyrolysis cycle in which there was a 60-sec delay after activation of the pyrolysis switch, allowing the gas chromatograph to stabilize. After the delay period, the jaw-style probe element which contained the lyophilized sample was energized for 12 sec to bring about complete pyrolysis at 1,200 C. The probe was fitted directly to the injection port of a Hewlett-Packard gas chromatograph (model 5750 B; F and M Scientific Division) by using a special adapter which allowed the pyrolysis products to be swept directly into the columns by the carrier gas after pyrolysis. The gas chromatograph was equipped with dual-flame ionization detectors and a Moseley 7128 A dual pen strip chart recorder. The operating parameters of the gas chromatograph were as follows: columns, 72 by $\frac{3}{4}$ inch (183 by 0.48 cm) copper tubing; sample size, 0.9 to 1.1 mg for spores and cells and 1.3 to 1.7 mg for DSF and control samples; coating, 15% high temperature-stabilized ethylene glycol adipate (Analabs, Hamden, Conn.); support, 90 to 100 mesh Anakrom ABS (Analabs); column conditioning, 240 C for 2 weeks; carrier gas, helium at a pressure of 60 psig (column A, 50 ml/min flow rate; column B, 67 ml/min flow rate); hydrogen, 12 psig; air, 33 psig; temperatures, 1,200 C for 12 sec for pyrolysis; 250 C for detectors; 230 C for injection port; column programmed at 10 C per min from 50 to 240 C; upper limit interval, variable; chart speed, 0.25 inch (0.64 cm) per min; sensitivity and attenuation, $10^8 \times 8$ for spores and cells, variable for DSF and control samples.

**RESULTS AND DISCUSSION**

Pyrograms of lyophilized cultures were interpreted by visual examination. Retention times, the presence or absence of peaks, and the ratio of peak heights with relationship to each other were particularly useful. No quantitation or identification of peaks was attempted in these preliminary studies.

Duplicate samples were grown for each culture, and two or more replicates of each sample were examined by PGLC. For purposes of simplifying the material presented in this paper, only representative pyrograms of each sample are shown, since results were consistent between both duplicate cultures and replicate pyrolysis determinations.

Considerable qualitative and quantitative differences were observed in pyrograms obtained from the various types and strains of *C. botulinum*. The quantitative differences appeared to be caused, in part, by an inability to accurately control the amount of sample being pyrolyzed, since the jaw-style probe limited the accuracy in weighing the sample size to the nearest 0.1 mg. The consistency of packing the sample into the probe also appeared to influence the pyrogram, since the pyrolysis products of samples packed too firmly were not swept instantaneously into the column after pyrolysis and those packed too loosely were often blown off the probe by the carrier gas. These variations in sample size can clearly be seen in Fig. 1. The pyrograms of 169B and 213B are essentially the same; however, more sample was pyrolyzed with 213B. The differences might also be caused by quantitative differences in the two strains, but this is impossible to predict with the type of probe that was employed in this experiment. Very accurate measurements of the sample pyrolyzed must be obtained before quantitative differences such as these can be considered.

Levy (4) also indicated the importance of the effect of sample size upon pyrolysis products. He observed that secondary reactions between radicals and fragments formed upon rupture of primary bonds during pyrolysis are affected by the quantity of material pyrolyzed. He also indicated that the rate of decomposition of the material pyrolyzed is dependent on the film thickness of the sample in relationship to the geometry of the source of heat. Ehrler and Frijouf (digest of a paper presented at the Pittsburgh Conf. of Anal. Chem. and Appl. Spectroscopy, Cleveland, Ohio, 1968) have further indicated that sample size as well as final temperature, rate of temperature rise, and residence time in the reactor greatly influence pyrolysis elution patterns. The final temperature and rate of temperature rise were readily controlled by the pyrolysis apparatus used in this study; however, residence time was influenced by sample packing and sample size was difficult to accurately control.

Pyrograms obtained upon PGLC analysis of vegetative cells of four strains of *C. botulinum* types A and B are shown in Fig. 1. Examination of these elution patterns revealed distinct differences in peaks e, h, o, and r. Strain 62A was distinguishable from strain 78A and strains of type B by the large peak o. The type B strains were not readily differentiated from each other; however, the larger quantity of material eluted in peaks e, h, and r allowed them to be differentiated from type A strains. All other variations, including those in peak d, failed to be consistent...
enough among duplicate samples as well as among replicates of the same sample to be used for identification purposes.

A representative pyrogram of vegetative cells of *C. botulinum* type E grown in TPSY medium is shown in part F of Fig. 2. Only quantitative variations were noted among the six strains of type E examined, and these differences appeared to be caused by variability in final population achieved in each culture. Those cultures giving higher direct microscopic counts (Table 2) produced larger concentrations of material in several elution peaks. No differences among type E strains were sufficiently consistent for identification purposes. Parts D and F of Fig. 2, however, indicated the ease with which the vegetative cells of type A could be differentiated from those of type E. Considerable differences were noted in peaks a through e, whereas peaks i and n were much larger in type A than in type E and an additional peak was present between peaks r and s in type E. Since type B was somewhat like type A, similar differences existed between types B and E. Thus, it was possible to differentiate between vegetative cells of *C. botulinum* at the type level; however, only the variation in strain 62 of type A was noted at the strain level.

Elution patterns obtained upon PGLC analysis of spores of types A and B are shown in Fig. 3. No consistent differences suitable for identification purposes occurred at the strain level for type A and B spores. Strain 78A did appear to yield pyrograms with larger quantities of material eluted in peaks g, i, and s. Sporulation of this culture (Table 3), however, was much better than that of the other type A and B strains examined, and these differences appeared to be related to sporulation. Differences in peaks w and z also failed to be consistent. Differentiation between spores of type A and type B was thus not possible. Spores of type E cultures (part E of Fig. 2) also failed to show any differences in pyrograms at the strain level suitable for identification purposes. Comparison of part E with part C of Fig. 2, which shows the pyrogram for a representative strain of type A spores, revealed distinct differences in elution patterns of type A and thus type B from type E. Differences which...
were always consistent and distinct occurred in peaks g, h, i, n, r, s, x, and z.

No studies have previously been undertaken to differentiate strains and types of cultures by pyrolysis of their spores. Differences in elution patterns, however, were expected since these spores were formed from the same vegetative cells in which differences were observed. Generally, the same type of difference was noted between spores of *C. botulinum* as between vegetative cells. Identification was possible at the type level and only occasionally at the strain level.

Spores and vegetative cells of *C. botulinum* type E grown in MSN medium also failed to provide pyrograms suitable for identification at the strain level. However, comparison of type E spore and vegetative cell pyrograms grown in MSN medium (parts A and B of Fig. 2) with those of type E grown in TPSY medium (Fig. 2E and F) revealed appreciable differences among elution patterns of the same cultures grown in the two different media. Differences in pyrograms of vegetative cells occurred in peaks c, k, m, n, q, s, and z. Peak i was also absent in MSN pyrograms, whereas there was a larger additional peak between peaks r and s in the TPSY pyrograms. Major differences in spore pyrograms occurred in peaks a, e, g, n, r, w, and z. Peak x was absent in spores grown in TPSY, and peak z was much larger in spores from TPSY.

Oyama and Carle (5) also indicated that the growth medium had an influence on the pyrograms, which they obtained when they grew *Candida pulcherrima* in malt extract and in Trypticase soy broth. They further observed that organisms grown in similar media give similar pyrograms.

The two media used in this experiment showed definite similarities as well as differences in pyrograms for the same culture. Since both media are rich in nitrogen, this might account for the similarities. The differences were probably caused by the presence of yeast extract as well as varying concentrations of other nitrogen-rich materials.

A comparison of spores and vegetative cells of *C. botulinum* types A and E is provided in Fig. 2. Consistent differences in peaks g, n, and z were common among all types studied. Type A (and B, similar to A) showed additional differences in peaks a through d and peaks w, x, and y. These differences between spores and cells were expected since they have been shown to be physi-
TABLE 2. Direct microscopic counts of vegetative cells of Clostridium botulinum types A, B, and E grown in two different media

| Type | Strain | Total count (organisms/ml) |
|------|--------|----------------------------|
|      |        | MSN | TPSY             |
| A    | 62     | 8.3 × 10⁸ | 7.3 × 10⁸ |
| A    | 78     | 5.9 × 10⁸ | 1.6 × 10⁸ |
| B    | 169    | 5.0 × 10⁸ | 1.3 × 10⁸ |
| B    | 213    | 1.1 × 10⁸ | 7.5 × 10⁸ |
| E    | VH     | 1.2 × 10⁸ | 1.5 × 10⁸ |
| E    | KAL    | 1.0 × 10⁸ | 7.5 × 10⁸ |
| E    | SF     | 1.0 × 10⁸ | 5.5 × 10⁸ |
| E    | A6247  | 6.0 × 10⁸ | 3.0 × 10⁸ |
| E    | 066BNT | 0.6 × 10⁸ | 2.0 × 10⁸ |

The presence of such materials as calcium and dipicolinic acid in spores and lysis of the sporangia and formation of new coat layers in spores made them completely different entities than the vegetative cells from which they were formed. In addition to sporulation, the age of the culture may also have an influence on the pyrogram obtained. The cultures undergo lysis as they grow older and many of the cell components may be released. This is particularly true for those cells that do not sporulate. The data collected in this experiment could not resolve the differences in pyrograms as being due to different sporulation percentages or age of the culture, since spores and cells of each strain were necessarily incubated for various times to obtain each cell type and various numbers of vegetative cells were present in sporulated cultures (Table 3).

An attempt was made to detect growth and toxin production of C. botulinum by using a modification of the dialysis sac technique described by Vinet and Fredette (9). Since most of the toxin should have been present in the DSF after growth and centrifugation of the cultures, it was proposed that this toxin might be detectable by PGLC. The dialysis sac technique was primarily used to limit and control the availability of nutrients to the cultures, thus preventing possible interference of these compounds with the pyrograms subsequently obtained. Because of the limited availability of nutrients to the cultures grown in the dialyzed medium, growth and

FIG. 3. Pyrograms from spores of four strains of Clostridium botulinum types A and B grown in TPSY medium. The capital letters denote the type and Att is the attenuation.
TABLE 3. Direct microscopic counts and per cent sporulation of cultures of Clostridium botulinum types A, B, and E grown in two different media

| Type | Strain | MSN | TPSY |
|------|--------|-----|------|
|      |        | Total count (organisms/ml) | Sporulation (%) | Total count (organisms/ml) | Sporulation (%) |
| A    | 62     | 1.20 × 10^8 (organisms/ml) | 54.8             | 9.5 × 10^8 (organisms/ml) | 94.8             |
| A    | 78     | 9.5 × 10^8 (organisms/ml)  | 94.8             | 9.0 × 10^8 (organisms/ml) | 65.6             |
| B    | 169    | 9.5 × 10^8 (organisms/ml)  | 85.4             | 9.5 × 10^8 (organisms/ml) | 65.6             |
| B    | 213    | 5.2 × 10^8 (organisms/ml)  | 93.5             | 5.2 × 10^8 (organisms/ml) | 93.5             |
| E    | VH     | 3.8 × 10^8 (organisms/ml)  | 76.3             | 5.8 × 10^8 (organisms/ml) | 93.1             |
| E    | KAL    | 3.8 × 10^8 (organisms/ml)  | 93.1             | 5.5 × 10^8 (organisms/ml) | 89.4             |
| E    | SF     | 2.0 × 10^8 (organisms/ml)  | 84.3             | 5.8 × 10^8 (organisms/ml) | 91.3             |
| E    | 317    | 2.0 × 10^8 (organisms/ml)  | 98.6             | 4.9 × 10^8 (organisms/ml) | 89.4             |
| E    | A6247  | 3.3 × 10^8 (organisms/ml)  | 72.5             | 5.5 × 10^8 (organisms/ml) | 89.4             |
| E    | 066BN  | 4.9 × 10^8 (organisms/ml)  | 78.4             | 5.5 × 10^8 (organisms/ml) | 89.4             |

Fig. 4. Comparison of pyrograms for samples consisting of (A) uninoculated dialysate supernatant fluid (DSF); (B) uninoculated DSF plus partially purified toxin; (C) 1.0 mg of partially purified toxin; (D) DSF of type E Clostridium botulinum; (E) DSF of type A C. botulinum; and (F) DSF of type B C. botulinum.

Sporulation was not as good as in cultures grown in normal TPSY medium. Toxin titers, however, were of the order 10^8 to 10^9 MLD per ml.

Representative pyrograms of the DSF of types E, A, and B of C. botulinum investigated are shown in parts D, E, and F of Fig. 4. Pyrograms were essentially the same among strains of each type, but differences were noted at the type level. Types A and B were distinguishable on the basis of peaks f, j, o, and p, whereas pyrograms of type
E were entirely different from those of type A or type B after peak f. These differences were probably caused by physiological differences in the three types. Since type A and type B were proteolytic, they assimilated and dissimilated different compounds than type E which was nonproteolytic and primarily saccharolytic. Types A and B are also markedly less saccharolytic than type E.

Parts A, B, and C of Fig. 4 illustrate pyrograms of control samples which consisted of (i) noninoculated DSF, (ii) noninoculated DSF plus partially purified toxin, and (iii) 1.0 mg of partially purified toxin, respectively. Comparison of the pyrograms obtained by PGLC of the control samples with those of the DSF from the cultures provided rather interesting results. Toxin production was not detectable, since practically all of the toxin was eluted in small peaks during the first 15 min (part C, Fig. 4). The toxin peaks appeared to be hidden by peaks produced by other pyrolytic products. The toxin plus DSF control failed to be of any significant differentiating value, since the presence of toxin seemed to cause little variation in the elution pattern (part B, Fig. 4). Detection of growth, however, was possible with this modified dialysis sac technique. The pyrogram for the noninoculated DSF control (part A, Fig. 4) was considerably different from those of the three types of C. botulinum. Peaks k and m of the type E pyrogram (part D, Fig. 4) were consistently different from those in the control, and the pyrograms of types A and B were completely different from the control after peak f.

Generally, PGLC appeared to show promise as an analytical tool for identification of C. botulinum at the type level. Further studies that include other species of Clostridium and other bacterial genera as well should be conducted. The investigation of other column materials and other operating parameters, however, is also necessary to determine the usefulness of this technique at the strain level since this study revealed only a few reliable differences. PGLC may also be extremely valuable for detection of growth of C. botulinum. Further studies must be conducted, however, to determine whether growth in actual food products can be detected.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant UI-00173-03 and Public Health Service training grant 5-T01-U1-01031 from the Consumer Protection and Environmental Health Service, National Center for Urban and Industrial Health.

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