Journal of Industrial Microbiology, 1 (1986) 165–170
Elsevier

SIM 021

Heterogeneity in the cellular protein profiles of *Clostridium botulinum* types A and B

Saumya Bhaduri*, Paul H. Demchick** and Charles N. Huhtanen

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118, U.S.A.

Received 28 October 1985
Revised 27 March 1986
Accepted 2 April 1986

Key words: Polyacrylamide gel electrophoresis; Cell protein; *Clostridium;* Clostridia; *Botulinum* type A, B

SUMMARY

Profiles of cellular proteins from 11 type A and 9 type B strains of *Clostridium botulinum* were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cellular protein profiles exhibited considerable heterogeneity among strains of the same biotype, as well as among strains of different biotypes. This study also demonstrated the reliability and usefulness of this rapid and inexpensive procedure to determine the variation which may occur among *C. botulinum* and related species.

INTRODUCTION

Polyacrylamide gel electrophoretic patterns of proteins in bacterial cell extracts have been extensively used to study differences among genera, species and strains of various bacteria. This technique appears to be useful for screening and identifying bacterial genera, such as *Streptococcus, Lactobacillus, Actinobacillus* and *Campylobacter* [4,8, 10,13,15,16]. A recent publication by Cato et al. [5] toxigenic microorganism composed of seven serotypes (A, B, C_α, D_β, E, F and G). Some of these are further separated into proteolytic and non-proteolytic types. A means of identifying these strains and differentiating them from the biochemically similar *Clostridium sporogenes* would be of considerable benefit. However, the work of Cato et al. [5] was limited to two or three strains of each *C. botulinum* serotype. Since types A and B (proteolytic) are most commonly associated with human food-borne botulism, it was of interest to determine whether these serotypes can be differentiated by polyacrylamide gel electrophoresis and if they are different from the non-toxigenic *C. sporogenes.*

* To whom correspondence should be addressed.
** Present address: Department of Biology, Indiana University, Bloomington, IN, U.S.A.
References to brand or firm names do not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Table 1

Principal protein bands of *C. botulinum* type A strains presented in Fig. 1

Lane	Designation of strain	Source of isolation	Origin of isolation	Principal protein bands on mm scale
1	17409-1	Center for Disease Control (CDC)	Fecal isolate during New York 1974 mushroom outbreak	9.5, 12*, 66, and 80
2	20 PLALC	CDC	Fermented beaver tail Alaska, 1975	9.5, 13, 41, 42, 48, 60, 64.5, 76, 81, and 104
3	1	Food Safety & Inspection Service (FSIS)	Unknown	9.5, 14, 39, 41, 42, 48, 60, 64.5, 71, 76, 80, 81, 85*, and 102
4	33	National Food Processor Association (NFPA)	Unknown	11, 13.5, 39, 41, 42, 48, 64.5, 66*, 76, 80, and 101*
5	B1218	Northern Regional Research Center, (NRRC) USDA	California	9.5, 13, 39, 41, 42, 48, 60, 64.5, 71, 76, 81, and 104
6	25763	American Type Culture Collection (ATCC)	Virgin soil	9.5, 13, 39, 41, 42, 48, 60, 64.5, 71, 76, 81, and 104
7	62A	Hooper Foundation	Cow liver	9.0*, 13.5, 39, 41, 42, 48, 60, 64.5, 71, 76, 81, and 104
8	69A	Food and Drug Administration (FDA), Received from Dr. H. Sugiyama	Unknown, 1975	10, 15, 39, 41, 42, 48, 60, 64.5, 76, 81, 86.5*, and 104
9	78A	FDA	Unknown	10, 15, 39, 41, 42, 48, 60, 64.5, 71, 76, 81, and 104
10	426A	FDA	Peppers	5*, 11, 15, 39, 41, 42, 48, 60, 64.5, 71, 76, 81, and 104
11	429A	FDA	Beets	11, 15, 39, 41, 42, 48, 60, 64.5, 76, 81, and 102
21	*C. sporogenes* B1219 (PA 3679)	NRRC	Unknown	8.5*, 14*, 28*, 43*, and 49*

* These bands are specific for each strain.

MATERIALS AND METHODS

Microorganisms. *C. botulinum* types A and B (proteolytic) and *C. sporogenes* (nontoxic) strains examined in this study are listed in Tables 1 and 2. Detailed description of the strains and sources are given elsewhere [9].

Culture conditions. All strains were grown under nitrogen for 24 h at 35°C in 5 ml of brain heart infusion (BHI) (Difco Laboratories, Detroit, MI) supplemented with 0.05% sodium thioglycollate. From each culture, 100 μl were transferred to 200 ml of supplemented BHI and were incubated at 35°C. Growth was monitored by measurement of absorbance at 600 nm. When absorbance reached 0.4 (approximately 16–18 h), cells were harvested and washed twice with phosphate-buffered saline [3].

Extraction of protein from bacterial cells. Protein extracts from washed cells were prepared using the acetone-sodium dodecyl sulfate (SDS) lysis technique of Bhaduri and Demchick [2] and were stored at −20 C in 0.5-ml portions. Chilling during harvesting and rupturing of the cells with 1% SDS prevented any proteolytic degradation of proteins in the cell-free extract as determined by assay for proteinase and peptidase activity (unpublished data). It was also found that storage of the protein ex-

Table 2

Principal protein bands of *C. botulinum* type B strains presented in Fig. 2

Lane	Designation of strain	Sourse of isolation	Origin of isolation	Principal protein bands on mm scale
12	4B	FSIS	Unknown	14*, 15.5*, 25*, 35*, 39*, 49*, 55*, 58*, 82*, and 90
13	B-APHIS 770	FSIS	Unknown	15, 19, 70*, 73, 85, 91, 95, and 104*
14	53B	NFPA	Unknown, 1961	15, 19, 33*, 69, and 89
15	7949B	ATCC	Unknown	15, 19, 69, 80, 89, 93*, 103*, and 109*
16	169B	FDA, Originally received from Dr. C. Schmidt	Unknown, 1965	15, 19, 69, 85, 91, 95, 100 and 105
17	383B	FDA	Can of mushrooms, 1972	12.5*, 15, 16*, 36*, 66, 71.5*, 75, 88*, 90, 95, 100, and 105
18	642B	FDA	Can of mushrooms, 1972	12, 62*, 66, 73, 75, 80, 85, and 101*
19	999B	FDA	Can of mushrooms, 1972	12, 36.5, 69, and 73
20	8688R	Walter Reed Hospital	Can of mushrooms, 1972	12, 36.5, 69, and 73
21	*C. sporogenes* B1219 (PA 3679)	NRRC	Unknown	10*, 28*, and 44*

* These bands are specific for each strain.

tracts at −20°C did not alter the protein profiles. The protein contents of the extracts were determined by the method of Lowry [14] using bovine serum albumin as a standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Vertical slab gel electrophoresis (gel size, 13.6 × 14 cm × 1.5 mm) of the bacterial protein extracts was carried out using a model SE600 Sturdier slab gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA). Stock solutions and the procedures for the preparation of 10% separating polyacrylamide gels and 3% stacking gels were essentially as described by Laemmli [11]. Samples (40 μl containing 50 μg of protein) were boiled for 2 min under reducing conditions (in 0.1 M β-mercaptoethanol) and then applied with a micropipettor to wells through the reservoir fluid on the top of the stacking gel. Six standard protein markers ranging in molecular weight from 12 300 to 200 000 (purchased from Bethesda Research Laboratory, Gaithersburg, MD) were run in one well to permit accurate comparison between different protein patterns. Electrophoresis buffer was layered over the sample, and electrophoresis was carried out at room temperature. A constant voltage of 60 V (35 mA) was maintained until the bromophenol dye front entered the separating gel, at which time the voltage was increased to a constant voltage of 120 V (48 mA). The electrophoresis was stopped when the bromophenol dye marker reached 1.5 cm from the bottom of the gel (6 h). The gel was then stained with Coomassie blue overnight at room temperature and destained with a methanol/acetic acid/water mixture [1]. Photographs of each gel were made for permanent records.

RESULTS AND DISCUSSION

This work compares the cellular proteins of *C. botulinum* types A and B by SDS-PAGE. Each of the 21 strains listed in Tables 1 and 2 was grown

and analyzed by SDS-PAGE at least three times. The protein profiles observed in all three experiments were similar. Electrophoretic patterns of the cellular proteins from each strain are shown in Fig. 1 (type A) and in Fig. 2 (type B). The gels presented contained 40–45 distinct bands having a molecular weight range of approximately 200 000 to 12 300. Individual bands from extracts of different strains, including bands with identical electrophoretic mobilities, often had different relative densities or other distinctive characteristics, such as sharp or tailing edges.

The protein profiles were distinctive for each strain and indicate significant differences between strains of *C. botulinum* (types A and B) and between *C. botulinum* and *C. sporogenes*. Definitive differentiation of these serotypes requires toxin neutralization tests in mice. All of the botulinal strains were toxic to mice; the toxin of each was neutralized by either of the specific antitoxins, A or B. The dissimilarity among the proteins in the M_r range between 200 000 to 18 400 is striking. The locations of the principal protein bands resolved by SDS-PAGE from strains of type A (Fig. 1) and type B (Fig. 2) are summarized in Tables 1 and 2, respectively. It is evident from Tables 1 and 2 that some of the protein bands are common to a particular group of strains, while there are other bands which are specific and distinct for a particular strain. Most strains of *C. botulinum* type A had protein bands

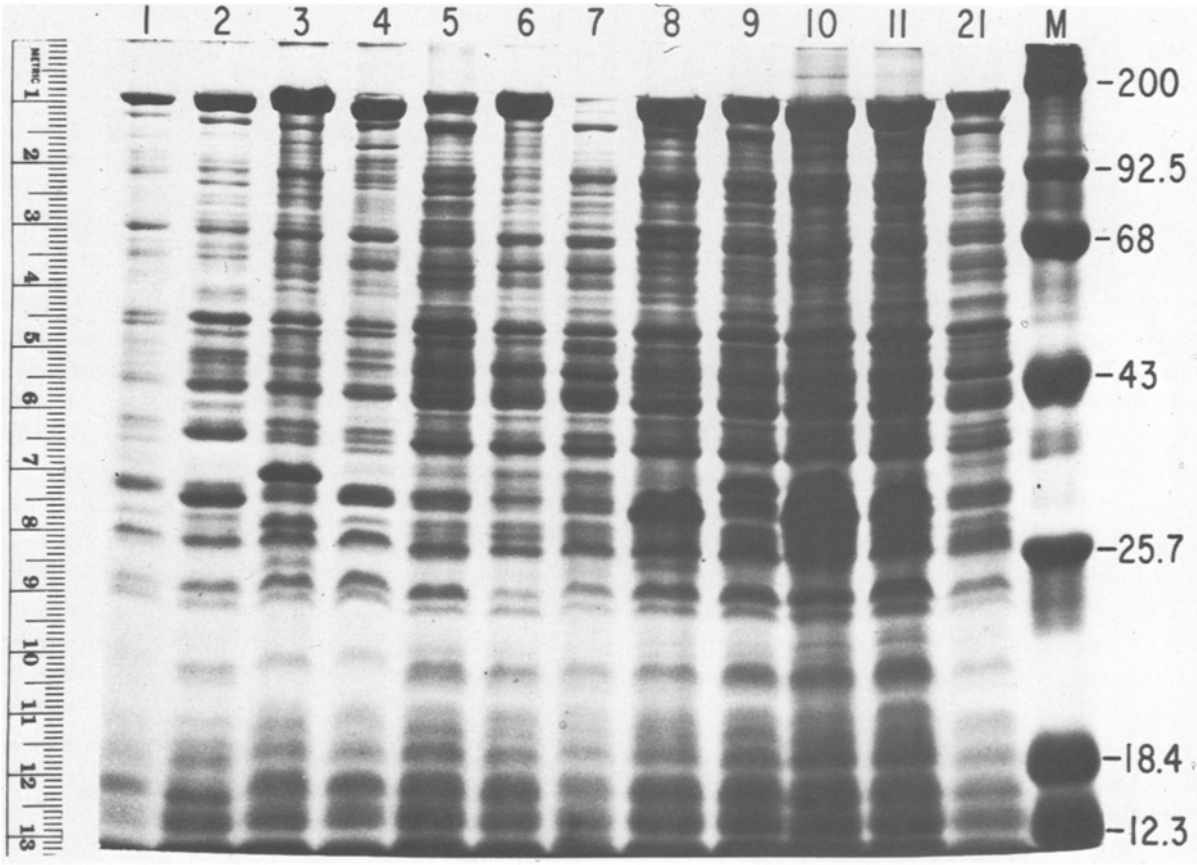

Fig. 1. SDS-PAGE of 11 strains of *C. botulinum* type A and one strain of *C. sporogenes* (lane 21) (see Table 1 for strain identification). Electrophoresis was carried out as described in Materials and Methods. Lane M, molecular weight markers: the numbers on the right indicate molecular weights ($\times 10^3$). The profiles presented here are the results of one of the three experiments; however, all showed similar patterns.

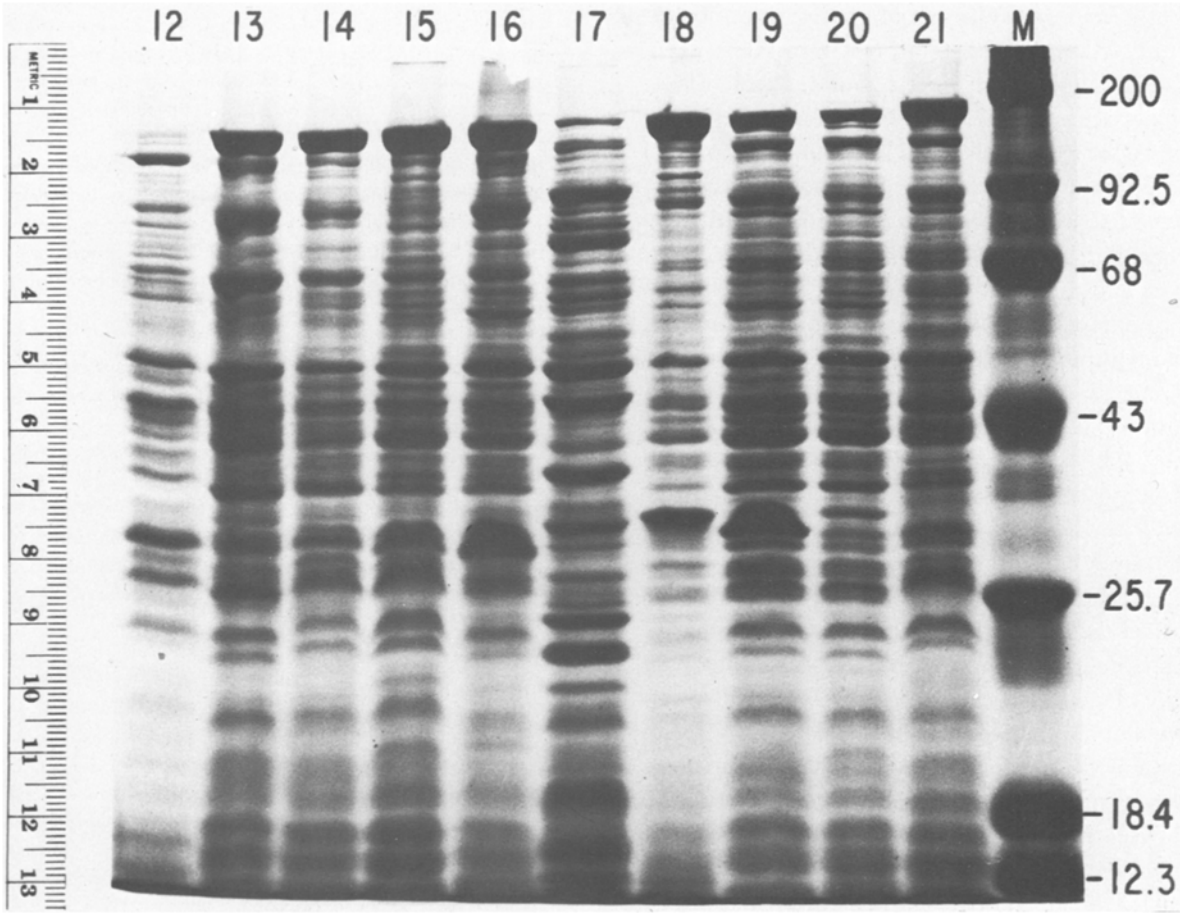

Fig. 2. SDS-PAGE of nine strains of *C. botulinum* type B and one strain of *C. sporogenes* (lane 21) (see Table 2 for strain identification). Electrophoresis was carried out as described in Materials and Methods. Lane M, molecular weight markers, the numbers on the right indicate molecular weight ($\times 10^3$). The profiles presented here are the results of one of the three experiments; however, all showed similar patterns.

at 39, 41, 42, 48, 60, 64.5 and 76 mm (Fig. 1, Table 1). Strain 17409-1 had a distinctively different profile including a specific band at 12 nm (Fig. 1, lane 1). Strain 20 PLALC (Fig. 1, lane 2) lacked the protein band at 39 mm, and strain 33 (Fig. 1, lane 4) lacked the 60 and 81 mm bands. The protein bands at 85 mm in strain 1 (Fig. 1, lane 3), at 66 and 101 mm in strain 33 (Fig. 1, lane 4), at 9.0 mm in strain 62A (Fig. 1, lane 7), at 86.5 mm in strain 69A (Fig. 1, lane 8) and at 5.0 mm in strain 426A (Fig. 1, lane 10) were distinctive for each of these strains. The bands at 8.5, 14, 28, 43 and 49 mm were present specifically in cultures of *C. sporogenes*. The protein patterns of *C. botulinum* type B in strain 4B (Fig. 2, lane 12), strain 7949B (Fig. 2, lane 15) and strain 383B (Fig. 2, lane 17) are the most dissimilar (See Table 2). The bands at 70 and 104 mm in strain B-APHIS 770 (Fig. 2, lane 13) at 33 mm in strain 53B (Fig. 2, lane 14) and at 62 and 101 mm in strain 642B (Fig. 2 lane 18) were specific for these strains. *C. sporogenes* can be distinguished from *C. botulinum* type B by the bands present at 10, 28 and 44 mm. Due to the variability of gel electrophoresis, the bands of *C. sporogenes* appear at different positions, but the actual migration of the proteins with respect to standard protein markers was the same.

This apparent variability in migration does not reflect the actual difference in protein profile.

The dissimilarity of electrophoretic protein patterns between strains from *C. botulinum* types A and B is not surprising, since Lee and Riemann [12] reported only 10–14% DNA homology among the strains of *C. botulinum* types A and B, and *C. sporogenes*. Farshy and Moss [6] reported that *C. sporogenes* and proteolytic strains of *C. botulinum* also can be differentiated by gas chromatographic patterns of trimethylsilyl derivatives of whole cell hydrolysates. Gutteridge et al. [7] reported similar observations using pyrolysis gas-liquid chromatography. This study shows that, in addition to heterogeneity between biotypes (data not shown), there is considerable heterogeneity among strains of the same biotype.

The results presented here do not agree with the report of Cato et al. [5] which indicated similarity among the electrophoretic patterns of proteolytic strains of *C. sporogenes* and types A and B of *C. botulinum*. The difference between our results and theirs may be due to differences in culture medium as well as differences in the procedures for protein extraction and electrophoresis.

Although the identities of specific proteins were not determined, the over-all protein profiles allow simultaneous examination of a large number of alleles at one time. The protein patterns presented here indicate marked differences among strains of *C. botulinum*. The results suggest further that protein profiling may be a useful technique for the characterization and identification of different isolates.

ACKNOWLEDGMENTS

We thank Dr. T. J. Montville of Rutgers University, New Brunswick, NJ, and Dr. S. Finver of the Wistar Institute of Anatomy and Biology, Philadelphia, PA, for reading the manuscript.

REFERENCES

1 Basch, J.J. and H.M. Farrell, Jr. 1979. Charge separation of protein complexed with sodium dodecyl sulfate by acid gel electrophoresis in the presence of cetyltrimethylammonium bromide. Biochim. Biophys. Acta 577: 125–131.

2 Bhaduri, S. and P.H. Demchick. 1983. Simple and rapid method for disruption of bacteria for protein studies. Appl. Environ. Microbiol. 46: 941–943.

3 Bhaduri, S., H.J. Raskas and M. Green. 1972. Procedure for the preparation of milligram quantities of adenovirus messenger ribonucleic acid. J. Virol. 10: 1126–1129.

4 Calhoon, D.A., W.R. Mayberry and J. Slots. 1981. Cellular fatty acid and soluble protein of *Actinobacillus actinomycetemcomitans* and related organisms. J. Clin. Microbiol. 14: 376–382.

5 Cato, E.P., D.E. Hash, L.V. Holdeman and W.E.C. Moore. 1982. Electrophoretic study of *Clostridium* species. J. Clin. Microbiol. 15: 688–702.

6 Farshy, D.C. and C.W. Moss. 1970. Characterization of clostridia by gas chromatography. Differentiation of species by trimethylsilyl derivatives of whole cell hydrolysates. Appl. Environ. Microbiol. 20: 78–84.

7 Gutteridge, C.S., B.M. Mackey and J.R. Norris. 1980. A pyrolysis gas-liquid chromatography study of *Clostridium botulinum* and related organisms. J. Appl. Bacteriol. 49: 165–174.

8 Hanna, J., S.D. Neil, J.J. O'Brien and W.A. Ellis. 1983. Comparison of aerotolerant and reference strains of *Campylobacter* species by polyacrylamide gel electrophoresis. Int. J. Syst. Bacteriol. 33: 143–146.

9 Huhtanen, C.N. 1979. Bile acid inhibition of *Clostridium botulinum*. Appl. Environ. Microbiol. 38: 216–218.

10 Jarvis, A.W. and J.M. Wolff. 1979. Grouping of lactic streptococci by gel electrophoresis of soluble cell extracts. Appl. Environ. Microbiol. 37: 391–398.

11 Laemmli, U.K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227: 149–154.

12 Lee, W.H. and H. Riemann. 1970. Correlation of toxic and nontoxic strains of *Clostridium botulinum* by DNA composition and homology. J. Gen. Microbiol. 60: 117–123.

13 Moore, W.E.C., D.E. Hash, L.V. Holdeman and E.P. Cato. 1980. Polyacrylamide slab gel electrophoresis of soluble proteins for studies of bacterial floras. Appl. Environ. Microbiol. 39: 900–907.

14 Schleiff, R.F. and P.C. Wensink. 1981. Practical Methods in Molecular Biology, pp. 74–77. Springer-Verlag, Inc., New York.

15 Seiter, J.A. and J.M. Jay. 1980. Application of polyacrylamide gel electrophoresis to characterization and identification of *Arthrobacter* species. Int. J. Syst. Bacteriol. 30: 460–465.

16 Tsai, C.M. and C.E. Frasch. 1980. Chemical analysis of major outer membrane proteins of *Neisseria meningitidis*: comparison of the serotypes 2 and 11. J. Bacteriol. 141: 169–176.