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Serological Studies of *Clostridium botulinum* Type E and Related Organisms

II. Serology of Spores

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Pure spore antigens for the immunization of rabbits were prepared by enzymic digestion of vegetative components and separation of the cleaned spores in polyethylene glycol. Spore antisera were prepared to strains representative of toxigenic *Clostridium botulinum* type E; nontoxigenic boticin E-producing variants; nontoxigenic nonproducers of boticin E; nontoxigenic "atypical" strains, which differ somewhat from *C. botulinum* type E in their physiology; *C. botulinum* types A and B; and *C. bifermentans*. They were tested against these and additional strains representative of the above groups, other types of *C. botulinum*, and other *Clostridium* species. There was no evidence of agglutination of flagellar or somatic antigens of vegetative cells by these antisera. Agglutination and agglutinin absorption tests showed common antigens among toxigenic type E strains and nontoxigenic variants, both producers and nonproducers of boticin E. Some nontoxigenic "atypical" strains varied in their ability to be agglutinated by type E antisera, and others did not agglutinate at all. Of those atypical strains that were not agglutinated, one was agglutinated by *C. bifermentans* antiserum. Antisera prepared against *C. botulinum* types A and B and *C. bifermentans* did not agglutinate the spores of type E or its variants nor share antigens common to each other. Similarly, antisera to type E, its nontoxigenic variants, and nontoxigenic atypical strains did not agglutinate other *C. botulinum* types or any other *Clostridium* species investigated.

We have previously shown (15) that the flagellar antigens of *Clostridium botulinum* type E and those of nontoxigenic organisms culturally identical to it were strain specific; i.e., antiflagellar serum prepared against one strain seldom agglutinated any of the other strains. Their somatic antigens, on the other hand, were common to all. Because of the narrow range of specificity of the flagellar antigens, it was impractical to use them to distinguish type E from other botulinum types or other *Clostridium* species. The somatic antigens on the other hand, having too broad a spectrum, could not be used to distinguish between nontoxigenic strains and toxigenic type E.

Spore antigens are distinct from those of the vegetative cell and have been employed to identify and classify aerobic (9, 12, 13, 14, 18, 21) and anaerobic (9, 16, 17, 19, 20, 25) sporeforming bacteria. The purpose of this investigation was to determine whether spore antigens could be used to distinguish toxigenic *C. botulinum* type E from nontoxigenic but culturally identical strains and to assess the relationship of type E to nontoxigenic "atypical" strains, other types of *C. botulinum*, and other *Clostridium* species.

MATERIALS AND METHODS

Strains. The strains studied are identified in Table 1. The toxigenic strains of *C. botulinum* type E are representative of widely separated geographical areas. The nontoxigenic strains are divided into two groups. The first group contains nontoxigenic variants of *C. botulinum* type E which, except for lack of toxin production, are culturally similar to toxigenic type E strains. This group is further separated into strains which produce boticin E (10) and those which do not. Members of the second group of nontoxigenic strains, tentatively designated "atypical," differ from toxigenic type E in several cultural characteristics; they also differ from each other. The last four strains in this group are OS (opaque sporulating) and TP (transparent proteolytic) strains, which are identified by the type of colonies they produce on brain heart agar (2). Strain KA91, originally described as a TP variant of toxigenic type E, was later culturally identified by L. Holdeman (*personal communication*) as *C. bifermentans*. It was included in this study for serological confirmation. A comparison of the cultural characteristics of the "atypical" strains was made with those of type E and the nontoxigenic variants (Table 2).

Other types of *C. botulinum* and other species of the genus *Clostridium* were also examined. The strain of *C. bifermentans* was isolated in this laboratory and

TABLE 1. *Identification of strains of Clostridium*

Strain designation	Source of isolation	Geographical area	Supplied by
C. botulinum type E			
Beluga	"Muktuk"	Alaska	C. F. Schmidt, Continental Can Co., Chicago
VH	Pickled herring	Vancouver, Canada	
Memanbetsu	"Izushi"	Hokkaido, Japan	H. Iida, Hokkaido, Institute of Pub. Health, Sapporo, Japan
1304, 211, 715	Sediments	Lake Abashiri, Japan	
4203, 4213	Sediments	Sweden	G. Hobbs, Torry Research Station, Aberdeen, Scotland
8E	Intestine of sturgeon	Russia	C. F. Schmidt (see above)
D8	Canned tuna	Exact area unknown	Food & Drug Administration
Minnesota	Smoked ciscoes	Great Lakes	Food & Drug Administration
066BTox, 070, 5191, 5192	Smoked whitefish chubs	Great Lakes	Food & Drug Administration
Kalamazoo	Smoked whitefish	Great Lakes	Food & Drug Administration
Sul, Su3	Viscera of sucker	Great Lakes	Food & Drug Administration
Nontoxigenic strains			
Nontoxigenic variants			
Produce boticin E			
S5	Viscera of smelt	Great Lakes	Food & Drug Administration
GB3, 28-2, 64 810, 811, 833	Sediments	Great Lakes	Food & Drug Administration
Produce no boticin E			
066 BNT	Derived from 066BTox	Great Lakes	Food & Drug Administration
38-1	Sediment	Great Lakes	Food & Drug Administration
4267, 4268	Bottom deposits	North Sea	G. Hobbs (see above)
Nontoxigenic atypical			
Serologically related to type E			
PM15	Potomac River sediment	Washington, D.C.	Food & Drug Administration
S9	Viscera of smelt	Great Lakes	Food & Drug Administration
Serologically unrelated			
42-2	Sediment	Great Lakes	Food & Drug Administration
KA89	OS variant of "VH"	Vancouver, Canada	L. Holdeman, Virginia Polytechnic Institute, Blacksburg, Virginia
KA91	TP variant		
KA94	OS variant of "Nanaimo"	Nanaimo, B.C.	
2087	OS variant of "Ajmal"	Great Britain	
Other botulinum types			
Type A (62A)	Cow liver		C. F. Schmidt, Continental Can Co,. Chicago
Type B (169B)			Food & Drug Administration
Type C (526C)			R. Lechowich, Michigan State Univ., E. Lansing
Type F (202F)	Marine sediment	Pacific coast	M. W. Eklund, Bureau of Commercial Fisheries, Seattle, Washington
Other *Clostridium* species			
C. perfringens	Chicken broth		Food & Drug Administration
C. sporogenes			Food & Drug Administration
C. bifermentans	Sediment	Great Lakes	Food & Drug Administration
C. sordelli (CN1734)			P. D. Walker, Wellcome Research Laboratories, Beckenham, England

TABLE 2. *Cultural characteristics of C. botulinum type E and nontoxigenic strains*

Property	*C. botulinum* type E 070	Nontoxigenic variants		Nontoxigenic atypical strains						
		Boticin +	Boticin −	PM15	S9	42-2	KA89	KA91	KA94	2087
Fermentation of:										
Glucose	AG[a]	AG	AG	AG	AG	AG	A−	A−	A−	A−
Fructose	AG	AG	AG	AG	AG	AG	A−	A−	A−	A−
Maltose	AG	AG	AG	AG	AG	AG	A−	A−	A−	A−
Sucrose	AG	AG	AG	−	−	−	A−	−	A−	A−
Ribose	AG	AG	AG	AG	AG	AG	A−	A−	A−	A−
Mannose	AG	AG	AG	AG	AG	AG	−	A−	−	−
Sorbitol	AG	AG	AG	AG	−	AG	−	A−	−	−
Trehalose	AG	AG	AG	AG	AG	AG	AG	−	A−	A−
Adonitol	AG	AG	AG	AG	−	−	−	−	−	−
Dextrin	−	−	−	AG	AG	AG	−	−	−	−
Mannitol	−	−	−	AG	−	−	−	−	−	−
Lactose	−	−	−	−	−	−	AG	−	A−	A−
Xylose	−	−	−	−	−	−	−	−	−	−
Toxicity	+	−	−	−	−	−	−	−	−	−
Lipolysis	+	+	+	−	+		−	−	−	−
Proteolysis	−	−	−	−	−	−	−	+	−	−
Nitrate reduction	−	−	−	−	−	−	−	−	−	−
Gelatin liquefaction	−	−	−	−	−	−	−	+	−	−
Opalescence on egg yolk	+	+	+	+	+	+	+	+	+	+
Pearly layer on egg yolk	+	+	+	+	+	+	−	−	−	−
Hemolysis (β)	+	+	+	+	+	−	+	+	+	+
Growth at 10 C	+	+	+	+	+	+	−	−	−	−
Colonial characteristics on:										
Liver Veal Agar	M[b]	M	M	OP[c]	M-OP	M-OP	OP	OP	OP	OP
Brain Heart Infusion Agar	M	M	M	OP	M-OP	M-OP	OP	OP	OP	OP
Sporulation[d]	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
Aerobic plate	−	−	−	−	−	−	−	−	−	−
Boticin activity	−	+	−	−	−		−	−	−	−

[a] Production of acid (A) and gas (G) in 3 days.
[b] Mosaic colonies.
[c] Opaque colonies.
[d] Sporulation in TPG.

characterized according to *Bergey's Manual of Determinative Bacteriology*. It is urease negative.

Methods for the propagation and characterization of all strains used in this study have been previously described (10). These methods have been used for characterizing known types and identifying new strains; thus, all strains have been subjected to the same scrutiny.

Production of spore suspensions. The media and the time and temperature of incubation were varied to meet the requirements of individual strains for maximal spore production. All strains of type E and nontoxigenic organisms resembling it, as well as *C. bifermentans* and *C. sordelli*, were grown in 15 ml of Trypticase (BBL)-peptone-glucose (TPG) broth of Schmidt et al. (24) containing 0.1% rather than 0.2% sodium thioglycolate. Satisfactory sporulation was obtained in 4 to 5 days at 26 C with all strains except that of *C. sordelli*, which required incubation at 35 C. *C. botulinum* types A and B, *C. sporogenes*, and *C. perfringens* were grown in a medium containing 4% Trypticase (BBL), 1 μg of thiamine hydrochloride per ml, 0.1% sodium thioglycolate, 0.1% dibasic potassium phosphate, and 0.05% sodium bicarbonate (L. E. Day, Thesis, Michigan State University, East Lansing, 1960). *C. botulinum* type F was grown in cooked meat medium (3) in 15-ml portions at 30 C for 5 days. *C. botulinum* type C was grown in TPG modified to contain twice the normal amount of Trypticase (BBL) and 2.5 times the normal amount of glucose (C. Schmidt, *personal communication*) in 15-ml portions at 30 C for 5 days.

Preparation of pure spore suspensions. Spore antigens free of vegetative components were produced by a combination of techniques. After maximal sporulation had occurred, the growth was collected by centrifuga-

tion and the sediment was resuspended in enough 0.0003 M phosphate buffer (*p*H 7.2) to give between 10 and 16% of the original. Vegetative components were digested by treatment with 100 μg of trypsin per ml plus 200 μg of lysozyme per ml (5). The mixture was incubated at 37 C for 6 to 8 hr with 1-min sonic treatments by means of a sonifier (Branson Sonic Power, Danbury, Conn.) at 50-w output at hourly intervals. The treatment of poorly sporulating strains was intensified by one or all of the following three measures: adding fresh enzymes, extending the incubation period, or sonically treating at higher intensity for more than 1 min. The treated suspension was refrigerated overnight, washed three times, and resuspended in sterile distilled water to the original volume of the suspension in buffer. The spores were then separated from the debris by extraction in a two-phase system, using polyethylene glycol (PEG). The method of Sacks and Alderton (23) was modified to allow extraction of large quantities of spores. The separating system consisted of the following: 168.00 ml of PEG solution (equal volumes of PEG 4,000 and 20,000, each dissolved at the rate of 500 g/liter of water), 170.50 ml of 3 M potassium phosphate buffer (*p*H 7.1), 25.00 ml of spore suspension, and distilled water to a total volume of 500 ml. The mixture was homogenized for 2 min in a Waring Blendor at high speed and centrifuged at 320 × *g* in 100-ml tubes at 5 C for 10 min. The upper phase, which consisted of PEG containing the spores, was transferred to 250-ml centrifuge bottles, with care not to disturb the interface which held the cellular debris. The centrifuge bottle was filled with distilled water to dilute the PEG, and the spores were sedimented in the cold at 8,000 × *g*. After three or four washings in this manner, spores free of cellular debris and PEG were obtained. The extracting system was reused by adding more of the same suspension and enough PEG to bring the volume back to 500 ml. This was then homogenized and carried through as before. This process was repeated until all the suspension had been extracted. Separations were most efficient when the spore suspensions were not too concentrated. Samples of the cleaned spore suspensions examined by phase-contrast and electron microscopy showed only spores with no evidence of vegetative cells or cellular debris.

Immunizing antigens. The pure spore suspensions were diluted to an optical density (OD) of approximately 0.5 on a Coleman colorimeter, model 8, at a wavelength of 655 nm. At this OD, the suspensions contained 10^6 to 10^7 viable spores per ml as determined by plate counts. Spore antigens were prepared by adding Formalin to the suspensions to give a 0.5% final concentration. The suspensions were incubated at 37 C for 4 to 7 days and tested for sterility. Immunization with spore antigens steamed at 100 C for 1.5 hr gave antisera the titers and specificities of which were comparable to those produced with the Formalin-treated antigens. Formalin attenuation was chosen on the assumption that it may result in less damage to the spore antigens.

Preparation of antisera. Hyperimmune antisera for agglutination tests were produced in New Zealand white rabbits. The rabbits were injected intravenously with 1.0 ml of antigen every 3 to 4 days for a total of six injections. Fourteen days later, they were given another 1.5-ml injection and were bled from the ear 10 days after this booster. Four more bleedings were obtained using this booster schedule. Antiserum was stored frozen without a preservative.

Agglutinating antigens. Preliminary experiments showed that pure spore suspensions agglutinated spontaneously and could not be used in the agglutination tests. Investigations with approximately 80 different diluents such as various concentrations of surface active agents and solutions of mono-, di-, and trivalent cationic and anionic salts failed to resolve the problem of autoagglutination. Crude spore suspensions containing approximately 50% spores and 50% vegetative cells were found satisfactory for agglutination tests. Organisms for these antigens were grown under their optimal conditions for sporulation but were harvested when approximately 50% sporulation was reached. The cultures were centrifuged, and the sediment was washed once with physiological saline containing 0.5% Tween 80 and resuspended in the same diluent. The suspension was adjusted to an OD of 0.5 on the Coleman colorimeter at a wavelength of 655 nm and used in the tube agglutination tests.

Agglutination tests. Agglutination tests were performed in 13 × 100-mm tubes by mixing 0.1-ml portions of the standardized antigen with a like amount of twofold dilutions of antiserum. The tubes were incubated for 3 hr in a 50 C water bath. Physiological saline containing 0.5% Tween 80 was the usual diluent for both the antiserum and the antigen, but in some instances it was necessary to increase the concentration of Tween 80 to as much as 1% to prevent autoagglutination. After incubation, 0.5 ml of diluent was added and the results were read immediately with the aid of a bright light against a black background, using a 3.5 × hand lens. Gently flicking the tubes facilitated the determination of end points. Titers were recorded as the reciprocal of the highest dilution giving definite agglutination.

Ability of spore antisera to agglutinate vegetative cells was tested in the same manner. For the flagellar agglutination tests, 18-hr TPG cultures grown at 26 C were used as antigens. For the somatic agglutination tests, similarly grown cultures were first steamed for 1 hr and the cells were concentrated in physiological saline to 10% of the original volume.

Agglutinin absorption. Crude spore preparations for agglutinin absorption were prepared from TPG cultures at their maximal sporulation stage. The cultures were centrifuged, and the sediment was washed once in physiological saline and packed by centrifugation. Portions (2 ml) of the undiluted antiserum were mixed with 1 to 2 ml of the packed spore preparation and incubated at 50 C for 1 hr with occasional shaking. After the sediment was removed by centrifugation, the antiserum was tested for antibody to the absorbing strain. If agglutinins to the absorbing strain remained, the antiserum was absorbed a second time. In all cases, an additional portion of the same antiserum was incubated as a control along with the one being absorbed.

RESULTS

High agglutinin titers to the homologous antigens were obtained from rabbits immunized with antigens containing only spores. All spore antisera were tested against the flagellar and somatic antigens of all the strains studied and gave no agglutination, nor did they agglutinate cellular debris prepared by ultrasonic disruption of 18-hr broth cultures of these strains. Thus the spore antisera were specific for spore antigens and contained no antibody to vegetative components.

Results of cross-agglutination tests among the strains investigated are summarized in Tables 3 and 4. Results of absorption experiments are presented in Table 5.

Toxigenic type E strains and nontoxigenic variants. All type E strains seem to have identical spore antigens. Antiserum produced against spores of any one of the strains agglutinated the spore antigens of all the others. Nontoxigenic variants of *C. botulinum* type E cannot be distinguished from type E on the basis of their spore antigens; the two groups of organisms share them in common. Antisera produced against the nontoxigenic variants agglutinated spore antigens of the toxigenic strains to full titer, and similar results were obtained in the reciprocal agglutinations in which the antisera were those prepared against the toxigenic strains (Table 3). Furthermore (Table 5), when spore antiserum of the toxigenic strain 070 was absorbed with spores of either a toxigenic strain or a nontoxigenic variant (first four absorbing strains), all antibody was removed; this also happened with sera to the non-

TABLE 4. *Agglutinin titers of spore antisera against spore antigens of atypical strains and other species of Clostridium*[a]

Antigens	Antiserum against[a]				
	Toxigenic type E strains	Nontoxigenic variants		Atypical strain, S9	*C. bifermentans*
		Boticin +	Boticin −		
Atypical strains					
PM15	2,560	2,560	2,560	160	
S9	320	320	320	2,560	
42-2				320	
KA89					
KA91					1,280
KA94					
2087					
Other species of *Clostridium*					
C. bifermentans					1,280
C. sordelli					640
C. perfringens					
C. sporogenes					

[a] Spore antisera to *C. botulinum* types A and B (Table 3) produced no agglutination at 1:20 against any of the antigens listed. Where no values appear, agglutination did not occur at 1:20. The same kinds and numbers of antisera were tested as in Table 3.

TABLE 3. *Agglutinin titers of spore antisera against spore antigens of Clostridium botulinum type E, nontoxigenic variants, and other types of C. botulinum*

Antigens	Antiserum against[a]					
	Toxigenic type E strains (7)[b]	Nontoxigenic variants		Atypical strain, S9	Other types of *C. botulinum*	
		Boticin + (3)[c]	Boticin − (1)[d]		A	B
Toxigenic type E strains (18)	2,560	2,560	2,560	320		
Nontoxigenic variants (11)						
Boticin + (7)	2,560	2,560	2,560	320		
Boticin − (4)	2,560	2,560	2,560	320		
Other types of *C. botulinum*						
Type A (62A)					5,120	
Type B (169B)						1,280
Type C (526C)						
Type F (202F)						

[a] *C. bifermentans* antiserum did not produce agglutination with any of the antigens listed. Values shown are mean titers with a range of one twofold dilution above and below. Where no values appear, agglutination did not occur at 1:20.

[b] Strains: Beluga, VH, Memanbetsu, 1304, 066BTOX, 070, and 5192. Numbers in parentheses indicate the numbers of strains tested.

[c] Strains: S5, GB3, and 28-2.

[d] Strain 066BNT.

TABLE 5. *Agglutinin titers of spore antisera absorbed with spore antigens*

Antiserum	Absorbing strain	Antigens					
		Toxigenic strains (18)[a]	Nontoxigenic variants (11)		Nontoxigenic atypical		
			Boticin + (7)	Boticin − (4)	PM15	S9	42-2
070	None	2,560	2,560	2,560	2,560	320	
	070						
	5192						
	S5						
	066BNT						
	PM15	640	640	640			
	S9	2,560	2,560	2,560	1,280		
1304	S9	2,560	2,560	2,560	2,560		
S5	None	2,560	2,560	2,560	2,560	320	
	S5						
	070						
	066BNT						
	S9	2,560	2,560	2,560	2,560		
066BNT	None	2,560	2,560	2,560	2,560	320	
	066BNT						
	070						
	S5						
	S9	2,560	2,560	2,560	1,280		
S9	None	640	640	640	160	2,560	320
	S9						
	070					2,560	320
	1304					2,560	320
	S5					2,560	320
	066BNT					2,560	320
	PM15	640	640	640		2,560	320

[a] Numbers in parentheses indicate the number of strains tested.

toxigenic variants S5 and 066BNT (first three absorbing strains).

Specificity of spore antigens. Spore antisera against toxigenic type E strains and nontoxigenic variants did not agglutinate spores of *C. botulinum* types A, B, C, or F. Similarly antisera against spores of *C. botulinum* types A and B did not agglutinate spores of toxigenic type E strains and nontoxigenic variants, type C, or type F, but agglutinated the homologous spores only (Tables 3 and 4).

Nontoxigenic atypical strains. Nontoxigenic atypical strains show considerable variation in their agglutination patterns both to type E strains and nontoxigenic variants and among themselves. Spore antiserum against toxigenic type E and nontoxigenic variants agglutinated spores of PM15 to full titer, those of S9 partially, and the other atypical strains not at all. Antiserum against S9 partially agglutinated spores of type E strains and nontoxigenic variants (Table 3) and spores of PM15 and 42-2 (Table 4) but not those of the other atypical strains. Strain 42-2 has not been agglutinated by any other antiserum. Absorption of the 070, S5, and 066BNT spore antisera with spores of strain S9 (Table 5) caused no reduction in their agglutinin titers to the toxigenic strains, the nontoxigenic variants, or the atypical strain PM15. Absorption of the 070 antiserum with spores of strain PM15 removed part of the antibody to the toxigenic strains and nontoxigenic variants and all of the antibody to strain S9, whereas absorption of S9 antiserum with PM15 spores did not affect its titer to any other strain. The only other agglutination of an atypical strain is that of KA91 by serum of *C. bifermentans* (Table 4).

Other Clostridium species. Other known *Clostridium* species were not agglutinated by antisera against type E strains, nontoxigenic variants, type A or type B (Table 4). The antiserum against *C. bifermentans* also agglutinated spores of *C. sordelli* and KA91 as noted above. When this antiserum was absorbed with any of the three strains, antibody to all was removed.

DISCUSSION

Spore antigens are distinct from those of the vegetative cell, since agglutination tests with spore antisera were uniformly negative when tested against antigens of the vegetative cell. This is consistent with the findings of other authors, although, due to some techniques used, it is not clear whether antibody to vegetative cells was stimulated by the spores themselves or by some other antigenic component of their spore preparations. In some cases, animals were immunized with live spores, which gave rise to antibody to both spores and vegetative cells (16). Such spores could have germinated in the animal, thus providing vegetative cells to stimulate antibody production. In other cases, the spores were not adequately separated from the vegetative components (9, 17, 20, 21), and immunization would result in antibody production to both stages. In such instances, the serum had to be absorbed to remove its vegetative components, in order to show that the spore antigens were distinct. Further complications may have arisen because of the antigenic disparity between spores and vegetative cells (9, 18) and because autolysis of a cell does not necessarily destroy its antigenicity (4). Note that in this investigation the immunizing antigens consisted of suspensions of cleaned spores, free of vegetative components or cellular debris. Since spores, and spores only, served to stimulate the production of antibody no subsequent absorptions were necessary. In addition, the use of pure spores as antigens may have accounted for the higher antibody titers obtained.

The nontoxigenic organisms included in this study, both those which produce boticin E and those which do not, have previously been shown to share cultural characteristics (10) and somatic antigens (15) with toxigenic *C. botulinum* type E. The present results show that the two groups of organisms have spore antigens which are identical by agglutination and agglutinin absorption tests. A suggestion of Oakley (22) leads to the logical conclusion that these strains are true nontoxigenic variants of *C. botulinum* type E.

The ecological and epidemiological significance of these variants can only be conjectured. The toxigenic strain 066BTOX, from which the nontoxigenic strain 066BNT was derived, regularly gave rise to both toxigenic and nontoxigenic colonies. Under laboratory conditions the nontoxigenic variant produces only nontoxigenic colonies, but, if this process could sometimes be reversed under natural conditions, a whole new view of the significance of nontoxigenic variants would be needed. Yet, since some of these variants produce a bacteriocin, boticin E, which is lethal for the vegetative cells of the toxigenic strains (10), their presence in nature and food products may serve to limit the hazard from toxigenic strains. Chemical characterization of both the type E toxin and the boticin E molecule might reveal a relationship between the production of these two substances, possibly that of phases in a sequence of variation.

The nontoxigenic atypical strains, however, must be regarded as a culturally (Table 2) and serologically heterogeneous group. They were included in this study because they are ubiquitous wherever type E is found, some because they were claimed to be nontoxigenic variants of *C. botulinum* type E, and some because they share common somatic antigens with both the toxigenic type E and nontoxigenic variants (15). No taxonomic claim is made for this group, but, rather, a station is provided from which they can be characterized and classified when sufficient information becomes available.

Three strains in this group best illustrate their heterogeneous character. Strains PM15, S9, and 42-2 share common somatic antigens with type E (15) but have spore antigens which differ with respect to both type E and each other. Strain PM15 was agglutinated by antisera of toxigenic type E and nontoxigenic variants to full titer, S9 only partially, and 42-2 not at all. Yet, the spore antigens they share among themselves are independent of those they share with type E and nontoxigenic variants. Here strain 42-2 is related to S9, S9 is related to PM15, and PM15 bridges the gap between these strains and the type E group. Thus absorption of 070 antiserum with spores of PM15, which removed only part of the 070 antibody, removed all the antibody to S9, but absorption of S9 antiserum with spores of PM15 left the S9 titer unaltered both to itself and to strain 42-2. Since the remaining atypical strains, including the OS and TP variants, were not agglutinated by antisera prepared against type E strains, they are probably not related to type E at all. One of these strains, KA91, described as a TP variant, shares spore antigens only with *C. bifermentans*. Similar findings have been reported by others (6, 8, 26).

There was no cross-agglutination among types A, B, and E though such crossings in the fluorescent-antibody technique have been reported (27). Antisera prepared against spores of either toxigenic type E strains or the nontoxigenic variants did not agglutinate the antigens of a limited number of strains of other types of *C. botulinum*. Spore antigens of *C. botulinum*, therefore, seem to be type specific rather than species specific as reported by Walker (25) who, to be sure, worked with other species of *Clostridium*.

One of the problems encountered in the preparation of spore antigens was the clumping of the spores. This has been found by others too (1, 20). Recent electron micrographs of type E spores (7, 11) have shown appendages radiating from the surface of the spore. Conceivably, they contribute to the autoagglutination of the purified antigens.

However, using suspensions made from cultures which were about 50% sporulated for agglutination antigens, we overcame the problem of autoagglutination. The validity of this approach was derived from previous tests which showed that spore antisera did not agglutinate vegetative cells or debris.

The many nontoxigenic organisms invariably accompanying the known types of *C. botulinum* call for a generalized scheme of classification of *C. botulinum*. Since cultural characteristics are often inconclusive and toxin production is not a stable character, serological classification based on cellular and spore antigens should provide a more reliable approach. This would require that the entire antigenic makeup including the flagellar, somatic, and spore antigens of these organisms be considered. Spore antigens seem to be a stable character, broad enough in their specificity to include both toxigenic and nontoxigenic forms of type E, yet narrow enough to exclude other types of *C. botulinum* and other *Clostridium* species. The flagellar antigens might be of value in subdividing the type E strains into several prototypic groups, each containing a limited number of strains.

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