Immunofluorescent study of the spore antigens of proteolytic strains of *Clostridium botulinum*

BY T. J. T. PRINCEWILL*

Department of Microbiology, Agricultural Science Building, The University, Leeds, LS2 9JT, England

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SUMMARY

By means of the spore fluorescent antibody technique, 31 strains of *Clostridium botulinum* types A (18 strains), B (10 strains) and F (3 strains) were found to belong to the same homogeneous group irrespective of their toxigenic types. Some strains of this species also cross-reacted with certain strains of *Clostridium sporogenes* types I, II and III and *Clostridium histolyticum* type II. By spore antigenic analysis it was found that *Clostridium parabotulinum* contained two components designated L and M, the former describing species specificity; the latter was the cross-reacting component shared by some strains of *Clostridium sporogenes* and *Clostridium histolyticum*. Following this, a scheme showing the distribution of spore antigenic components among various species of *Clostridium* was given.

INTRODUCTION

Strains of *Clostridium botulinum* are divided into six types (A–F) according to the antigenic specificity of the toxins they produce, whereas their biochemical activity separates them into proteolytic and non-proteolytic groups. Thus, proteolytic types A, B and F and non-proteolytic types B, C, D, E and F of *Cl. botulinum* exist. Sharing of somatic antigens among strains of proteolytic types A, B and F has been reported by Walker & Batty (1964), Solomon, Lynt, Kautter & Lilly (1971) and Lynt, Solomon & Kautter (1971). Partial cross-agglutination of *Cl. sporogenes* with the somatic antisera of the proteolytic group of *Cl. botulinum* has also been observed by Mandia (1955), Solomon *et al.* (1971), and Lynt *et al.* (1972). Except by means of the toxigenicity test it is impossible to distinguish *Cl. botulinum* from *Cl. sporogenes* on the basis of physiological or biochemical characteristics (Lynt *et al.* 1972).

Princewill (1979a) divided *Cl. sporogenes* into types by means of the spore antigens. The antisera obtained in that study have been used to determine the immunological relationship between *Cl. sporogenes* and the proteolytic strains of *Cl. botulinum* by the indirect fluorescent antibody test (FAT).

* Present address: Nigerian Institute for Trypanosomiasis Research, Vom, via Jos, Nigeria.

Table 1. *Strains and sources of Cl. botulinum cultures used*

(a, Originally from Dr D. Berkowitz, U.S. Army, Natick, Mass.; b, originally from National Canners Association (NCA), California, U.S.A.; c, originally from National Collection of Type Cultures (NCTC), Colindale, London; d, originally from Dr L. V. Holderman, Virginia Polytechnic Institute, Blacksburg, Virginia.)

Serial number	Toxigenic types	Type and number in original collection		Origin
168	A	CN	751	Wellcome Research Laboratories, Langley Court, Beckenham, Kent
169	A	CN	5008	
170	A	CN	640	
171	A	CN	1354	
172	B	CN	1356	
173	B	CN	5009	
174	A		117	Dr H. Meisel, Serum Research Institute, Warsaw
175	A		190	
176	A		365	
177	B		140	
178	B		366	
179	B		924	
180	A		33a	Dr T. A. Roberts, Meat Research Inst., Langford, Bristol
181	A		62b	
182	A		387c	
183	A		1192b	
184	A		2012c	
185	A		2916c	
186	A		3805c	
187	A		3806c	
188	A		4587c	
189	A		7272c	
190	A		9837c	
191	B		53a	
192	B		213b	National Collection of Industrial Bacteriology, Aberdeen
193	B		751c	
194	B		3807c	
195	B		7273c	
196	F		Fd	
197	F	T-15		
198	F	T-42		

MATERIALS AND METHODS

These were similar to those used in previous reports (Princewill, 1978, 1979*a, b*), with the addition of the following:

Cl. botulinum strains. Of the 31 strains (Table 1), 18 were of type A, 10 of type B and 3 of type F; all were proteolytic. Four of the type A were from the Wellcome Research Laboratories, Langley Court, Beckenham, Kent, England; 3 from the late Dr H. Meisel, Serum Research Institute, Warsaw, Poland; and 11 from Dr T. A. Roberts, The Meat Research Institute, Langford, Bristol, England. Two of the type B cultures were from Beckenham, 3 from Warsaw and 5 from Bristol. Of the 3 strains of type F, one was obtained from Bristol and the

Table 2. *Fluorescent antibody (FA) reactions of spores of proteolytic types A, B and F of Cl. botulinum with spore antisera* of Cl. sporogenes*

		Number of positive reactions† with <i>Cl. sporogenes</i> antisera against strains of:						
Type	No. of strains tested	Type I					Type II	Type III
		2	17	37	75	98	59	60
A	18	5	2	0	2	6	7	5
B	10	1	1	2	1	1	2	4
F	3	0	0	0	0	0	0	0

* Antisera used at 1/100 dilution.

† Fluorescent intensity 3+ or 4+.

other 2 from the National Collection of Industrial Bacteria (NCIB), Torry Research Station, Aberdeen, Scotland.

These strains have been given serial numbers which have been used in this investigation.

RESULTS

Screening with Cl. sporogenes spore antisera

To determine the extent of sharing of spore antigens by strains of *Cl. sporogenes* and *Cl. botulinum*, spores were prepared from the 31 strains of *Cl. botulinum* and screened with the spore antisera prepared against strains of *Cl. sporogenes* (Princewill, 1979a) by FAT.

The results summarized in Table 2 show that there was no regular pattern of reaction between the spore antisera of *Cl. sporogenes* and the spores of strains of *Cl. botulinum*. Even antisera of the same type of *Cl. sporogenes* did not always react with the same strains of *Cl. botulinum*. Moreover, the cross-reactions among the strains of *Cl. botulinum* did not agree with the toxigenic type; there was no reaction with the 3 strains of type F tested and some of the strains of types A and B.

Cross-fluorescence tests

In the light of the results of the preliminary screening tests, one strain each of *Cl. botulinum* type A (strain 188) and type B (strain 172) was selected for the production of spore antisera. A strain of type F could not be included, because none of the strains sporulated sufficiently to provide spores for immunization. The two spore antisera obtained were then used along with the *Cl. sporogenes* antisera in cross-fluorescence tests.

The results (Table 3) show that both antisera to *Cl. botulinum* types A and B fluoresced to full titre with all the strains of homologous and heterologous types. This confirms that the spore antigens of *Cl. botulinum* are not related to the toxins produced by this organism. The cross-fluorescence of strains of *Cl. sporogenes* was of a low titre and the pattern of reaction did not follow any regular order, some strains of a type reacting whereas others did not.

Table 3. *Fluorescent antibody (FA) titres of spore antisera of Cl. sporogenes and Cl. botulinum*

(Antisera 172 and 188 reacted to full titre with spores of the 3 strains of *Cl. paratubulinum* type F; they also reacted partially with spores of *Cl. histolyticum* type II; but they did not react with spores of *Cl. bifermentans*, *Cl. butyricum* and *Cl. histolyticum* type I).

Strains	FA titres, against strains in column 1, of								
	<i>Cl. sporogenes</i> antisera against strains of							<i>Cl. botulinum</i> antisera against strains	
	Type I					Type II	Type III		
	2	17	37	75	98	59	60	188A	172B
<i>Cl. sporogenes</i>									
Type I									
2	51 200	25 600	12 800	25 600	51 200	—	—	—	—
17	25 600	25 600	25 600	25 600	12 800	—	—	160	160
37	25 600	25 600	25 600	12 800	25 600	—	—	1 000	1 000
75	51 200	25 600	12 800	51 200	25 600	—	—	—	—
98	51 200	25 600	25 600	25 600	51 200	—	—	1 000	160
Type II									
9b	—	—	—	—	—	6 400	—	400	400
27	—	—	—	—	—	6 400	—	160	—
42	—	—	—	—	—	3 200	—	—	160
59	—	—	—	—	—	6 400	—	1 000	1 000
Type III									
60	—	—	—	—	—	—	6 400	2 000	2 000
<i>Cl. botulinum</i>									
Type A									
169	160	—	—	—	160	320	—	10 000	10 000
171	—	—	—	—	—	320	—	10 000	10 000
174	320	320	—	320	320	—	—	10 000	10 000
175	320	320	—	320	320	—	—	10 000	10 000
176	—	—	—	—	—	—	160	10 000	10 000
181	—	—	—	—	—	—	160	10 000	10 000
182	—	—	—	—	—	—	320	20 000	10 000
183	160	—	—	—	160	320	—	10 000	10 000
184	—	—	—	—	—	320	—	10 000	10 000
187	—	—	—	—	—	160	160	10 000	10 000
188	160	—	—	—	160	320	—	20 000	10 000
189	—	—	—	—	160	160	—	10 000	10 000
190	—	—	—	—	—	—	160	10 000	10 000
Type B									
172	—	160	—	—	—	160	—	20 000	20 000
173	—	—	—	—	—	—	160	10 000	10 000
177	—	—	—	—	—	—	160	10 000	10 000
178	—	—	320	—	—	—	—	20 000	20 000
179	320	—	320	320	320	—	—	10 000	10 000
191	—	—	—	—	—	320	320	20 000	20 000
194	—	—	—	—	—	—	160	10 000	10 000

— = No fluorescence at 1/100 dilution of antiserum.

Table 4. *Fluorescent antibody (FA) titres of spore antiserum to Cl. sporogenes strain 2 cross-absorbed with spores of Cl. sporogenes and Cl. botulinum*

FA titres, of antiserum against spores of <i>Cl. sporogenes</i> 2, against:										
Absorbing spore antigen	<i>Cl. sporogenes</i> antigens					<i>Cl. botulinum</i> antigens				
	Type I		Type II		Type III	Type A			Type B	
	2	98	9b	59	60	174	175	188	172	179
None	51 200	51 200	40	40	40	320	320	160	—	320
<i>Cl. sporogenes</i>										
2	—	—	—	—	—	—	—	—	.	—
98	—	—	—	—	—	—	—	—	.	—
9b	1 600	1 600	—	—	—	—	—	—	.	—
59	3 200	1 600	—	—	—	—	—	—	.	—
60	800	1 600	—	—	—	—	—	—	.	—
<i>Cl. botulinum</i>										
174	12 800	12 800	—	—	—	—	—	—	.	—
188	6 400	6 400	—	—	—	—	—	—	.	—
172	6 400	12 800	—	—	—	—	—	—	.	—
179	12 800	12 800	—	—	—	—	—	—	.	—

— = less than 10.

. = test not done.

Table 5. *Fluorescent antibody (FA) titres of spore antiserum to Cl sporogenes strain 59 cross-absorbed with spores of Cl sporogenes and Cl. botulinum*

FA titres, of antiserum against spores of <i>Cl. sporogenes</i> 59, against:										
Absorbing strain	<i>Cl. sporogenes</i> antigens					<i>Cl. botulinum</i> antigens				
	Type I		Type II		Type III	Type A			Type B	
	2	98	9b	59	60	174	175	188	172	179
None	80	20	6 400	6 400	40	40	40	320	160	80
<i>Cl. sporogenes</i>										
2	—	—	800	1 600	—	—	—	—	—	—
98	—	—	800	800	—	—	—	—	—	—
9b	—	—	—	—	—	—	—	—	—	—
59	—	—	—	—	—	—	—	—	—	—
60	—	—	1 600	800	—	—	—	—	—	—
<i>Cl. botulinum</i>										
174	—	—	3 200	3 200	—	—	—	—	—	—
188	—	—	1 600	3 200	—	—	—	—	—	—
172	—	—	1 600	1 600	—	—	—	—	—	—
179	—	—	3 200	1 600	—	—	—	—	—	—

— = less than 10.

Table 6. *Fluorescent antibody (FA) titres of spore antiserum to Cl. sporogenes strain 60 cross-absorbed with spores of Cl. sporogenes and Cl. botulinum*

FA titres, of antiserum against spores of <i>Cl. sporogenes</i> 60, against:										
Absorbing strain	<i>Cl. sporogenes</i> antigens					<i>Cl. botulinum</i> antigens				
	Type I		Type II		Type III	Type A			Type B	
	2	98	9b	59	60	174	175	188	172	179
None	20	20	20	40	6400	80	40	160	40	80
<i>Cl. sporogenes</i>										
2	—	—	—	—	1600	—	—	—	—	—
98	—	—	—	—	1600	—	—	—	—	—
9b	—	—	—	—	800	—	—	—	—	—
59	—	—	—	—	800	—	—	—	—	—
60	—	—	—	—	—	—	—	—	—	—
<i>Cl. botulinum</i>										
174	—	—	—	—	3200	—	—	—	—	—
188	—	—	—	—	1600	—	—	—	—	—
172	—	—	—	—	1600	—	—	—	—	—
179	—	—	—	—	1600	—	—	—	—	—

— = less than 10.

Table 7. *Fluorescent antibody (FA) titres of spore antiserum to Cl. botulinum strain 188 (type A) absorbed with spores of Cl. sporogenes and Cl. botulinum*

FA titres, of antiserum against spores of <i>Cl. botulinum</i> 188, against:										
Absorbing strain	<i>Cl. sporogenes</i> antigens					<i>Cl. botulinum</i> antigens				
	Type I		Type II		Type III	Type A			Type B	
	2	98	9b	59	60	174	175	188	172	179
None	40	1000	400	1000	2000	10000	10000	20000	20000	10000
<i>Cl. sporogenes</i>										
2	—	—	—	—	—	8000	8000	8000	8000	4000
98	—	—	—	—	—	4000	4000	8000	8000	4000
9b	—	—	—	—	—	8000	8000	4000	8000	4000
59	—	—	—	—	—	8000	8000	8000	4000	8000
60	—	—	—	—	—	8000	8000	8000	8000	4000
<i>Cl. botulinum</i>										
174	—	—	—	—	—	—	—	—	—	—
188	—	—	—	—	—	—	—	—	—	—
172	—	—	—	—	—	—	—	—	—	—
179	—	—	—	—	—	—	—	—	—	—

— = less than 10.

Table 8. *Fluorescent antibody (FA) titres of spore antiserum to Cl. botulinum strain 172 (type B) absorbed with spores of Cl. sporogenes and Cl. botulinum*

FA titres, of antiserum against spores of <i>Cl. botulinum</i> 172, against:										
Absorbing strain	<i>Cl. sporogenes</i> antigens					<i>Cl. botulinum</i> antigens				
	Type I		Type II		Type III	Type A			Type B	
	2	98	9b	59	60	174	175	188	172	179
None	20	160	400	1000	2000	10 000	10 000	10 000	20 000	10 000
<i>Cl. sporogenes</i>										
2	—	—	—	—	—	8 000	8 000	4 000	8 000	4 000
98	—	—	—	—	—	4 000	8 000	8 000	8 000	4 000
9b	—	—	—	—	—	4 000	8 000	4 000	4 000	4 000
59	—	—	—	—	—	4 000	8 000	8 000	4 000	8 000
60	—	—	—	—	—	4 000	8 000	8 000	8 000	8 000
<i>Cl. botulinum</i>										
174	—	—	—	—	—	—	—	—	—	—
188	—	—	—	—	—	—	—	—	—	—
172	—	—	—	—	—	—	—	—	—	—
179	—	—	—	—	—	—	—	—	—	—

— = less than 10.

Table 9. *Distribution of spore antigens in species of proteolytic clostridia*

(+ , present; — , absent; ± , present in some strains; (+) , possibly present.

Organism	Spore antigenic components										
	A	B	C	D	E	F	G	J	K	L	M
<i>Cl. sporogenes</i>											
I	+	—	—	+	—	—	+	—	—	—	±
II	—	+	—	+	—	—	—	—	—	—	±
III	—	—	+	+	—	—	—	—	—	—	±
<i>Cl. histolyticum</i>											
I	—	—	—	—	+	+	—	—	—	—	—
II	—	—	—	—	—	+	+	—	—	—	±
<i>Cl. bifermentans</i>	—	—	—	—	—	—	—	+	—	—	—
<i>Cl. butyricum</i>	—	—	—	—	—	—	—	—	+	—	—
<i>Cl. botulinum</i>											
A	—	—	—	—	—	—	—	—	—	+	+
B	—	—	—	—	—	—	—	—	—	+	+
F	—	—	—	—	—	—	—	—	—	+	(+)

Immunofluorescence cross-absorption tests.

To observe the degree of sharing of spore antigens, cross-absorption tests were conducted on some of the antisera with a few selected strains of both *Cl. sporogenes* and *Cl. botulinum*.

The results (Tables 4–8) show that the cross-reacting antibodies could be removed by absorption. Thus, in the sporogenes–antisporogenes system (Tables 4–6) homologous absorption removed all the spore antibodies in the serum whereas heterologous absorption removed a substantial proportion of spore fluorescent

antibodies. In the botulinum-antibotulinum system (Tables 7, 8), homologous and heterologous absorptions removed all the spore antibodies in the serum thus confirming that the proteolytic strains of *Cl. botulinum* types A and B have identical spore antigens. In the botulinum-antisporogenes (and vice versa) cross-absorption systems, spores of *Cl. botulinum* types A and B removed the low titre cross-reacting antibodies in *Cl. sporogenes* antisera, thus rendering the antisera specific for *Cl. sporogenes* types; the reverse held good when *Cl. botulinum* antisera were absorbed with spores of *Cl. sporogenes*.

DISCUSSION

Cl. botulinum is divided into six types (A-F) on the basis of antigenically distinct toxins that they produce but their biochemical activity separates them into proteolytic and non-proteolytic groups. Because of serological differences in their vegetative antigens, the two biochemical groups have been considered as two separate species. Thus, the proteolytic group, consisting of *Cl. botulinum* types A, B and F have been considered as representing or belonging to *Cl. parabotulinum* whilst the non-proteolytic types B, C, D, E and F are members of *Cl. botulinum* (Bengtson, 1924). In this investigation we have not followed this nomenclature rigidly, but have used both names when appropriate. However, since our other organism of study is *Cl. sporogenes*, which is a strongly proteolytic species, the comparative studies were made between *Cl. sporogenes* and *Cl. parabotulinum* although we have continued to use the term *Cl. botulinum* for these.

On morphological, cultural and biochemical grounds, non-toxigenic *Cl. parabotulinum* is indistinguishable from *Cl. sporogenes*. Somatic and flagellar cross-agglutination between the two species has been reported (Mandia, 1951, 1955; Solomon *et al.* 1971; Lynt, Solomon & Kautter, 1972). Meisel & Rymkiewicz (1959), however, did not find any cross-reactions by spore agglutination among strains of *Cl. sporogenes* and *Cl. botulinum* types A and B (proteolytic). This is not surprising as they used only a small number of strains (three) of each species. In this study, FAT was used to examine 31 strains of *Cl. parabotulinum* (types A, 18; B, 10; and F, 3). Table 2 shows that the spores of the strains fluoresced in *Cl. sporogenes* spore antisera but there was no regular pattern of reactions; antisera of the same type of *Cl. sporogenes* did not consistently react with the same strains of *Cl. botulinum*; the cross-reacting strains of *Cl. botulinum* did not correspond to the toxigenic types; the three strains of *Cl. botulinum* type F and some strains of types A and B showed no reaction with any of the *Cl. sporogenes* antisera. These latter results are reminiscent of the experience of Meisel & Rymkiewicz (1959).

A two-way cross-fluorescence test (see Table 3) shows that the two antisera to *Cl. botulinum* types A (Strain 188) and B (strain 172) fluoresced to full titre with all the strains of the homologous and heterologous types. This again shows that there is no relation between the spore antigens of *Cl. botulinum* and the potential toxin production by types of this organism. This result does not agree with the findings of Meisel & Rymkiewicz (1959), who did not show any cross-agglutination between spores of *Cl. botulinum* types A and B. This is surprising since sharing of vegetative antigens among the proteolytic *Cl. botulinum* (i.e. *Cl. parabotulinum*)

has been reported by Mandia (1951) and others. It is possible that Meisel & Rymkiewicz (1959) were dealing with examples of incomplete antibodies which failed to bring about agglutination of the spore suspensions; FAT might have given a positive reaction. The two-way cross-fluorescence tests show a great deal of cross-reaction among strains of *Cl. sporogenes* and *Cl. parobotulinum* types A and B, even though the cross-reacting titres are lower than those given by specific homologous antisera.

Cross-absorption of the antisera suggests the presence of more than one antigenic component (Tables 4–8). The different types of *Cl. sporogenes* share a component with *Cl. parobotulinum* types A and B; this component removes the cross-reacting antibody thereby rendering the botulinum antisera specific for this species. It is also responsible for the slight lowering of the titre in the sporogenes antisera when absorbed with botulinum spores. Another component is shared exclusively by types of *Cl. botulinum* including the three type-F strains (not shown in the Tables) and this is responsible for species specificity. We name this L and the group-specific component M (following the sequence of the spore antigenic components described by Princewill, 1979*a, b*), which is possessed by some strains of *Cl. sporogenes*. Some strains of *Cl. histolyticum* type II (Princewill, 1979*b*) also possessed component M which was absent from the strains of *Cl. bifermentans* and *Cl. butyricum* studied by Princewill (1979*b*). Table 9 shows the distribution of spore antigenic components among strains of the species of *Clostridium* studied in the series.

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