SPORE ANTIGENS IN THE CLASSIFICATION OF SOME CLOSTRIDIA

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ALTHOUGH *Clostridium sporogenes* and *C. histolyticum* show obvious cultural and biochemical differences, several workers have adduced evidence for the sharing of vegetative and spore antigens by these two species (Smith, 1937; Mandia, 1955; Meisel and Rymkiewicz, 1959; Lynt, Solomon and Kautter, 1972). In their attempts to classify the proteolytic clostridia by means of vegetative antigens, Mandia (1951; 1952*a*; 1952*b*; 1955) and Mandia and Bruner (1951) did not include *C. bifermentans* which is an important proteolytic species. Meisel and Rymkiewicz (1959) showed that the spore antigens of this organism did not cross-react with spore antigens of the other proteolytic species they studied. However, cross reactions were reported by Walker (1963) between the spore antigens of *C. bifermentans* and *C. sordelli*, which are closely related species (Brooks and Epps, 1959). Princewill (1979) used the spore antigens to serotype strains of *C. sporogenes*. That study has now been extended to investigate the taxonomic relationship between *C. sporogenes*, *C. histolyticum*, *C. bifermentans* and *C. butyricum*.

MATERIALS AND METHODS

Strains of clostridia. The 84 strains of *C. sporogenes* used in this study and their origin have been described elsewhere (Princewill, 1978). The 69 strains of *C. histolyticum* were: nos. 119, 294, 646 from Dr H. Meisel, Serum Research Institute, Warsaw, Poland; CN nos. 919, 920, 948, 949, 950, 1693, 3056, 3252, 3254, 3255, 3256, 3257, 3258, 3259, 3260, 3261, 3265, 3266, 3267, 3268, 3269, 3270, 3271, 3272, 3273, 3274, 3276, 3277, 3278, 3279, 3280, 3281, 3282, 3283, 3284, 3285, 3286, 3287, 3288, 3289, 3290, 3292, 3293, 3294, 3295, 3296, 3297, 3298, 3300, 3301, 3302, 3303, 3304, 3305, 3306, 3307, 3308, 3309, 3310, 3311, 3312, 3313, 3786, 4760, 6304 from the Wellcome Research Laboratories, Langley Court, Beckenham, Kent; and from The National Collection of Industrial Bacteria no. NCIB503, Torrey Research Station (NCIB), Aberdeen. Other species were: *C. bifermentans* NCIB strains nos. 506, 2912, 2929, 6800, 6928 from NCIB; *C. beijerinckii* no. NCIB9362 from NCIB; *C. tyrobutyricum* no. CNRZ500 and *C. saccharobutyricum* no. CNRZ555 from Dr J. L. Bergère, INRA, Jouy-en-Josas, France; *C. butyricum* no. CNRZ5328 from Dr Bergère, and *C. butyricum* no. SA II from Dr J. W. Classens, Bloemfontein, South Africa.

Lyophilised cultures were reconstituted, grown in cooked-meat medium (CMM from Southern Group Laboratory, Hither Green Hospital, London) and cultured on fresh horse-blood agar. After incubation at 37°C for 48 h, single colonies were isolated and their identity was checked by routine methods (Princewill, 1978).

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Some members of the butyric group did not grow in the above media and were cultured in tomato-juice medium (Sarathchandra, 1973), which consisted of tomato puree 6.0 g, Peptone (Oxoid) 10.0 g, Tryptone (Difco) 10.0 g, cysteine hydrochloride 0.5 g, distilled water 1 litre; the pH was adjusted to 6.1 and the medium was autoclaved at 121°C for 15 min.; it was used either liquid or solidified with agar.

Production of spores. The method of producing the spore suspensions was similar to that described by Princewill (1979). The cultures were harvested and sonicated and the spores were freed from the vegetative debris by repeated washings. Clean spore suspensions were then prepared, containing 10^8 – 10^9 spores/ml as determined in a counting chamber by phase-contrast microscopy. Spore-antigen suspensions for immunisation were prepared by incubating the clean spore suspensions with formaldehyde (0.2% v/v) at 37°C for 7 days. The spore antigens were then stored at 4°C. The antigen for the agglutination test was prepared from the spore-antigen suspensions by washing once with preservative saline—physiological saline containing thiomersal 0.005% (w/v) and sodium azide 0.5% (w/v)—to remove the formalin.

Precipitation of spore antigens. Acid extracts of spore antigens were prepared by the method of Lamanna (1942); 10 ml of clean spore suspension was centrifuged and the spore sediment was resuspended in 1.0 ml of 0.05N HCl in preservative saline and heated for 10 min. in boiling water. After cooling, the suspension was centrifuged and the pH of the supernatant was adjusted to 7.0 with 1N NaOH and phenol-red indicator.

Vegetative-cell antigens. Broth cultures were incubated for 18 h then examined by phase-contrast microscopy to confirm the presence of vegetative cells and the absence of spores. For H-O antigens, the cultures were inactivated by incubation with formaldehyde (0.2% v/v) at 37°C for 3–4 days; for somatic (O) antigens the cultures were heated at 100°C for 1 h, cooled and then preserved with formaldehyde (0.1% v/v). Both H-O and O suspensions were adjusted to contain 10^8 – 10^9 vegetative cells/ml as described for the spore suspensions.

Spore antiserum. Male New Zealand white rabbits (2–3 kg) were given weekly subcutaneous injections of 1.0 ml of spore-antigen suspensions. Samples of blood were taken before the first injection, on the 3rd and 7th day and at weekly intervals until 3 weeks; 7–10 days later the rabbits were bled and the sera were tested for the presence of agglutinins against the immunising antigens. If satisfactory titres were obtained—at least 800, except the antiserum to strain 81 of which the titre was 160—the animals were bled under anaesthetic. The antisera were absorbed with homologous vegetative antigens to remove H and O antibodies. Ten ml of the H-O and O vegetative-cell suspensions were centrifuged; the cell deposit of the H-O suspension was resuspended in 1.0 ml of the undiluted serum, incubated in a shaking water bath at 37°C for 45 min and then centrifuged. The supernatant serum was absorbed with the cell deposit of the O suspension. If vegetative antibodies were still detected in the serum, the absorptions were repeated until there was no agglutination with the absorbing suspensions.

Agglutination tests. Twofold dilutions of antiserum were prepared in 0.1-ml volumes in saline starting from 1 in 10 in Wasserman test tubes. Equal volumes of the appropriate spore-antigen suspension were added and, after mixing, the tubes were incubated in a shaking water bath at 60 shakes/min. and 37°C for 45 min.; 0.8 ml of saline was added to each tube and the agglutination was observed immediately with the aid of a bright light against a black background. The highest dilution showing visible agglutination was taken as the end point. Normal rabbit serum and saline controls were included. (Noble, 1927; Lamanna, 1940; Norris and Wolf, 1961; Walker, 1963).

Fluorescent-antibody technique (FAT). Smears prepared on clean slides from the spore-antigen suspensions used in the agglutination test were dried in air and fixed by gentle heat. The smears were overlaid with one drop of a twofold dilution of spore antiserum and held in a moist chamber for 30 min. at 37°C. The slides were rinsed, washed twice in 3×10^{-4} M phosphate buffered saline, pH 7.2 for 20 min. and dried in air. Finally, the smears were overlaid with fluorescein-labelled sheep anti-rabbit immunoglobulin (Burroughs Wellcome) diluted 1 in 20, held in a moist chamber as before and then rinsed and washed. The stained smears were dried in air and examined with an M41 Photoplan Fluorescence Microscope (Vickers Instruments Ltd). The intensity of fluorescence was assessed on a scale of – to 4+; a reaction of 2+ or greater was scored as positive.

Precipitation ring test. The acid extract of spore antigens was carefully layered on the antiserum in narrow tubes (2 mm internal diameter) and the results observed at intervals up to 1 h. A precipitation ring usually developed within a few minutes.

RESULTS

Spore-agglutination tests

Antisera against two strains of *C. histolyticum* (strains 119 and 646) and one strain each of *C. bifermentans* (strain NCIB506) and *C. butyricum* (strain CNRZ528) were diluted 1 in 100, and used in agglutination tests with spore suspensions of each of the 84 strains of *C. sporogenes*, 69 strains of *C. histolyticum* and five strains each of *C. bifermentans* and the butyric group. The seven antisera prepared against the three types of *C. sporogenes* (Princewill 1979) were also included in these tests. The results are shown in table I. None

TABLE I

Agglutination reactions of spore antisera prepared against strains of Clostridium sporogenes, C. histolyticum, C. bifermentans and C. butyricum

Antigen strains	Number of strains tested	Number of strains agglutinating with antisera* against					
		<i>C. sporogenes</i> type			<i>C. histolyticum</i> strain		<i>C. bifermentans</i> strain NCIB506
		I†	II	III	119	646	
<i>C. sporogenes</i> : I	66	66	0	0	0	66	0
type II	17	0	17	0	0	0	0
type III	1	0	0	1	0	0	0
<i>C. histolyticum</i>	69	0	0	0	69	3	0
<i>C. bifermentans</i>	5	0	0	0	0	0	5
Butyric group	5	0	0	0	0	0	5

* Diluted 1 in 100.

† The 5 spore antisera (2, 17, 37, 75 and 98) prepared by Princewill (1979) were used.

of the seven *C. sporogenes* antisera agglutinated the spores of the 69 strains of *C. histolyticum* and the five strains each of *C. bifermentans* and the butyric group. Furthermore, there were no cross reactions between the three types of *C. sporogenes*. One of the antisera prepared against *C. histolyticum* (strain 119) agglutinated all the 69 strains of that species; the other antiserum (strain 646) reacted only with the homologous strain and two other strains. On the other hand, this latter antiserum reacted with spores of the 66 strains of *C. sporogenes* type I but not with spores of types II and III. The antisera prepared against *C. bifermentans* (strain NCIB506) and *C. butyricum* (strain CNRZ528) reacted only with strains of the homologous species and showed no cross reactions.

To determine the extent of the sharing of spore antigens between *C. sporogenes* type I and *C. histolyticum*, the agglutinins in the antisera were absorbed and titrated. The results (table II) showed that in general the

TABLE II
Agglutinin titres of *C. sporogenes* and *C. histolyticum* spore antisera absorbed with spores of other strains of these species

Antiserum prepared against strain no.	Agglutinin titre against										
	<i>C. sporogenes</i> type I, strain					<i>C. histolyticum</i> , strain					
	2	17	37	75	98	119	294	NCIB 503	CN 6304	646	CN 3288 3300
2 { 98 119 646	6400	3200	1600	3200	6400	<20	<20	<20	<20	<20	<20
	<20	<20	<20	<20	<20	...	...	...	...	...	...
	<20	<20	<20	<20	<20	...	...	...	...	...	...
	6400	3200	1600	3200	6400	...	...	...	...	...	...
	6400	3200	1600	3200	6400	...	...	...	...	...	...
119 { 2 98 119 646	<20	<20	<20	<20	<20	160	160	160	160	80	80
	...	...	...	...	...	160	160	160	160	80	80
	...	...	...	...	...	160	160	160	160	80	80
	...	...	...	...	...	<20	<20	<20	<20	<20	<20
	...	...	...	...	...	20	20	20	20	<20	<20
646 { 2 98 119 646	160	160	160	160	160	<20	<20	<20	<20	2560	1280
	<20	<20	<20	<20	<20	...	...	...	...	640	320
	<20	<20	<20	<20	<20	...	...	...	...	640	320
	80	80	40	80	80	...	...	...	...	1280	640
	<20	<20	<20	<20	<20	...	...	...	...	<20	<20

... = Not tested.

antigens were species specific; the only cross reaction occurred with antiserum prepared against *C. histolyticum* strain 646, which agglutinated spores of some strains of *C. sporogenes* type I to a relatively low titre (160). Absorption of this antiserum with spores of *C. sporogenes* type I strains 2 and 98 removed these antibodies and absorption with *C. histolyticum* strain 119 also slightly reduced the titre against the homologous strain. In addition, there was a distinct difference between strains 119 and 646 of *C. histolyticum*; the antiserum prepared against 119 agglutinated the spores of all strains of *C. histolyticum* tested (table I), but this strain did not react with antiserum against strain 646 which agglutinated spores of only three strains of *C. histolyticum* (646, CN3288 and CN3300).

Absorption of antiserum against strain 646 with spores of strain 119 did not remove the antibody. On the other hand, absorption of antiserum against strain 119 with spores of strain 646 removed all the antibodies reacting with it and with the other two strains (CN3288 and CN3300), but did not remove all the antibodies reacting with the homologous strain (119) or with three other strains (294, NCIB503 and CN6304). A low level of antibody was left even after repeated absorptions. This suggests that there are at least two types of *C. histolyticum*; type I represented by strain 119 and type II by strain 646 which cross reacted with *C. sporogenes* type I.

To detect whether cross reactions occurred at low titres of antisera among strains of *C. sporogenes*, *C. bifermentans* and the butyric group, quantitative agglutination tests were performed. The results are presented in table III. There was no cross reaction among the three species of *Clostridium*. The only cross reactions were at low titres (20–40) among the three types of *C. sporogenes*.

Fluorescent-antibody technique (FAT)

To examine whether the antisera giving negative results in spore-agglutination tests contained incomplete antibodies, spores of the strains of the other four species were treated with the antisera and the indirect FAT was applied. The results were similar to those of the agglutination tests; the only difference was a positive fluorescence given by spores of strain CN3288 of *C. histolyticum* with antisera to strains of *C. sporogenes* type I.

Precipitation reactions

To study the taxonomic significance of spore precipitinogens among strains of *C. sporogenes*, *C. histolyticum*, *C. bifermentans* and the butyric group, acid spore extracts were tested against the spore antisera in the capillary-tube (ring) test. The results showed complete species specificity with no inter-species cross reactions among the four groups.

The only cross reactions were among the different types of *C. sporogenes* and *C. histolyticum*. This confirms once more the taxonomic value of the spore precipitinogens (Norris and Wolf, 1961; Princewill, 1979).

TABLE III
Agglutination of titres of spore antisera against various strains of *C. sporogenes*, *C. bifermentans* and *C. butyricum*

Antigen, spores of	Agglutinin titre, in tests against the stated spore antigen, of antisera raised against				
	<i>C. sporogenes</i> strain (type)			<i>C. bifermentans</i>	<i>C. butyricum</i>
	2(I)	59(II)	60(III)	strain NCIB506	strain CNRZ528
<i>C. sporogenes</i>					
type I, strain: 2	6400	20	40	< 20	< 20
17	3200	< 20	20	< 20	< 20
37	1600	20	40	< 20	< 20
75	3200	< 20	< 20	< 20	< 20
98	6400	< 20	20	< 20	< 20
type II, strain: 9b	20	800	20	< 20	< 20
27	< 20	800	< 20	< 20	< 20
42	20	800	< 20	< 20	< 20
59	< 20	800	20	< 20	< 20
type III, strain: 60	20	< 20	800	< 20	< 20
<i>C. bifermentans</i>					
strain:					
NCIB506	< 20	< 20	< 20	1280	< 20
NCIB2912	< 20	< 20	< 20	1280	< 20
NCIB2929	< 20	< 20	< 20	1280	< 20
NCIB6800	< 20	< 20	< 20	1280	< 20
NCIB6928	< 20	< 20	< 20	640	< 20
Butyric group					
strain:					
NCIB9362	< 20	< 20	< 20	< 20	1280
CNRZ500	< 20	< 20	< 20	< 20	1280
CNRZ528	< 20	< 20	< 20	< 20	1280
CNRZ555	< 20	< 20	< 20	< 20	1280
SA II	< 20	< 20	< 20	< 20	1280

DISCUSSION

The differentiation of *C. sporogenes* from *C. histolyticum* is based on their cultural and biochemical characters. However, several reports cite a serological relationship involving their H (Mandia and Bruner, 1951), O (Lynt *et al.*, 1972) and spore antigens (Meisel and Rymkiewicz, 1959). A comparison of the spore antigens of *C. sporogenes* and *C. histolyticum* therefore was considered relevant. The two strains of *C. histolyticum* selected for the production of antisera were two of the three strains used by Meisel and Rymkiewicz (1959) in their classical study of the spore agglutination of seven proteolytic *Clostridium* species; in that investigation, the only cross reaction was between *C. sporogenes* and *C. histolyticum*; these workers also found evidence for the existence of at least three types of *C. histolyticum*. In general, our results agree with theirs, that there is a cross reaction between *C. sporogenes* and *C. histolyticum* on the basis of spore agglutination; there is also evidence of more than one type of *C. histolyticum*.

The antisera prepared against *C. histolyticum* strains 119 and 646 behaved differently in tests with spores of *C. sporogenes* (see table I). Antiserum against strain 119 was species specific, reacting only with 69 strains of *C.*

histolyticum, whilst antiserum against strain 646 reacted with all the 66 strains of *C. sporogenes* type I and only with three of the strains (646, CN3288 and CN3300) of *C. histolyticum*. This result emphasises the importance of the selection of strains for immunological studies.

For an inter-species study strain 646 is a valuable organism, whereas it is of little value for studying the relationship between strains of *C. histolyticum*. On the other hand, if strain 119 alone had been selected, the cross reaction between *C. sporogenes* and *C. histolyticum* would not have been demonstrated; the antigens of strain 119 are, however, more widely distributed among the strains of *C. histolyticum* than those of strain 646.

Cross-absorption tests revealed the existence of at least three types of spore antigens in strains of *C. histolyticum* (see table II). The species can therefore, be divided into two types: type I, exemplified by strain 119, possesses two antigenic components, one of which (E) determines the type specificity whilst the other (F) is shared by all strains of the species. The designation of these components follows the sequence of the spore antigenic components A, B, C and D of *C. sporogenes* reported by Princewill (1979). Type II, represented by strain 646, also possesses two antigens: the group component (F) is shared by all strains, and an additional factor (G) is responsible for the cross reaction with strains of *C. sporogenes* type I. Component (G) is also present in strains of *C. sporogenes* type I, but it failed to elicit the production of spore antibodies as shown by the non-reactivity of antisera prepared against *C. sporogenes* type I with the strains of *C. histolyticum*. This was presumably because of either its haptenic nature or of steric factors. A one-way cross reaction between *C. sporogenes* and *C. histolyticum* was also observed by Mandia and Bruner (1951) and Mandia (1952*b*) in their studies on the vegetative antigens of proteolytic clostridia. Although such one-way reactions might be attributable to the presence of incomplete antibody, the results of the FAT in the present study do not bear this out.

C. bifermentans can be identified by cultural and biochemical tests, but in this investigation an attempt was made to classify it within the serological scheme of the proteolytic clostridia. The antiserum (against strain 646) that was used indicated that the organism had no serological relationship with the strains of *C. sporogenes* examined by means of spore agglutination, FAT and precipitation. Indeed, *C. bifermentans* showed no cross reaction with any of the other species of *Clostridium* studied and the results indicate that the five strains of *C. bifermentans* form a single type; this confirms the findings of Meisel and Rymkiewicz (1959) and Walker (1963). The number of strains examined in this study was small but the results appear to be due to the presence of a single antigenic component (J) shared by spores of strains of *C. bifermentans* tested. 'H' and 'I' have been omitted from the nomenclature of the spore antigens to avoid confusion with flagellar antigen (H) and the Roman numeral (I).

C. butyricum and other members of the butyric group can also be identified by cultural and biochemical tests; they were included in this study as non-proteolytic organisms and as type species of the genus. As in the case of *C.*

bifermentans, the antiserum against spores of *C. butyricum* strain CNRZ528 was species specific. There was no cross reaction in the spore agglutination, FAT or precipitation tests with strains of *C. sporogenes* or with the other species of *Clostridium* examined. Although only five members of the butyric group were examined, we conclude that there is a single antigenic component (K) which is shared by spores of all the strains tested. The five strains studied therefore form a single group; this finding agrees with those of other workers who studied the serological relationship among members of *C. butyricum* with respect to the H and O antigens (McCoy and McClung, 1938).

SUMMARY

The spore antigens of *Clostridium sporogenes*, *C. histolyticum*, *C. bifermentans* and the butyric group were compared. By spore agglutination and fluorescent-antibody technique (FAT) the 69 strains of *C. histolyticum* were divided into two types: serum raised against type I (66 strains) reacted with all strains of this species but showed no cross reaction with any of the three types of *C. sporogenes*; serum raised against type II (three strains) did not react with strains of *C. histolyticum* type I but showed cross reaction with all the 66 strains of *C. sporogenes* type I. Thus, by antigenic analysis, spores of *C. histolyticum* type I were found to possess two components designated E and F; E was a type-specific component whilst F was shared by strains of type II. In addition, strains of *C. histolyticum* type II possessed a second component, G, which was shared by strains of *C. sporogenes* type I. There was no cross reaction between the precipitinogens of the spores of the two species. Five strains of *C. bifermentans* formed a homogeneous group as judged by spore agglutination, FAT and precipitation reactions. There was no cross reaction with any of the other proteolytic species studied. Five butyric strains also formed a homogeneous group. Two antigenic components were therefore assigned to the spore antigens of the two groups: J, for *C. bifermentans* and K, for the butyric strains.

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