REINVESTIGATIONS OF THE RELATIONSHIP BETWEEN SPORULATION, HEAT RESISTANCE AND SOME BIO-CHEMICAL PROPERTIES IN STRAINS OF *CLOSTRIDIUM PERFRINGENS*

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IN a previous paper (Nishida, Seo and Nakagawa, 1969) we demonstrated that heat selection was one of the main elements to give rise to or to demonstrate diversity in the biological properties of *Clostridium perfringens*; salicin-fermenting and gelatinase-negative strains of *C. perfringens* were frequently isolated from soil samples preheated at 100°C for 1 hour or more. We recently found that there appeared to be concurrent variation in some other properties of isolates obtained from heated samples. In the present investigation we attempt to establish a correlation between the concurrent conversion of biochemical properties and conditions for preheating in studies with a number of *C. perfringens* isolates. In the course of these studies, it was proved that sporulation of these organisms frequently occurs in the presence of a slowly fermentable sugar. This finding led us to a further study of the effects of sugars on sporulation of *C. perfringens*.

MATERIALS AND METHODS

Strains. A total of 94 strains of *C. perfringens* was used in this study. Most of the strains were newly isolated from soil according to the method described by Yamagishi, Ishida and Nishida (1964). The soil samples were collected from places at least 50 metres apart in the vicinity of our Medical School. One isolate was taken from one soil sample. Typing of these isolates was not performed. Fourteen stock strains (Nishida *et al.*, 1969) were also used.

Biochemical tests. Routine biochemical tests were performed according to Sterne and van Heyningen (1965). All cultures were incubated for 1 week anaerobically in H₂ in an anaerobic jar (Tominaga Ltd, Tokyo, Japan) with Deoxo pellets (Baker Platinum Ltd, London) as the catalyst; the cultures were then examined for their biochemical properties. Hydrolysis of aesculin was detected by adding 2-3 drops of 1% ferric ammonium citrate solution to the culture. Hydrolysis of arbutin was detected by adding 0.5 ml 2M sodium carbonate solution to a 1-ml volume of the culture and standing the mixture at room temperature for 60 min. Development of a black colour was recorded as a positive result in both cases.

Sporulation medium. The medium of Duncan and Strong (1968) was used (D-S medium). To examine effects of sugars on sporulation, 0.4 ml of a 10% Seitz-filtered solution of the test sugar was added to a 10-ml volume of D-S medium without starch and the following procedure was performed. An active culture was obtained by overnight growth of each strain in 10 ml Fluid Thioglycollate Medium (FTG; Nissan Co., Tokyo, Japan). Two subsequent serial transfers into 10-ml volumes of fresh FTG, with a 10% inoculum and a 4-hour incubation period at 37°C, were then made. A 1-ml volume of the last culture was inoculated into 10 ml D-S medium that had been boiled and cooled immediately before use, and this

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culture was incubated at 37°C for 40 hours, when the sporulating frequency was determined. These cultures in D-S and FTG media were not incubated in an anaerobic jar.

Determination of sporulating frequency. This was estimated by two different methods.

1. *Viable counts before and after pasteurisation.* The total number of colony-forming units (viable count, V) in a culture was determined by plating 0.1-ml volumes of serial ten-fold dilutions of the culture in 0.85% NaCl solution on Brain-Heart Infusion Agar (Nissan Co., Tokyo, Japan) supplemented with 1% glucose. The total spore population (S) was determined by the same procedure after heating the culture at 75°C for 15 min. Plates were incubated anaerobically in H₂ at 37°C for 40 hours. The sporulation frequency was recorded as the ratio S/V.

2. *Enumeration of cells and spores by microscopy.* A portion of the culture was centrifuged and a smear of the sediment was stained by the method of Wirtz (1908). Bacterial cells and green spores in a microscopic field were enumerated; at least 1000 cells were counted and the sporulation frequency was measured as the ratio of the number of spores to the total cell count.

RESULTS

In a preliminary experiment, seven *C. perfringens* strains from each of two groups, (i) strains isolated from unheated soil samples (unheated group), and (ii) strains from those heated at 100°C for 60 min. (100-60 group), were examined for their ability to ferment the following 14 carbohydrates: cellobiose, dextrin, fructose, galactose, glucose, inositol, lactose, mannose, maltose, melibiose, raffinose, salicin, sucrose and starch. Distinct differences between the two groups could be found only in the fermentation of salicin, cellobiose and inositol; all strains of the unheated group fermented inositol, but none fermented salicin and cellobiose, whereas all strains of the 100-60 group fermented salicin and cellobiose, but none attacked inositol.

Further experiments were undertaken to establish a correlation between strains isolated by a heating procedure and the ability to ferment salicin, cellobiose and inositol. Soil samples were heated at 70, 90 and 100°C for 10 min., and at 100°C for 1, 2, 3 and 5 hours respectively. A total of 80 *C. perfringens* isolates were obtained from soil samples in this way. The longer the preheating, the greater the number of isolates that fermented salicin and cellobiose. No isolate from samples heated at 100°C could ferment inositol, although all of the unheated group could ferment inositol. Out of 50 isolates from samples heated at 100°C, 28 fermented both salicin and cellobiose, only 3 fermented salicin or cellobiose alone, and the remaining 19 fermented neither (table I).

We further investigated the sporulating abilities of isolates that had been subjected to heat treatment at 100°C for more than 1 hour; a total of 27 *C. perfringens* strains belonging to groups VI, VII and VIII (table I) were used. The results shown in table II indicate that the longer the period of preheating at 100°C, the weaker the sporulating abilities of the isolates. The findings summarised in tables I and II suggest a close correlation between attenuation of sporulating ability and the above-mentioned fermentation patterns. When the 27 isolates of groups VI, VII and VIII (table I) were graded according to their sporulating abilities and examined for their fermentation of cellobiose and salicin, a very clear correlation emerged (table III).

Salicin and cellobiose both have a β -glucoside bond in their structures. We accordingly tested the cellobiose- and salicin-positive strains for their

TABLE I
Effects of preheating on biochemical properties of Clostridium perfringens isolates*

Group	Preheating exposure	isolates used	Number of			
			cellobiose- and salicin-positive isolates	cellobiose-positive and salicin-negative isolates	cellobiose-negative and salicin-positive isolates	inositol-positive isolates
I	none	10	0	1	1	10
II	70°C	10	0	0	2	7
III	for 10 min. 90°C	10	2	0	0	6
IV	for 10 min. 100°C	9	3	0	0	0
V	for 1 hour 100°C	14	5	1	1	0
VI	for 2 hours 100°C	10	6	0	1	0
VII	for 3 hours 100°C	10	7	0	0	0
VIII	for 5 hours 100°C	7	7	0	0	0

* This denotes that a heat treatment of a soil sample was used for the isolation of the organism.

TABLE II
Effects of heat treatment of soil samples on sporulating abilities of isolates therefrom

Heat-treatment group*	Number of isolates used	Number of isolates with a sporulation frequency† of more than		
		0.1 %	0.09-0.01 %	0.009-0.001 %
VI	10	4	2	4
VII	10	3	1	6
VIII	7	0	0	7

* Grouping according to conditions of preheating of soil sample (see table I).

† Measured by a viable count procedure (see *Methods*); the sporulation medium was that of Duncan and Strong (1968).

ability to attack two other sugars, aesculin and arbutin, that have a β -glucoside bond. Eighteen strains each of salicin- and cellobiose-positive and salicin- and cellobiose-negative *C. perfringens* strains were selected from our collection; all were isolated from soil samples heated for 1 hour or more. The results given

in table IV show that all strains of the former group hydrolysed aesculin and arbutin, whereas none of the latter group hydrolysed the two glucosides.

TABLE III
Relationship between sporulation frequency and fermentation of cellobiose and salicin

Grade of sporulation*	Number of isolates used	Number of cellobiose- and salicin-positive isolates
+	7	0
±	3	0
—	17	17

*Sporulation frequency was determined by a viable count procedure; the following scoring system was used: + = >0.1%; ± = 0.09–0.01%; — = <0.009%.

We then examined the influence of various sugars on sporulation. In a preliminary study with two strains of *C. perfringens*, the effects of 9 sugars, namely glucose, maltose, fructose, lactose, sucrose, salicin, raffinose, melibiose and starch, were examined. Raffinose and melibiose appeared to promote sporulation, but other sugars were ineffective and starch was relatively ineffective. Table V gives the results of a detailed study of the effects of raffinose,

TABLE IV
Difference in hydrolysis of aesculin and arbutin between cellobiose- and salicin-positive and cellobiose- and salicin-negative isolates

Category of isolate	Number of isolates used	Number (and percentage) of isolates hydrolysis-positive for	
		aesculin	arbutin
Cellobiose- and salicin-positive	18	18 (100)	18 (100)
Cellobiose- and salicin-negative	18	0 (0)	0 (0)

melibiose and starch in tests with 21 isolates of *C. perfringens* from samples exposed to different conditions of preheating. These data confirm that raffinose and melibiose influence sporulation of these strains under the conditions of our tests. The findings also indicate further differences in behaviour of isolates from samples heated at 100°C and isolates from samples heated at less than 100°C. None of the isolates from samples preheated at 100°C sporulated in the sugar-free D-S medium although most of them could still sporulate in the presence of melibiose and raffinose, and less markedly in the presence of starch.

However, most of the isolates recovered from samples preheated at less than 100°C sporulated in the sugar-free medium and their sporulation was enhanced by melibiose and raffinose but depressed by starch.

DISCUSSION

Bergey's manual (Breed, Murray and Smith, 1957) records that *C. perfringens* rarely ferments salicin; there is no comment on the utilisation of cellobiose or the hydrolysis of aesculin and arbutin by this species. The present

TABLE V
Effect of various sugars on sporulation of C. perfringens

Isolate number	Conditions of heating used for isolation	Sporulation frequency (percentage)* in the presence of			
		melibiose	raffinose	starch	no carbohydrate
6	unheated	—†	—	—	0.6
7		7.8	3.1	0.2	7.2
8		11.2	4.7	—	1.2
9		—	—	—	1.8
12	70°C, 10 min.	—	—	—	—
14		5.8	3.2	—	0.5
15		6.0	5.4	—	—
16		6.9	9.0	—	3.0
18		12.1	3.6	—	9.7
112-4	100°C, 10 min.	3.8	1.3	—	—
22	100°C, 1 hour	38.0	24.0	0.1	—
23		0.7	30.3	1.3	—
25		1.7	—	—	—
35	100°C, 2 hours	—	—	—	—
36		0.7	12.8	0.9	—
38		—	1.9	—	—
40	100°C, 3 hours	—	—	—	—
44		29.0	8.7	—	—
45		10.8	9.6	0.9	—
47	100°C, 5 hours	26.9	—	—	—
55		—	—	—	—

* Sporulation frequency was determined microscopically (see *Methods*).

† — = <0.1%.

study confirms that strains that can ferment salicin, cellobiose or arbutin are rarely isolated from unheated soil samples or from samples heated at less than 100°C, although more than half of these strains were able to hydrolyse aesculin. It was found that the longer the preheating of soil samples at 100°C, the greater the number of isolates that could attack all four of these carbohydrates.

Two hypotheses may be postulated to account for the appearance of strains with these variations in which heat resistance—as judged by survival of a pre-heating step during their isolation—appears to be paradoxically related to attenuated sporulating ability and to extended fermentation ability. Attenuated sporulating abilities of strains recovered from samples heated at 100°C for more than 1 hour give rise to a prolonged vegetative stage of the organism and,

consequently, (i) their normally undetectable enzyme activities may be expressed, or (ii) the frequency of the appearance of mutants that can attack these carbohydrates may be increased. Our recent findings suggest that the second postulate is the more reasonable. However, the genetic or biochemical process whereby this biochemical diversity is expressed remains to be determined and the apparent difference in heat resistance of the two groups is unexplained.

Most of the sugars tested inhibited sporulation of *C. perfringens*, although these sugars enhanced growth. The present study reveals, however, that *C. perfringens* could sporulate in the presence of some slowly fermented sugars more abundantly than in the absence of sugars or in the presence of rapidly fermented sugars. Ellner (1956), and Duncan and Strong (1968) employed starch, and Ellner recommended raffinose for a sporulation medium although he did not give any data on this. We have found that melibiose and raffinose may promote more reproducible sporulation than starch, a commercial product that usually differs in its properties according to the material from which it was prepared. We examined three kinds of starch prepared from respectively wheat, potato or corn (Wako, Tokyo, Japan) but none was effective in promoting sporulation of *C. perfringens*.

Nishida *et al.* (1969) stated that strains of *C. perfringens* in the 100-60 group were definitely weaker in their sporulating ability than the strains of the unheated group; these observations were based on studies with Nishida and Seo's medium (Nishida *et al.*, 1969) which did not include any sugar. Our present findings in studies with the sugar-free medium of Duncan and Strong (1968) support that view, but disclose that most of the heat-treated strains could still sporulate in the medium containing raffinose or melibiose.

SUMMARY

Isolates of *Clostridium perfringens* obtained from soil samples heated at 100°C and from samples heated at less than 100°C exhibited markedly different fermentation properties in tests with inositol, salicin, cellobiose, arbutin and aesculin. None of the *C. perfringens* isolates obtained from samples heated at 100°C for various lengths of time could ferment inositol, whereas all of the isolates obtained from unheated samples could ferment this sugar. The longer the preheating period at 100°C, the greater the number of aberrant isolates that attacked salicin, cellobiose, aesculin and arbutin, and the weaker were the sporulating abilities of these isolates. Most of the "heat-resistant" strains with attenuated sporulating abilities, however, could sporulate well when raffinose or melibiose was the only carbohydrate present in a test medium.

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