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Response of *Clostridium perfringens* Spores and Vegetative Cells to Temperature Variation[1]

JAMES C. CANADA, DOROTHY H. STRONG, AND LELIA G. SCOTT[2]

Department of Foods and Nutrition, University of Wisconsin, Madison, Wisconsin

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ABSTRACT

CANADA, JAMES C. (University of Wisconsin, Madison), DOROTHY H. STRONG, AND LELIA G. SCOTT. Response of *Clostridium perfringens* spores and vegetative cells to temperature variation. Appl. Microbiol. **12**:273-276. 1964.—The vegetative cells and spores of four strains of *Clostridium perfringens* were examined to determine the effect of lowered and elevated temperatures. Spores were produced by following the method of Ellner, and vegetative cells were obtained from thioglycolate cultures. After exposure to freezing or refrigeration temperatures (−17.7 and 7.1 C, respectively), only small numbers of the vegetative cells were recovered. After similar treatment, 16 to 58% of the spores were recovered. Essentially no vegetative cells and few spores survived holding at 80 C for 10 min. Although all strains were isolated from food, only one strain of the four studied had its origin in a food-poisoning outbreak, and it had been carried on laboratory media for approximately 10 years.

It has been observed that certain strains of *Clostridium perfringens* produce heat-resistant spores, whereas others do not. The existence of both types of spores and their ubiquitous occurrence has been stressed by many workers, including Collee, Knowlden, and Hobbs (1961).

Hall et al. (1963) described the characteristics of *C. perfringens* strains associated with food and food-borne disease. Two types of spores were delineated, one of which was "atypical—spores that caused a distinct swelling of the cell." The other type was described as small, and did not swell the cell. The type of spores produced by a strain of *C. perfringens* appeared to be governed, in some degree, by the medium in which they were produced. The morphologically atypical spores occurred more frequently in Ellner's (1956) and modified Ellner's broth than in other media. The authors also reported a difference in the degree of heat resistance, the "atypical" spores being less heat-resistant than the other. The source of the strain appeared to have a bearing on the thermal resistance of the spores, because heat resistance was not found in spores of *C. perfringens* isolated from normal feces, soil, or pathological specimens. Strains which produced heat-resistant spores were obtained from food-poisoning incidents in England, Europe, Japan, the United States, and from edible foods in the United States. However, not all the spores from these sources were heat-resistant.

Hobbs (1960) found that spores survived boiling for several hours when they had been isolated promptly during food-poisoning outbreaks. Hall and Angelotti (1962) observed that the spores of only 5 of 19 American food-poisoning strains demonstrated heat resistance. The spores of *C. perfringens* isolated by Dam-Mikkelsen, Petersen, and Skovgaard (1962) from food-poisoning incidents in Denmark did not survive heating at 100 C for 2 min. Yamamoto et al. (1961) were also unable to demonstrate heat resistance in spores isolated during a random survey of feces and livers of market poultry.

Raj and Liston (1961) noted little change in the population of *C. perfringens* when it had been frozen and stored for extended periods. Kemp, Proctor, and Browne (1962) reported that the freezing of bacteriological specimens containing *C. perfringens* may make recovery of the organism extremely difficult. They suggested that epidemiologists investigating food-poisoning outbreaks, presumably due to *C. perfringens*, should refrigerate rather than freeze food specimens.

This study represents a further effort to gain information concerning the effects of elevated and lowered temperatures on selected strains of *C. perfringens*.

MATERIALS AND METHODS

Four type-A strains of *C. perfringens* were studied. Two of the strains (108; 142 A) had been isolated from raw meat, one (65) from raw vegetables, and the fourth (8799F 1546/52) from a food-poisoning incident. The latter strain had been carried on laboratory media for approximately 10 years. Three different diluents [phosphate buffer (pH 7.2; American Public Health Association, 1960), 0.1% peptone water (Straka and Stokes, 1957), or 2% sucrose solution] served as suspending media in preparing the cell or spore suspensions. The effect of elevated (100 and 80 C) and lowered temperatures (−17.7 and 7.1 C) was studied. The lowered temperatures correspond, respectively, to those usually attained in household freezers or refrigerators.

Stock cultures were maintained in veal broth at room temperature and were transferred monthly. Vegetative cells were obtained by centrifugation from a 4-hr culture

[1] Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

[2] Present address: Department of Horticulture, University of Kentucky, Lexington.

actively growing in thioglycolate medium containing glucose. The packed cells were washed and centrifuged three times in the desired suspending medium. To obtain spores, the procedure and medium described by Ellner (1956) were employed. Thus, 1 ml of an actively growing thioglycolate (without sugar) culture was inoculated into the sporulating medium and incubated at 37 C for 22 hr. The spores also were harvested by a centrifuging and washing procedure.

The organisms, in either vegetative or spore states, were diluted in the suspending media to an approximate concentration of 10^3 or 10^6 per ml. Samples of these suspensions were further diluted and then plated in duplicate to determine the population of the suspensions. The samples were prepared for exposure to the temperature at test by placing 1 ml of a suspension in a sterile section of soft glass tubing (outer diameter, 6 mm; approximately 15 cm in length) which had been sealed at one end. Immediately after filling, the open end was also sealed with heat.

Tubes containing the suspensions to be frozen were immersed in 95% ethyl alcohol maintained at a temperature of −17.7 C. The holding time was 48 hr. A similar procedure was followed for suspensions held at 7.1 C. Tubes containing suspensions to be heated at either 80 or 100 C were immersed in propylene glycol maintained at these respective temperatures. The time required for the temperature of the suspension to equilibrate with that of the propylene glycol was determined by the use of a probe thermometer. The holding time was exactly 10 min after the probe thermometer indicated that the desired temperature of 80 or 100 C had been reached. The tubes were removed from the heating bath and rapidly cooled to room temperature.

The population of the suspensions that had been exposed to the temperatures was determined by the following procedure. A sealed glass tube was aseptically broken in a vial containing 9 ml of the suspending medium. After a minimum of agitation to insure thorough mixing, a 1-ml portion was further diluted in the suspending medium. Duplicate plates were prepared from each of the diluted suspensions. The plating medium used throughout this series of experiments was SPS agar described by Angelotti et al. (1962), except that the antimicrobial agents, polymyxin B sulfate and sodium sulfadiazine, were omitted. The plates were incubated at 37 C in an atmosphere of 90% nitrogen and 10% CO_2. *C. perfringens* reduced sulfite to sulfide and, in the presence of iron, black colonies developed. These colonies were observed and counted after both 24 and 48 hr of incubation. Populations of the suspensions prior and subsequent to the test temperatures were thus compared. Results were expressed as per cent survival of the original population. Three replications of the experimental design described above were performed.

RESULTS AND DISCUSSION

Tables 1 and 2 present a summary of the results obtained when vegetative cells or spores of four strains of *C. perfringens* were submitted to various temperature treatments. Survival percentage is shown for two different size-of population (10^3 and 10^6 cells per ml) and for cells suss pended in three different diluents during the temperature treatments. The survival percentage rates of the individual strains were averaged. Table 3 presents an example of the data obtained for each strain.

Examination of the average values indicates that, under conditions of these experiments, very small numbers of vegetative cells were recovered after freezing. Likewise, except in the case in which 0.1% peptone water was used as a diluent, small numbers of the vegetative cells were recovered subsequent to holding at a temperature of 7.1 C for 48 hr.

TABLE 1. *Percentage of survival of Clostridium perfringens vegetative cells at four temperatures in three suspending diluents*

Holding temp	Original population[a]	Per cent of original population surviving in					
		Phosphate buffer (pH 7.2)		Peptone-water (0.1%)		Sucrose solution (2.0%)	
		Avg[b]	Range	Avg	Range	Avg	Range
C							
−17.7[c]	10^3	0.0	—	0.4	0.0–2.6	<0.1	0.0–0.3
	10^6	0.4	0.1–4.9	0.2	0.0–0.8	2.4	0.0–29.4
7.1[c]	10^3	0.6	0.0–3.2	4.0	0.0–33.4	0.3	0.0–2.8
	10^6	0.3	0.0–2.8	9.6	0.0–36.9	1.1	0.0–13.2
80.0[d]	10^3	3.8	0.0–21.6	0.0	—	0.0	—
	10^6	0.0	—	0.0	—	0.0	—
100.0[d]	10^3	0.0	—	0.2	0.0–2.5	0.0	—
	10^6	0.1	0.0–0.8	0.0	—	0.0	—

[a] Expressed as approximate number of cells per ml.
[b] Average values derived from dual platings in three replications for each of four strains.
[c] Holding time was 48 hr.
[d] Holding time was 10 min.

TABLE 2. *Percentage of survival of Clostridium perfringens spores produced in Ellner's medium held at four temperatures in three suspending diluents*

Holding temp	Original population[a]	Per cent of original population surviving in					
		Phosphate buffer (pH 7.2)		Peptone water (0.1%)		Sucrose solution (2.0%)	
		Avg[b]	Range	Avg	Range	Avg	Range
C							
−17.7[c]	10^3	26.6	0.0–65.0	44.1	0.0–89.0	58.3	0.0–100
	10^6	16.8	0.0–47.8	16.2	0.3–41.8	42.4	0.0–100
7.1[c]	10^3	31.8	0.0–100.0	26.0	0.0–100	41.1	0.0–100
	10^6	34.5	<0.1–91.4	25.9	0.1–100	25.1	0.1–52.9
80.0[d]	10^3	5.8	0.0–52.4	15.8	0.0–60.7	1.2	0.0–7.5
	10^6	4.8	0.0–19.1	8.0	0.0–22.9	6.4	0.0–19.4
100.0[d]	10^3	0.0	—	1.1	0.0–8.0	0.3	0.0–3.7
	10^6	0.0	—	0.0	—	0.0	—

[a] Expressed as approximate number of cells per ml.
[b] Average values derived from dual platings in three replications for each of four strains.
[c] Holding time was 48 hr.
[d] Holding time was 10 min.

When the spore suspensions were held at −17.7 C, average values of approximately 16 to 58% recovery of the organism were obtained; 25 to 41% of the spores survived holding at 7.1 C.

Except in one instance, vegetative cells were not recovered when the culture had been maintained at 80 C for 10 min. The average percentage of recovery from spores heated at 80 C for 10 min was greater than that of vegetative cells, and ranged from 4.8 to 15.8%. After 10 min at 100 C, few vegetative cells or spores appeared to survive. There seemed to be a slight trend toward greater recovery when the suspending medium was 0.1% peptone water.

Kemp et al. (1962) reported that their own experience as well as that of Smith indicated difficulty in recovery of *C. perfringens* subsequent to "undue harshness" in freezing. The values presented here indicate considerable recovery from spores which had been frozen. Because no special procedures were utilized in our experiments to assure maximal recovery of cells which may have been damaged by freezing, it is possible that the values presented for recovery are minimal. Evidence that the use of thawing agents and diluents of increased osmotic strength yielded higher plate counts for *Escherichia coli* which had been frozen was presented by Bretz and Hartsel (1959). Similar special treatment of frozen vegetative cells and spores of *C. perfringens* may be needed to obtain maximal recovery after freezing.

The vegetative cell or spore suspension which made up each sample was only 1 ml, and the tubes into which the suspensions were placed were small in diameter; hence, the time required for the temperature of the total quantity of suspension to equalize with its environment was very short. This factor, plus possible protection to organisms offered by components of food, probably makes refrigeration for both cells and spores appear to be more destructive than would be true under usual conditions of food storage.

Regardless of the stress applied, there was considerable difference in the percentage of recovery between the cells grown in thioglycolate medium and those produced in Ellner's (1956) medium. The results of the tests for heat resistance of spores indicated values which did not appear to be comparable to the heat-resistant strains reported by Hall et al. (1963). In their work, however, more than half of the strains studied were classified as not being heat-resistant. The lesser heat resistance noted in our studies may be a reflection of strain difference, or it may be confirmatory of the possibility that spores produced in Ellner's medium are not satisfactory for maximal heat-resistance studies. The value for heat resistance of spores produced in Ellner's medium by Hall et al. (1963) was not given. The effect of medium on the heat resistance of spores is currently being studied in some detail in this laboratory.

Under the conditions of our experiments, strain F 1546/52 did not appear to be as heat-resistant as was reported by Collee et al. (1961). The English workers showed, however, that, when measuring thermal resistance, variation in procedure markedly affects the results obtained with this strain. It is highly probable that differences in methodology explain the differences between the results of Collee et al. and those obtained here.

TABLE 3. *Comparison of effect of temperature on four strains of Clostridium perfringens**

Holding temp and time	Inoculum†	Per cent of original population which survived temperature treatment			
		Strain 108	Strain 142 A	Strain 65	Strain F 1546/52
−17.7 C	Vegetative Cells	0.8	0.0	0.0	0.9
48 hr	Spores	46.6	43.1	56.8	29.7
80.0 C	Vegetative Cells	0.0	0.0	0.0	0.0
10 min	Spores	24.3	13.2	25.2	0.6

* Values derived from dual platings of three replications. Original population was approximately 10^3 cells per ml. Suspending diluent was 0.1% peptone water.

† Vegetative cells produced in thioglycolate medium with sugar; spores produced in Ellner's (1956) medium.

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