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Effects of Animal Alimentary Passage on the Heat Resistance of *Clostridium perfringens*[1]

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ABSTRACT

CANADA, JAMES C. (University of Wisconsin, Madison), AND DOROTHY H. STRONG. Effect of animal alimentary passage on the heat resistance of *Clostridium perfringens*. Appl. Microbiol. **13**:788–792. 1965.—The resistance to heat, as measured by *D* values and phantom thermal death time curves, was observed to increase for one of three strains of *Clostridium perfringens* type A subsequent to animal passage. Animal passage was accomplished by the force-feeding of germ-free mice with bacterial suspensions of the organism, followed by the force-feeding of additional gnotobiotic mice with the contaminated feces. For the one strain in which an increase in heat resistance was noted, the result could not be attributed to mouse feces per se, since the presence of sterile germ-free mouse feces in a suspending medium did not protect *C. perfringens* spores from elevated temperature destruction.

Results from separate research studies have led to two apparently contradictory conclusions concerning the basis for different degrees of heat resistance which have been observed among strains of *Clostridium perfringens*. (i) Food-poisoning strains of *C. perfringens* are more heat-resistant soon after their isolation from mammalian feces than after an extended maintenance on laboratory media (Hobbs et al., 1953), and (ii) the resistance to the lethal effects of heat is genetically controlled for each strain of the organism and is not an adaptive mechanism (Collee, Knowlden, and Hobbs, 1961). Because of the uncertainty suggested by these reports, it appeared further investigation was justified. Experiments were designed to determine whether the heat resistance of laboratory strains of *C. perfringens* type A would be increased after the strains were associated with feces through animal passage.

MATERIALS AND METHODS

Three strains of *C. perfringens* type A were used in the study: S-45 (Hall et al., 1963), 214D (Strong and Canada, 1964), and 65 (Canada, Strong, and Scott, 1964; Strong and Canada, 1964). These strains of the organism had been carried in veal broth for several months.

The research plan was as follows. A standardized bacterial suspension (I) was prepared with a stock culture inoculum. The resistance that the spores of this suspension displayed toward elevated temperature stress was measured. Bacterial suspension I was also used to contaminate orally a germ-free mouse (Ha/ICR, Schmidt). After an incubation period of 48 hr, the feces from this mouse were used to contaminate orally another germ-free mouse. After a total of six such alimentary passages through mice, the organism was recovered from feces in Thioglycollate Medium (Difco). Another standardized bacterial suspension (II) was prepared with the feces-inoculated Thiogylcollate culture as inoculum. The resistance of the spores of bacterial suspension II to elevated temperature stress was measured, and the resistance values were compared with those obtained from suspension I.

Bacterial suspension I was prepared by inoculating a tube containing 20 ml of Fluid Thioglycollate Medium (Difco) with 1 ml of stock culture grown in veal broth. Bacterial suspension II differed only in being seeded with a single dropping from a *C. perfringens*-exposed mouse after six transfers. After 24 hr of incubation at 37 C, 1 ml of the Fluid Thiogylcollate Medium culture was transferred to 10 ml of tubed Thioglycollate Medium without sugar. After incubating for 4 hr at 37 C, 1 ml of this second culture was transferred to each of two tubes containing 10 ml of SEC sporulating broth (Hall et al., 1963). The SEC broth cultures were allowed to incubate for 40 hr at 37 C. They were then combined and diluted in cold SEC broth to an optical density (OD) of 0.06

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TABLE 1. *Scheme for the identification and treatment of thermal death time tubes*

Identification no.	Treatment
1	Entered into germ-free mouse isolator
2	Entered into germ-free mouse isolator
3	Not pasteurized (total count)
4	Not pasteurized (total count)
5	Not pasteurized (total count)
6	Pasteurized (spore count)
7	Pasteurized (spore count)
8	Pasteurized (spore count)
9	Heated at 80 C for 20 min
10	Heated at 80 C for 25 min
11	Heated at 80 C for 30 min
12	Heated at 85 C for 7 min
13	Heated at 85 C for 15 min
14	Heated at 85 C for 25 min
15	Heated at 90 C for 2 min
16	Heated at 90 C for 5 min
17	Heated at 90 C for 10 min
18	Heated at 95 C for 1 min
19	Heated at 95 C for 5 min
20	Heated at 95 C for 10 min

(660 mμ), as measured by a Spectronic-20 colorimeter (Bausch & Lomb, Inc.). This OD value approximated 10,000,000 viable cells per milliliter. The diluted bacterial suspension was then chilled in an ice-water bath. A 1-ml amount of the chilled suspension was injected into each of 20 thermal death time (TDT) tubes (Canada et al., 1964). The tubes were sealed with flame, numbered, and immersed in ice water. A scheme, identifying each TDT tube and its treatment, is presented in Table 1.

TDT tubes numbered 1 and 2 were transported to the supplier of the germ-free mice. The exterior of the sealed tubes was sterilized with peracetic acid, and then the tubes were aseptically entered into a film isolator containing seven 6-week-old, female, germ-free mice. Other sterile material provided in the isolator included forceps, pliers, 1-ml syringes, needles, cloth toweling, screw-capped tubes of Fluid Thioglycollate Medium, a vial containing 70% ethyl alcohol, mouse cages, drinking water, feed (Purina 5010 Diet; Ralston Purina Co.), and bedding.

The first of the seven mice was orally inoculated with the bacterial suspension. The process was accomplished by snipping off one end of a TDT tube with pliers. A sample (0.5 ml) of the suspension was withdrawn with a needle and syringe, and a portion of this sample was then force-fed to the mouse.

After 48 hr, a freshly collected fecal dropping from the first mouse was force-fed to the second mouse. Additional mice, through mouse numbered six, were similarly force-fed at 2-day intervals. Mouse number seven was used in the passage studies only if one of the other mice died before its feces had been collected.

The total viable count of the bacterial suspension used for the initial contamination of the mice was obtained by aseptically breaking each of the three TDT tubes (3, 4, and 5; Table 1) into a 0.1% peptone-water dilution blank. The contents of the TDT tubes were then further diluted and plated in duplicate in SPS agar (Angelotti et al., 1962). The antimicrobial agents, polymyxin B and sulfadiazine, were omitted from the plating medium. The plates were incubated at 37 C in an atmosphere of 90% N and 10% CO_2. Resulting colonies were observed and recorded after 24 and 48 hr of incubation.

The spore count of the bacterial suspension was obtained by pasteurizing the contents of three TDT tubes (6, 7, and 8; Table 1) for 20 min in propylene glycol maintained at 75 C. After the heating period, the tubes were immediately cooled in ice water. The contents of the tubes were diluted and plated in manner similar to the total count procedure.

Twelve TDT tubes (9 to 20; Table 1) were heated at elevated temperatures for various times. The heating medium was propylene glycol in a thermostatically controlled oil bath. Prior to and immediately after the heating period, the tubes were held in an ice-water bath. Populations were determined by plate count after the heated bacterial suspensions were diluted in 0.1% peptone-water.

The results of the heating experiments at 80, 85, 90, and 95 C were used to calculate D values, "phantom" TDT curves, and z values. These terms were defined by Townsend et al. (1956) as follows: D value = time in minutes at a known constant temperature to destroy 90% of the organisms present; "phantom" TDT curve = plot on semilog paper of D values on the logarithmic scale versus temperature on the linear scale; and z value = slope of the "phantom" TDT curve through one log cycle. D values were calculated from the equation:

$$D = \frac{U}{\log a - \log b}$$

where D = death rate in minutes; U = adjusted heating time in minutes (the nonlethal log time as calculated from heat penetration curves was subtracted from the actual holding time); a = initial spore population, and b = surviving population at heating time U. The "phantom" thermal death time curves were plotted by linear regression. The before-animal-passage D values, "phantom" TDT curves, and z values were compared with those obtained after passage. The procedures heretofore described were followed twice for each of the three strains of *C. perfringens*.

The composition of the medium in which bacteria are heated affects the survival response. Factors may be present in mouse feces that would enable *C. perfringens* to better withstand the lethal effects of elevated temperature exposure. SEC broth cultures of the three strains were

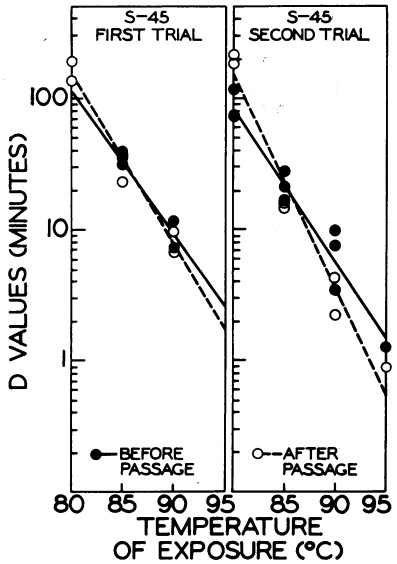

FIG. 1. *Effect of animal passage on the "phantom" thermal death time curve of Clostridium perfringens strain S-45.*

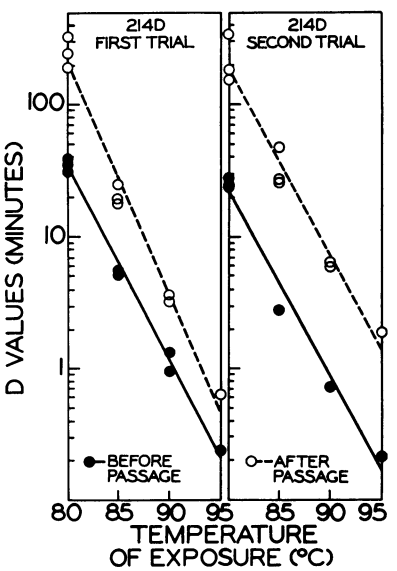

FIG. 2. *Effect of animal passage on the "phantom" thermal death time curve of Clostridium perfringens strain 214D.*

diluted in chilled SEC broth so that the total counts were ca. 10^7 per milliliter. Pooled, sterile feces from germ-free mice were then added in a ratio of 1 g of feces to 100 ml of SEC broth to a portion of each bacterial suspension. Total counts, spore counts, and temperature heat-resistance studies were carried out on suspensions containing

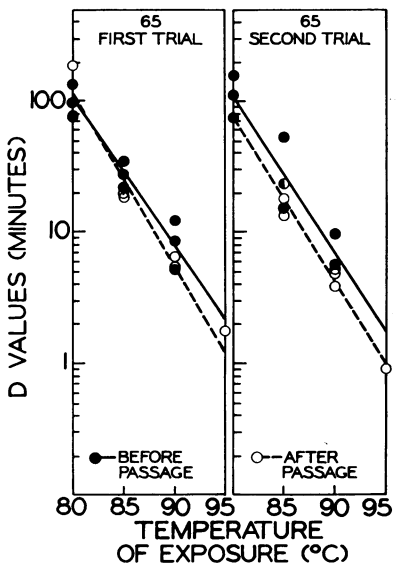

FIG. 3. *Effect of animal passage on the "phantom" thermal death time curve of Clostridium perfringens strain 65.*

and not containing mouse feces in a manner identical to the animal passage experiments.

RESULTS

The "phantom" TDT curves obtained for the three strains of *C. perfringens* are reproduced in Fig. 1, 2, and 3. These curves were plotted by linear regression from calculated *D* values. The before- and after-animal-passage curves show little difference from each other for strains S-45 and 65. A striking difference is noted, however, when the before-passage curves for strain 214D are compared with their corresponding after-passage curves. The after-passage *D* values are greater than those before passage, thus elevating the after-passage "phantom" thermal death curves. For each of the three strains, the second trial curves are in good agreement with those from the first trial.

D values at 85 and 90 C are presented in Table 2. These values were obtained from the "phantom" thermal death time curves. Animal alimentary passage did not appear to greatly influence the heat resistance of either strain S-45 or 65. Most (90%) of the S-45 strain spores were reduced in 28 min when heated at 85 C and in 6 to 7 min when heated at 90 C. Similar results were obtained with strain 65. A significant difference was noted when the before- and after-passage *D* values were compared for strain 214D. The after-passage *D* values at both 85 and 90 C

TABLE 2. *D_{85} and D_{90}* values for the spores of three strains of Clostridium perfringens, both previous and subsequent to animal passage*†

Strain	D_{85} value		D_{90} value	
	Before passage	After passage	Before passage	After passage
	min	*min*	*min*	*min*
S-45	27.0	29.0	7.4	5.6
214D	5.3	31.5	1.0	5.2
65	27.5	21.0	7.2	4.7

* Minutes of exposure at either 85 or 90 C required to effect a 90% reduction in numbers.

† In each case, values are averages, derived from two "phantom" thermal death time curves.

TABLE 3. *z_D* values for the spores of three strains of Clostridium perfringens, both previous and subsequent to animal passage*†

Strain	z_D value	
	Before passage	After passage
	F	*F*
S-45	15.9	12.5
214D	12.5	11.3
65	15.5	14.0

* Slope of the "phantom" thermal death time curve (F).

† In each case, values are averages, derived from two "phantom" thermal death time curves.

were from five to six times greater than their corresponding before-passage *D* values.

The data also served as a basis for calculating before- and after-animal-passage *z* values for each of the three strains of *C. perfringens*. These values are presented in Table 3. In all cases, the after-passage values were somewhat lower than the before-passage values. For strain S-45, in which the demonstrated difference was greatest, the before- and after-passage *z* values varied by 3.4 F.

D values for bacterial suspensions with and without added sterile mouse feces were calculated from adjusted heating times at 80, 85, 90, and 95 C. "Phantom" TDT curves were plotted by linear regression from the *D* values and are presented in Fig. 4. Germ-free mouse feces neither increased nor decreased the elevated temperature stress resistance for any of the strains of *C. perfringens*. When compared, good agreement for both suspensions of each strain existed for the *D* value points and "phantom" TDT curves. The *z* values for both types of suspension appear

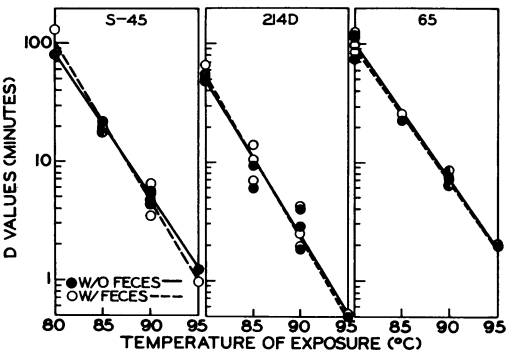

FIG. 4. *Effect of added feces from germ-free mice on the "phantom" thermal death time curves of three strains of Clostridium perfringens.*

to be almost identical, since the "phantom" TDT curves are nearly superimposed.

DISCUSSION

In this study spores have been differentiated from vegetative cells only by the process of pasteurization. The time and temperature (20 min at 75 C) was that recommended by Gibbs and Hirsch (1956) in their study on spore formation by *Clostridium* species. Those cells which were viable after heating for 20 min at 75 C were considered spores, and others not surviving such treatment were considered vegetative cells. This assumption, while useful for experimentation and data analysis, is arbitrary and has, no doubt, been the source of some experimental error.

Spores were produced in SEC broth; however, the production was minimal. Average spore percentage of the total count for each strain was: S-45, 0.34; 214D, 0.52; and 65, 0.22. While spore stain smears from each SEC bacterial suspension were prepared, and spores were always observed, it was not possible to quantitate accurately the spores by microscopic examination, owing to their small numbers within the microscopic field. Hall et al. (1963) encountered this same difficulty.

Animal passage did not appear to influence either the "phantom" TDT curves or the *D* values of strains S-45 and 65 in the temperature range examined. Several explanations are offered for this lack of demonstrable increase in heat resistance. (i) Strain variation may exist. The heat resistance property of some strains may be adaptive, whereas for others it may be genetically controlled (Collee et al., 1961) and not subject to adaptation. (ii) The length of time that the strains were associated with feces was not sufficient for the adaptation to take place. (iii) The heat resistance was increased but subsequently

decreased during the preparation of the SEC bacterial suspensions. Hobbs et al. (1953) reported that the heat resistance of some strains is lost even after the first subculture from feces to laboratory media.

The heat resistance of strain 214D was appreciably increased for a specified temperature range after it had been passed through mice. The results obtained with strains 214D support the observations by Hobbs et al. (1953): strains of *C. perfringens* newly isolated from mammalian feces were more heat-resistant than were the same strains when carried on laboratory media.

The *z* values obtained from the after-passage "phantom" TDT curves were in each case lower than their corresponding before-passage *z* values. The difference, however, was small and probably falls within statistical limits of experimental error. Vinton, Martin, and Gross (1947) demonstrated a reduced heat resistance in the spores of P.A. 3679 after growing the cells in raw meat as compared with growing the cells in sterilized meat. Although the growth substrate influenced the heat resistance, it was found to have no effect on the slope of the TDT curves.

Paired bacterial suspensions with and without added sterile mouse feces were subjected to heat-resistance experiments similar to those used in the animal passage studies. Feces from germ-free mice in a 1% concentration did not influence the elevated temperature stress resistance of *C. perfringens*. *D* values, for both types of suspensions, were in good agreement, as were the "phantom" TDT curves. Under certain conditions, changes in the heating substrate will alter heat resistance of bacterial spores. The *D* values would possibly have been changed if either a higher ratio of mouse feces to SEC bacterial suspension had been used, or feces from a source other than germ-free mice were used.

ACKNOWLEDGMENT

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