APPLIED MICROBIOLOGY, Sept. 1973, p. 282-287

Vol. 26, No. 3
Printed in U.S.A.

Inactivation of *Clostridium perfringens* Type A Spores at Ultrahigh Temperatures[1]

D. M. ADAMS

Department of Food Science, North Carolina State University, Raleigh, North Carolina 27607

Received for publication 24 April 1973

The inactivation of *Clostridium perfringens* type A spores (three strains of different heat resistances) at ultrahigh temperatures was studied. Aqueous spore suspensions were heated at 85 to 135 C by the capillary tube method. When survivors were enumerated on the standard plating medium, the spores appeared to have been rapidly inactivated at temperatures above 100 C. The addition of lysozyme to the plating medium did not affect the recovery of spores surviving the early stages of heating, but lysozyme was required for maximal recovery of spores surviving extended heat treatments. The percentage of survivors requiring lysozyme for colony formation increased greatly with longer exposure times or increasing treatment temperature. Time-survivor curves indicated that each spore suspension was heterogeneous with respect to the heat resistance of spore outgrowth system or in the sensitivity of the spores to lysozyme. Recovery of survivors on the lysozyme containing medium revealed greater heat resistance for one strain than has been reported for spores of many mesophilic aerobes and anaerobes. The spores of all three strains were more resistant to heat inactivation when suspended in phosphate buffer, but a greater percentage of the survivors required lysozyme for colony formation.

The presence of *Clostridium perfringens* spores in food is a potential health hazard because cooking can heat activate the spores and mishandling of the food can result in germination, outgrowth, and vegetative cell growth. The incidence of *C. perfringens* food poisoning in the United States has been high in recent years (3, 4), yet little attention has been given to the inactivation of *C. perfringens* spores. This may result from a belief that *C. perfringens* spores are not particularly heat resistant. However, Roberts (14) observed considerable resistance of spores of certain food poisoning strains to heat treatments at 80 to 100 C. More recent studies (8, 9) indicated that *C. perfringens* spores are injured by heat treatments in this temperature range. The enumeration methods used by Roberts may not have detected injured spores; therefore, *C. perfringens* spores may be more heat resistant than previously recognized.

Ultrahigh temperature (UHT) processing offers higher product quality and longer shelf life and may be more widely used in the future, but inactivation of spores at UHT can present unique problems. The increase in the rate of inactivation for a given increase in treatment temperature can be smaller (6, 11), and the rate and extent of spore injury can be greater in the UHT range (11) than at lower temperatures. Therefore, the actual inactivation characteristics of *C. perfringens* spores at UHT may be very different than those expected on the basis of heat treatments at lower temperatures.

The objective of this study was to evaluate the thermal inactivation characteristics of *C. perfringens* spores at UHT. Preliminary results have been presented previously (D. M. Adams and M. L. Speck, Abstr. Annu. Meet. Amer. Soc. Microbiol., Philadelphia, p. 4, 1972).

MATERIALS AND METHODS

Test organisms. The test organisms included *C. perfringens* type A strains NCTC 8798 (Hobbs 9), NCTC 10240 (Hobbs 13), and ATCC 3624, producing heat-resistant, intermediate, and heat-sensitive spores, respectively (C. L. Duncan, personal communication). The cultures were obtained from C. L. Duncan (University of Wisconsin, Madison) and were grown in cooked meat medium (BBL) at 35 C for 24 h and stored at room temperature.

Media. The sporulation medium was that of Duncan and Strong (10), but peptone (Difco) was substituted for proteose-peptone. This modification greatly improved the extent of sporulation of strain

[1] Paper no. 4036 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, North Carolina.

3624 and did not affect heat resistance of the spores of any of the strains used. The standard enumeration medium (TYCS) contained (per liter) the following: Trypticase, 15 g; yeast extract, 10 g; ferric citrate, 0.5 g; sodium sulfite, 0.05 g; and agar, 15 g. Lysozyme (18,000 U/mg; Schwarz-Mann, Orangeburg, N.Y.) was added to a final concentration of 1 μg/ml to make TYCS plus lysozyme. Sodium sulfite and lysozyme were added to the autoclaved medium just prior to pouring into petri dishes.

Preparation of spore suspensions. Spores were obtained by the method of Duncan and Strong (10). Spores were separated from vegetative cells by repeated centrifugation and washing with cold, sterile, distilled water, and were stored in sterile distilled water at 2 C.

Heat treatments. Portions of the stock spore suspension (10^7–10^9 spores per ml) were heat activated at 75 C for 20 min, cooled, and UHT treated by the capillary tube method (7). When the heating menstruum was 60 mM sodium phosphate buffer, the spores were heat activated in water, centrifuged, suspended in buffer, and UHT treated. Equilibration of the spore suspensions to treatment temperature required 8.7 s. Treatment times were corrected (12) for inactivation during temperature equilibration, using the z_D values determined in this study, and were expressed as equivalent times at treatment temperature.

Enumeration of survivors. Duplicate capillary tubes containing UHT-treated spores were washed, rinsed in sterile distilled water, and crushed in 99 ml of 0.1% peptone (Difco) water containing 0.02% (wt/vol) Antifoam B (Sigma Chemical Co., St. Louis, Mo.). The spores were diluted in peptone water, and portions of the diluted suspension were pipetted into petri dishes and mixed with TYCS or TYCS plus lysozyme. The solidified agar was overlaid with the same medium and incubated in a 90% N_2-10% CO_2 atmosphere in Anaero-Jars (Pfizer, Inc., New York) at 35 C for 48 h.

RESULTS

Inactivation kinetics. When survivors were enumerated on TYCS without lysozyme, the spores of all three strains (initially 10^7 to 10^9/ml) appeared to have been rapidly inactivated. At temperatures greater than 107 C for strain 8798 and 97 C for strains 10240 and 3624, the decimal reduction times (D values: times required for a 90% reduction in viable spores) were less than 1 min. The time-survivor curves for strain 8798 spores generally were linear and unbroken (Fig. 1). There was no evidence of tailing, even when the number of survivors was reduced to less than 0.0001%. Similar results were obtained for strains 10240 and 3624, although the inactivation rates were much greater.

The recovery of survivors and therefore the apparent inactivation kinetics were markedly different when heated spores were plated on TYCS plus lysozyme (Fig. 2). The presence of lysozyme in the medium did not affect the colony counts of non-UHT-treated spores or the recovery of spores surviving the early stages of heating. The initial reductions in survivors were at rates similar to those observed for recovery on TYCS. For treatment temperatures above 95 C, however, lysozyme improved the recovery of spores surviving extended UHT treatments, and the initial reductions in survivors were followed by decreased rates of inactivation. No lysozyme effect was observed for the lower temperatures even after extensive inactivation, e.g., strain 8798 spores heated at 95 C to less than 0.00001% survivors. Similar results were obtained for strains 10240 and 3624.

Regardless of the treatment temperature, if a lysozyme effect was observed, the portion of the time-survivor curve indicating a response to lysozyme (decreased rate of inactivation) extrapolated to an ordinate intercept representing 1 to 2% of the non-UHT-treated spore population. This was observed for strains 8798 (Fig. 2), 10240, and 3624.

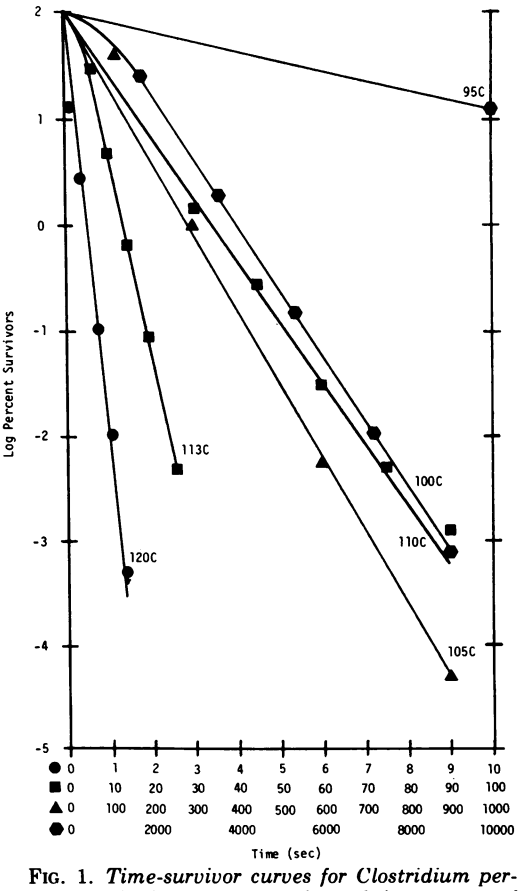

FIG. 1. *Time-survivor curves for Clostridium perfringens NCTC 8798 spores heated in water and plated on TYCS.*

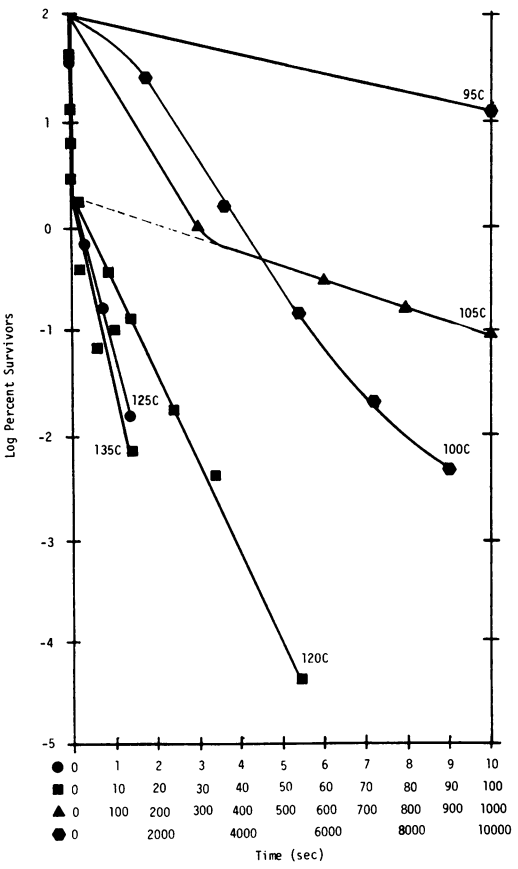

FIG. 2. *Time-survivor curves for Clostridium perfringens NCTC 8798 spores heated in water and plated on TYCS plus 1 μg of lysozyme per ml.*

The influence of treatment temperature on the apparent inactivation rates for spores plated on TYCS with or without lysozyme is illustrated by the decimal reduction time curves in Fig. 3. If a lysozyme effect was observed, the D value was calculated from the portion of the time-survivor curve indicating a response to lysozyme. The z_D values (changes in temperature producing a 10-fold change in the D value) were similar for the three strains but varied between plating media. Average z_D values were 9.7 C (9.1–10 C) or 5.8 C (5.2–6.2 C) for enumeration on TYCS with or without lysozyme, respectively. No lysozyme effect was observed for the lower treatment temperatures, but the D values were more consistent with the z_D of 9.7 C than with the z_D of 5.8 C.

Effect of heat activation on heat resistance. Less than 1% of the non-heat-activated strain 8798 spores formed colonies on TYCS (Fig. 4). The presence of lysozyme in TYCS increased the colony counts of unheated spores by approximately 1.4% of the total viable count. The unheated spores were activated during the early stages of UHT treatment. The apparent rates of inactivation were unaffected by the prior heat activation treatment.

Effect of phosphate in the heating menstruum. Strain 3624 spores suspended in water and heated at 90 C exhibited a much larger D value than had been reported for these spores heated in phosphate buffer (9, 10). When the strain 3624 spores used in this study were heated at 90 or 100 C in 60 mM phosphate buffer (pH 7), the D values for enumeration on

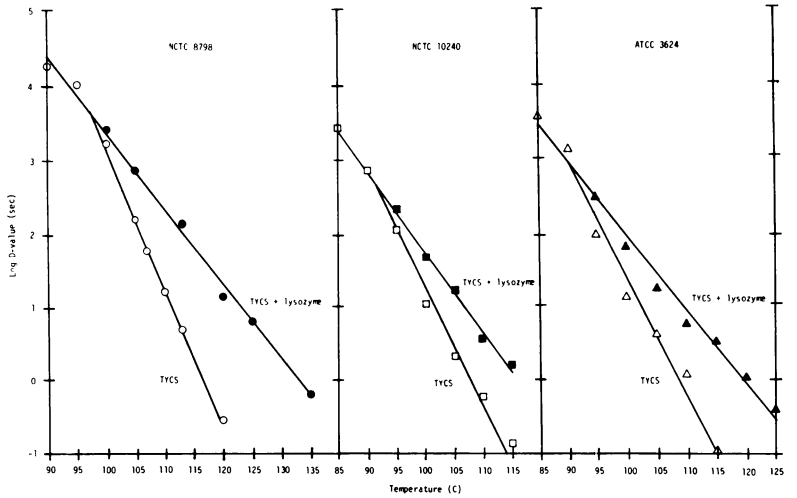

FIG. 3. *Decimal reduction time curves for Clostridium perfringens spores heated in water. Survivors were plated on TYCS, open symbols, or TYCS plus 1 μg of lysozyme per ml, closed symbols. Curves were fitted by the method of least squares.*

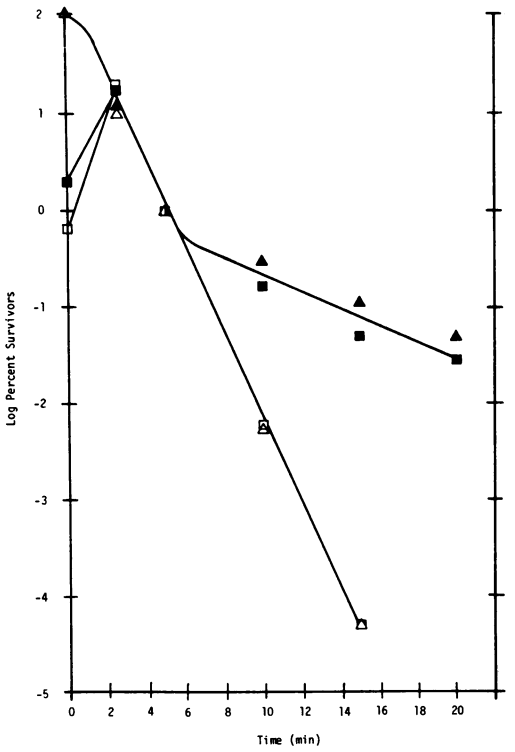

FIG. 4. *The influence of heat activation treatment on the heat resistance of Clostridium perfringens NCTC 8798 spores. No heat activation, squares; heat activated before UHT treatment, triangles. Heated (105 C) spores were plated on TYCS, open symbols, and TYCS plus 1 μg of lysozyme per ml, closed symbols.*

TYCS were much smaller (Table 1). The D values for the other strains were also lower. The D values for spores responding to lysozyme, however, were more than doubled.

DISCUSSION

C. perfringens spores are not generally considered to be particularly heat resistant and would not be expected to be important in UHT-treated foods. However, Roberts (14) observed that spores of several food poisoning strains exhibited considerable resistance to heat treatments at 80 to 100 C. The results obtained in this study for *C. perfringens* spores heated at 90 to 135 C are similar to those reported by Roberts and also indicate a high degree of heat resistance (Fig. 5). The high heat resistance does not appear to involve heat adaptation (1) during heat activation or UHT treatment. Also shown in Fig. 5 are inactivation data reported by others for various types of spores. Strain 8798 spores are more heat resistant than spores of five mesophilic *Bacillus* spp., including the highly heat resistant *B. subtilis* A. The resistance of strain 8798 spores also appears to be greater than that of the other food poisoning sporeformers, *B. cereus* and *Clostridium botulinum* types A and B (Fig. 5, reference 17), and similar to or greater than the heat resistance of putrefactive anaerobe 3679 NCA strain which is commonly used for thermal process evaluation.

TABLE 1. *Influence of phosphate on the inactivation of Clostridium perfringens spores*

Strain	Temperature (C)	Water		Phosphate[a]	
		D_{-L}[b] (s)	D_{+L} (s)	D_{-L} (s)	D_{+L} (s)
8798	105	150	750	110	1,800
8798	120	0.3	13	0.3	31
10240	90	930	ND[c]	460	1,300
10240	100	12	50	2.9	100
3624	90	1,650	ND	40	2,600
3624	100	13	70	0.5	180

[a] 60 mM sodium phosphate buffer, pH 7.

[b] Survivors enumerated on TYCS without (−L) or with (+L) lysozyme. When the influence of lysozyme was detected, the D value was calculated from the portion of the time-survivor curve indicating a response to lysozyme.

[c] ND, no influence of lysozyme detected.

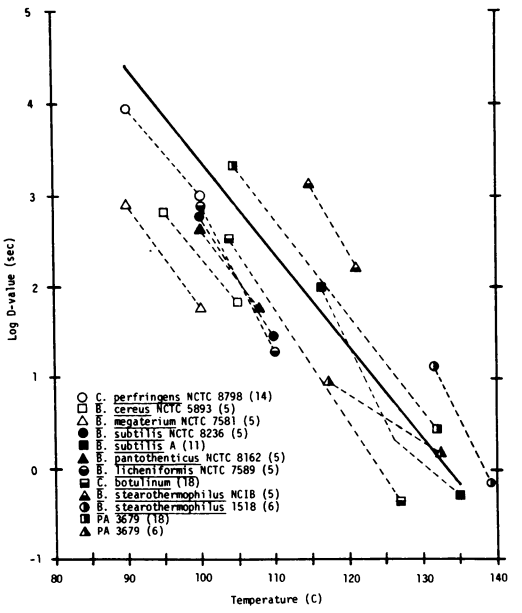

FIG. 5. *Decimal reduction time curves for spores of Clostridium perfringens NCTC 8798 and other organisms. Strain 8798 data obtained in this study (solid line) taken from Fig. 3 (TYCS + lysozyme). All other data taken from references cited in legend. Abbreviation: PA, putrefactive anaerobe.*

The greater heat resistance in phosphate buffer also observed for other spores suggests that *C. perfringens* spores suspended in certain foods may be even more heat resistant than indicated in Fig. 5.

Maximal recovery of heated *C. perfringens* spores required lysozyme in the plating medium. Similar findings have been reported for these and other strains of *C. perfringens* spores heated at lower temperature (8, 9), but the extent of injury is much greater after heating in the UHT range (Fig. 3). Duncan et al. (9) attributed the higher recovery to lysozyme mediated germination of spores sublethally damaged (injured) during heating. Development of thermal processes that will lower the total number of viable *C. perfringens* spores in food to an acceptable level and monitoring the effectiveness of such heat treatments requires the incorporation of lysozyme into the recovery medium to allow the detection of injured as well as uninjured spores. This is especially important for evaluation of UHT processes. None of the media recommended (2) for the specific enumeration of *C. perfringens* in foods or for the examination of canned foods contains lysozyme. Two media (13, 16) contain egg yolk emulsion which may provide sufficient lysozyme (8).

Enumeration of survivors on TYCS plus lysozyme yielded biphasic-concave time-survivor curves. Similar inactivation kinetics have not been reported for *C. perfringens* spores heated at lower temperatures and plated on lysozyme containing media (8, 9). However, the survival data reported by Duncan et al. (9) for strains 3624 and 8798 heated for one time and temperature agree with the inactivation kinetics and parameters presented here.

Ordinate intercepts for the second phase of the biphasic time-survivor curves were similar for all treatment temperatures. This is characteristic of mixed spore suspensions, the two types of spores typically differing in heat resistance (15). These kinetics were observed only when lysozyme was added to the plating medium to germinate injured spores (9), suggesting that 1 to 2% of the spores possess uniquely heat resistant outgrowth systems. Alternatively, the spores may be equally heat resistant but differ in their sensitivity to lysozyme, and the time-survivor curves describe the rapid inactivation of the heat sensitive germination system and the slower inactivation of the heat resistant outgrowth system common to all the spores but expressed only by the spores germinated by lysozyme. This would be consistent with (i) the D and z_D values obtained for spores heated at the lower treatment temperatures and enumerated with or without lysozyme (Fig. 3) and, (ii) the increase in colony counts of non-heat-activated spores when lysozyme was added to TYCS (Fig. 4). This requires further clarification. If some surviving spores are not detected because they are insensitive to lysozyme, survival data obtained using lysozyme containing media could still be in error by 50-to 100-fold.

The data presented here indicate that some *C. perfringens* strains produce spores of considerable heat resistance. These spores could be very important in establishing thermal process requirements. Accurate evaluation of thermal processes, especially UHT processes, requires that current recovery methods by modified by supplementation of the recovery medium with lysozyme and possibly sensitization of surviving spores to lysozyme.

ACKNOWLEDGMENTS

This research was supported by grant FD 00051 from the Food and Drug Administration.

I gratefully acknowledge the technical assistance of Mabel Cardellino.

LITERATURE CITED

1. Alderton, G., P. A. Thompson, and N. Snell. 1964. Heat adaptation and ion exchange in *Bacillus megaterium* spores. Science **143**:141-143.
2. Anonymous. 1969. Bacteriological analytical manual, 2nd ed. Food and Drug Administration, U. S. Dept. of Health, Education, and Welfare, Washington, D.C.
3. Anonymous, 1971. Foodborne outbreaks. Center for Disease Control, U. S. Dept. of Health, Education and Welfare, Atlanta.
4. Anonymous, 1972. Foodborne outbreaks. Center for Disease Control, U. S. Dept. of Health, Education and Welfare, Atlanta.
5. Briggs, A. 1966. The resistance of spores of the genus *Bacillus* to phenol, heat, and radiation. J. Appl. Bacteriol. **29**:490-504.
6. Busta, F. F. 1967. Thermal inactivation characteristics of bacterial spores at ultrahigh temperatures. Appl. Microbiol. **15**:640-645.
7. Busta, F. F., and D. M. Adams. 1972. Identification of a germination system involved in the heat injury of *Bacillus subtilis* spores. Appl. Microbiol. **24**:412-417.
8. Cassier, M., and M. Sebald. 1969. Germination lysozyme-dépendante des spores de *Clostridium perfringens* ATCC 3624 apres traitement thermique. Ann. Inst. Pasteur (Paris) **117**:312-324.
9. Duncan, C. L., R. G. Labbe, and R. R. Reich. 1972. Germination of heat-and alkali-altered spores of *Clostridium perfringens* type A by lysozyme and an initiation protein. J. Bacteriol. **109**:550-559.
10. Duncan, C. L., and D. H. Strong. 1968. Improved medium for sporulation of *Clostridium perfringens*. Appl. Microbiol. **16**:82-89.
11. Edwards, J. L., F. F. Busta, and M. L. Speck. 1965. Thermal inactivation characteristics of *Bacillus subtilis* spores at ultrahigh temperatures. Appl. Microbiol. **13**:851-857.
12. Halvorson, H. Orin. 1958. The physiology of the bacterial spore. The Technical University of Norway, Trondheim.

13. Harmon, S. M., D. A. Kautter, and J. T. Peeler. 1971. Improved medium for enumeration of *Clostridium perfringens*. Appl. Microbiol. **22**:688-692.
14. Roberts, T. A. 1968. Heat and radiation resistance and activation of spores of *Clostridium welchii*. J. Appl. Bacteriol. **31**:133-144.
15. Roberts, T. A., and A. D. Hitchens. 1969. Resistance of spores, p. 615-616. *In* G. W. Gould and A. Hurst (ed), The bacterial spore. Academic Press Inc., London.
16. Shahidi, S. A., and A. R. Ferguson. 1971. New quantitative, qualitative, and confirmatory media for rapid analysis of food for *Clostridium perfringens*. Appl. Microbiol. **21**:500-506.
17. Stumbo, C. R. 1965. Thermobacteriology in food processing. Academic Press Inc., New York.
18. Stumbo, C. R., J. R. Murphy, and J. Cochran. 1950. Nature of thermal death time curves for P. A. 3679 and *Clostridium botulinum*. Food Technol. **4**:321-326.