

# HEAT RESISTANCE OF *CLOSTRIDIUM PERFRINGENS* VEGETATIVE CELLS AS AFFECTED BY PRIOR HEAT SHOCK<sup>1</sup>

V.K. JUNEJA<sup>2</sup> and J.S. NOVAK

*U.S. Department of Agriculture  
Agricultural Research Service  
Eastern Regional Research Center  
600 East Mermaid Lane  
Wyndmoor, Pennsylvania 19038*

B.S. EBLEN

*Food and Drug Administration  
Center for Food Safety and Applied Nutrition  
200 C. Street SW  
Washington, D.C. 20250*

AND

B.A. MCCLANE

*Department of Molecular Genetics and Biochemistry  
University of Pittsburgh School of Medicine  
Pittsburgh, PA 15261*

Received for Publication December 21, 2000

Accepted for Publication February 5, 2001

## ABSTRACT

*This study quantified the heat resistance and the effects of a heat shock on the subsequent heat resistance of 10 strains of Clostridium perfringens. Beef gravy samples inoculated with vegetative cells of the pathogen were subjected to sublethal heating at 48C for 10 min, and then heated to a final temperature of 58C using a submerged-coil heating apparatus. Heat-treated samples were spiral plated on Shahidi-Ferguson Perfringens agar to determine surviving bacterial*

<sup>1</sup>Mention of brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

<sup>2</sup>Corresponding author: TEL: (215)233-6500; FAX: (215)233-6551; E-mail: vjuneja@arserrc.gov.

*population. No correlation between the heat resistance and the origin of the C. perfringens could be established due to significant variations in the heat resistance among strains. Inactivation kinetics of both heat-shocked and nonheat-shocked samples exhibited log-linear decline in the number of surviving cells with time. D-values at 58C for C. perfringens vegetative cells ranged from 1.21 to 1.60 min. Heat shocking allowed the organism to survive longer and the increase in heat resistance was as high as 1.5 fold. Also, heat shock resulted in the overexpression of proteins exhibiting epitopic and size similarity to E. coli GroEL and B. subtilis small acid soluble proteins. Increased heat resistance due to heat shock must be considered while designing cooking/reheating regimes that ensure safety of ready-to-eat foods contaminated with high numbers of C. perfringens vegetative cells.*

## INTRODUCTION

*Clostridium perfringens* is a continuing concern to the foodservice industry. The ability of this pathogen to form heat-resistant spores and grow at a very rapid rate at relatively high temperatures are major contributing factors leading to food poisoning. The temperature range for growth of *C. perfringens*, 6 to 52.3C, is well documented (Hall and Angelotti 1965; Johnson 1990; Shoemaker and Pierson 1976; Juneja *et al.* 1999). Optimum growth occurs typically between 43-46C at which a generation time of 7.1 min in autoclaved ground beef has been reported (Willardsen *et al.* 1978; Labbe and Juneja 2001). The short generation time of the organism in the rapid growth temperature range implies that > 1 million-fold increase in population densities can be fostered within 2 h. The potential for *C. perfringens* growth and adverse food safety conditions are likely to occur in institutional settings or large gatherings where food is prepared in large quantities a day or more in advance of the meal and the food is not adequately refrigerated or is temperature abused for extended periods. Bean and Griffin (1990) reported that food poisoning with *C. perfringens* is usually associated with temperature abuse during post-process handling and storage. Moreover, the potential for *C. perfringens* growth is increased in view of the reports revealing that holding temperatures of retail cabinets and consumer refrigeration units are commonly > 10C (Anon. 1989; Bryan 1988; Daniels 1991; Wyatt and Guy 1980; Van Grade and Woodburn 1987).

Unsafe levels of *C. perfringens* vegetative cells, resulting from the germination and outgrowth of spores, in precooked, ready-to-eat foods must be killed by sufficient reheating before consumption to guard against food poisoning. Conversely, inadequate reheating of such products before consumption would lead to food poisoning. In a study by Juneja and Marmer (1998), when *C. perfringens* cells inoculated in beef were heated at 65C for 1 min, complete inactivation of 8 log<sub>10</sub> of cells was reported. While the study by Juneja and Marmer (1998)

quantified the time and temperature for an adequate degree of protection against the hazards associated with *C. perfringens* in beef, there may be conditions when the vegetative cells become more heat resistant. Such conditions include sublethal environmental stresses occurring prior to cooking such as heat-shocking conditions, including cyclic and static temperature abuse of the cooked products. As a result of such heat-shock conditions, a physiological response is triggered in organisms leading to the synthesis of a specific set of proteins known as heat-shock proteins (HSPs). Synthesis of HSPs have been observed in bacterial as well as mammalian cells (Lindquist and Craig 1988). In the work reported here, the objectives were to determine heat-shock effects on the subsequent thermotolerance of *C. perfringens* vegetative cells, and to detect the increased expression of molecular chaperones, such as *Escherichia coli* GroEL or DNA-protecting small acid soluble proteins (SASPs), such as *Bacillus subtilis* SspC. The heat-shock response and the induced thermotolerance defined in this study could be used to establish re-heating protocols that would minimize the potential danger of *C. perfringens* food poisoning.

## MATERIALS AND METHODS

### Bacteria

Ten strains of *Clostridium perfringens* originating from foodborne illness, veterinary or human clinical cases, were used in the study (Table 1). The strains were kindly provided by Dr. Bruce McClane (University of Pittsburgh School of Medicine, Pittsburgh, PA). Stock cultures were maintained at 4C in cooked-meat medium (Difco Laboratories, Detroit, MI). Rabbit antiserum raised against *B. subtilis* SASP, SspC, was provided as a generous gift from Dr. Peter Setlow at the University of Connecticut (Farmington, CT). Rabbit polyclonal antibodies raised against *E. coli* chaperonin, GroEL, were purchased from StressGen Biotech. Corp. (Victoria, B.C., Canada).

### Preparation of Inoculum

To prepare inocula, 0.1 mL of the stock cultures were individually transferred to 9.9 mL of freshly prepared fluid thioglycollate medium (FTG, Difco). The inoculated medium was then heat shocked at 75C for 20 min and incubated at 37C for 6 h. Approximately 1 mL of the 6-h culture was transferred to 9 mL of FTG and incubated for 18 h. The cells were harvested by centrifugation at room temperature for 10 min at  $7,700 \times g$ , the cell pellet washed twice and finally resuspended in sterile 0.1% peptone water (w/v) to a target levels of  $9 \log_{10}$  cfu/mL. The population densities in each inoculum were enumerated by spiral plating (Spiral Biotech, Bethesda, MD; Model D) appropriate dilutions (in 0.1% peptone

water), in duplicate, on to agar dishes containing Tryptose-sulfite-cycloserine (TSC) agar without cycloserine, i.e., Shahidi-Ferguson Perfringens (SFP) agar with no added egg yolk enrichment. The SFP agar plates were overlaid with an additional 10 mL of SFP agar. After overlaying, the agar was allowed to solidify before the dishes were placed in a Bactron anaerobic chamber (Sheldon Manufact. Inc., Cornelius, OR). Vegetative cells counts were determined after 48 h of incubation at 37C in the anaerobic chamber.

### **Beef Gravy Formulation**

The model beef gravy used in the present study consisted of 1.5% proteose peptone, 5.0% beef extract, 0.5% yeast extract and 1.7% soluble starch. All ingredients were obtained from Difco Laboratories (Detroit, MI). The gravy was sterilized by autoclaving prior to use.

### **Inoculation, Heat Shock and Thermal Inactivation**

Appropriate dilution of each bacterial suspension (0.1 mL) was added to 10-mL beef gravy in sterile test tubes to give a final concentration of approximately 5 - 6 log<sub>10</sub> cfu/mL. The test tubes containing beef gravy were vortexed to ensure even distribution of the organisms. The bacterial suspensions were heat-shocked by submerging the tubes in a 48C temperature controlled water bath for 10 min and then cooled. Thereafter, both heat-shocked and nonheat-shocked samples (controls) were heated at 58C using a submerged coil heating apparatus (Cole and Jones 1990). It comprises a stainless steel coil fully submerged in a thermostatically-controlled water bath which allows microbial suspensions to be heated between 20-90C with a short time to temperature equilibrium. During the heating procedure, samples (0.2 mL) were removed at predetermined time intervals. Where low cell numbers were expected, 0.6-mL aliquots were removed. Samples were cooled rapidly to room temperature in peptone (0.1% w/v) water. After removal, samples were analyzed within 30 min.

### **Enumeration of Survivors**

For determination of the number of surviving bacteria, decimal serial dilutions were prepared in 0.1% peptone water and appropriate dilutions were surface-plated, in duplicate, onto SFP agar as described above. Uninoculated samples were plated as controls. When increased sensitivity was required, 0.1 mL of undiluted suspension was surface-plated. For each replicate experiment performed, an average cfu/g of two platings of each sampling point was used to determine the D-values.

## HEAT RESISTANCE OF *CLOSTRIDIUM PERFRINGENS*

TABLE 1.  
THE EFFECT OF PRIOR EXPOSURE OF *CLOSTRIDIUM PERFRINGENS* VEGETATIVE  
CELLS IN MODEL BEEF GRAVY AT 48C FOR 10 MIN (HEAT SHOCK) ON HEAT

Isolate	Origin	D-value (r <sup>2</sup> ) <sup>B</sup>	
		No heat shock	Heat Shock
NCTC 8238	1950s European food poisoning	1.36±0.00 (0.94) <sup>abc,1</sup>	1.64±0.01 (0.96) <sup>b,1</sup>
NCTC 8239	1950s European food poisoning	1.31±0.00 (0.94) <sup>abc,1</sup>	1.95±0.08 (0.88) <sup>a,2</sup>
NCTC 10239	1950s European food poisoning	1.58±0.06 (0.99) <sup>ab,1</sup>	2.25±0.00 (0.98) <sup>a,2</sup>
153	1990s North American veterinary	1.22±0.06 (0.98) <sup>bc,1</sup>	1.42±0.01 (0.99) <sup>b,1</sup>
222	1990s North American veterinary	1.32±0.07 (0.92) <sup>abc,1</sup>	1.47±0.21 (0.99) <sup>b,1</sup>
FD 1041	1980s North American food poisoning	1.31±0.10 (0.94) <sup>abc,1</sup>	1.52±0.21 (0.91) <sup>b,1</sup>
C - 1841	1980s North American food poisoning	1.21±0.08 (0.99) <sup>bc,1</sup>	1.76±0.04 (0.99) <sup>b,2</sup>
F 4969	1980s European: clinical	1.60±0.01 (0.91) <sup>a,1</sup>	2.23±0.00 (0.82) <sup>a,2</sup>
NB 16	1980s European: clinical	1.48±0.00 (0.98) <sup>abc,1</sup>	1.95±0.10 (0.99) <sup>ab,2</sup>
B 40	1980s European: clinical	1.15±0.10 (0.99) <sup>c,1</sup>	1.73±0.04 (0.95) <sup>b,2</sup>

<sup>A</sup>D-values shown are the means of two replicate experiments, each performed in duplicate and expressed as mean ± standard deviation.

<sup>B</sup>Correlation coefficients in parenthesis.

<sup>abc</sup>values in the same column followed by the same alphabet are not significantly different (p < 0.05).

<sup>1,2</sup>values in the same row followed by the same number are not significantly different (p < 0.05).

### Determination of D-values

D-values (time for 10-fold reduction in viable cells), expressed in minutes, were determined by plotting the log<sub>10</sub> number of survivors against time for the heating temperature using Lotus 1-2-3 Software. The line of best fit for survivor plots was determined by regression analysis (Ostle and Mensing 1975); a regression equation of the type  $y = a + bx$  was derived, where b is the slope of the best straight line and, when inverted and the sign changed from - to +, gives the D-value in minutes for the specific temperature.

## Statistical Analysis

The heat resistance data were analyzed by analysis of variance (ANOVA) using SAS (SAS 1989) to determine if there were statistically significant differences among the treatments. Bonferroni mean separation test was used to determine significant differences ( $p < 0.05$ ) among means (Miller 1981).

## Vegetative Cell Lysate Preparation

Vegetative cells of control and heat-shocked samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 4% (w/v) acrylamide stacking gel; 15% (w/v) acrylamide resolving gel; 30:0.8 acrylamide:bis acrylamide and a modified Tris-glycine buffer system; Lugtenberg *et al.* 1975) analysis immediately following heat shocking. Cell pellets on ice were resuspended in gel-loading buffer (Sambrook *et al.* 1989) and lysed at 100C in a boiling water bath for 5 min. These were then stored at -70C until needed. Duplicate samples were resuspended in similar volumes of distilled water for quantitation of total protein using a revised Lowry method (Lowry *et al.* 1951; Markwell *et al.* 1978).

## Analysis of Proteins Expressed Following Heat Shock

Proteins were electrophoresed using standard SDS-PAGE procedures [4% (w/v) acrylamide stacking gel, 15% (w/v) acrylamide resolving gel; 30:0.8 acrylamide:bis acrylamide] and a modified Tris-glycine buffer system (Lugtenberg *et al.* 1975). For immunoblots, denatured proteins were transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Fisher Scientific, Pittsburgh, PA) using a Panther semidry electroblotting apparatus model HEP-1 (Owl Separation Systems, Portsmouth, NH). Blots were prewashed with PBS-0.05% (v/v) Tween-20 (PBS-Tween) as previously described (Sambrook *et al.* 1989). Dehydrated nonfat milk (5%) (w/v) in PBS-Tween was used as a blocking agent overnight. Antibodies raised against specific proteins were diluted in PBS-Tween as follows: a-SspC (1:3,000) and a-GroEL (1:5,000). The blots were incubated with agitation for 1 h at room temperature (Novak and Tabita 1999). A colorimetric detection assay was used with secondary antibody consisting of goat anti-rabbit IgG conjugated to alkaline phosphatase (GIBCO BRL Life Technologies, Grand Island, NY) that was diluted 1:3,000 in PBS-Tween and incubated for 2 h prior to chromogenic substrate development (Sambrook *et al.* 1989).

## RESULTS AND DISCUSSION

Fluid thioglycollate medium was used as a growth medium because this

medium supports only vegetative growth (Juneja *et al.* 1993). Surviving populations of *C. perfringens* vegetative cells per mL of beef gravy were counted and logarithms were plotted against exposure time at 58C to calculate D-values. *C. perfringens* cells heated at 58C in beef gravy exhibited log-linear decline in the number of surviving cells with time. No obvious lag periods or shoulders and tailing were evident in any of the survivor curves of bacteria heated in this menstruum. Such linear survival curves would suggest that the pathogen population was homogenous in heat resistance. A representative example of the destruction of *C. perfringens* vegetative cells in beef gravy at 58C is depicted in Fig. 1. Data are expressed as the log of the ratio of count at time t (N) and initial count ( $N_0$ ), which was calculated by subtracting the log initial count before cooking ( $\log N_0$ ) from the log final count after cooking ( $\log N$ ). These resulting data yielded the log numbers of *C. perfringens* colonies per gram of gravy destroyed by the heat treatment. For nonheat-shocked samples of beef gravy (controls) heated at 58C, the *C. perfringens* counts decreased by 2.6 logs (5.60  $\log_{10}$  CFU/g to 3.0  $\log_{10}$  CFU/g) within 3 min and by 4.9 logs (0.70  $\log_{10}$  CFU/g) after 7 min of heating (Fig. 1). As observed with nonheat-shocked beef gravy (controls), exposing beef gravy containing *C. perfringens* to 48C for 10 min prior to heating at 58C also resulted in log-linear decline in surviving cells with time. This observation contradicts previous published reports in which the authors reported a decrease in the slope of the straight line portion of the survivor curves and an increase in the duration of shoulder after a heat shock of *Listeria monocytogenes* or *Escherichia coli* O157:H7 (Pagan *et al.* 1997; Juneja *et al.* 1997). Heating heat-shocked *C. perfringens* cells at 58C for 3 and 5 min resulted in 1.85 and 2.68 log reductions in *C. perfringens* colony counts per mL, respectively, from an initial inoculum of 5.57  $\log_{10}$  CFU/mL, and after 7 min at 58C the log destruction was 3.8  $\log_{10}$  CFU/g. Thus, when *C. perfringens* in gravy was heated at 48C for 10 min before exposure to heat at an internal temperature of 58C, the heat resistance of the cells was substantially increased and the organism survived longer than nonheat-shocked cells.

In the present study, no correlation between the heat resistance at 58C and the origin of the *C. perfringens* vegetative cells (food poisoning, veterinary or human clinical) could be established due to significant variations in the heat resistance among strains (Table 1). This observation concerning the lack of correlation between the heat resistance at 58C and the origin of the *C. perfringens* isolates is inconsistent with a previous report in the literature. In a study by Sarker *et al.* (2000), when the heat resistance of 13 isolates varying considerably with respect to their geographical origins and dates of isolation was determined, a strong association of the food poisoning isolates and increased heat resistance was reported. The authors reported that the D-values at 55 or 57C for the *C. perfringens* chromosomal *cpe* isolates were significantly higher ( $p < 0.05$ ) than the D-values of the *C. perfringens* isolates carrying a plasmid *cpe* gene; however, differences in heat resistances were not observed at higher temperatures.

Nevertheless, understanding these variations in heat resistance is certainly necessary in order to design adequate cooking regimes to eliminate *C. perfringens* vegetative cells in ready-to-eat foods. The thermal resistance (D-values in min) of *C. perfringens* cells heated in beef gravy at 58C ranged from 1.21 min (C - 1841 isolate) to 1.60 min (F 4969 isolate). Regression curves calculated for 58C fit with an  $r^2$  value of  $> 0.90$ . Heat shock resulted in statistically significantly increase ( $p < 0.05$ ) in D-values at 58C for 6 out of 10 isolates (Table 1). Compared to the control (no heat shock), the increase in heat resistance ranged from 1.2-fold (B 40 isolate) to 1.5-fold (NCTC 8239 isolate).

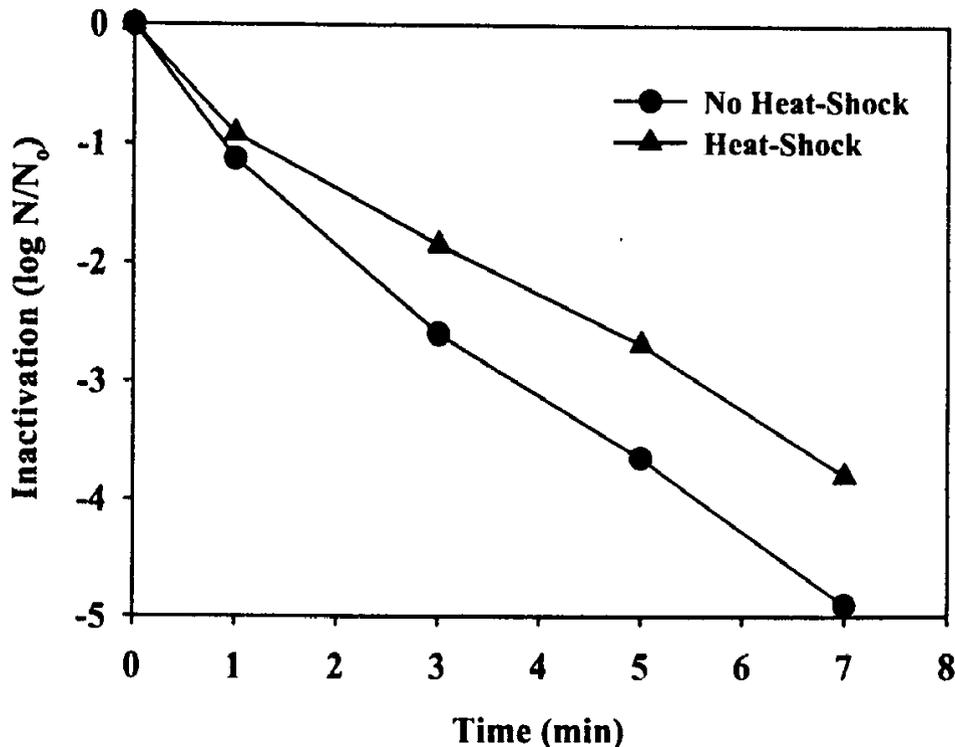


FIG. 1. DESTRUCTION OF *CLOSTRIDIUM PERFRINGENS* (NB 16) IN BEEF GRAVY MODEL AT 58C

The organism in beef gravy was either not heat shocked or heat shocked at 48C for 10 min before exposing to 58C. The data have been expressed as log of the ratio of count at time t (N) and initial count (N<sub>0</sub>).

It is feasible to compare the thermal inactivation data obtained in this study with those reported in the published literature on the heat resistance of *C. perfringens* vegetative cells. The thermal inactivation data reported in this study were, in general, consistent with those reported elsewhere. In a study by Heredia *et al.* (1997), when heat resistance of *C. perfringens* vegetative cells, grown at 43C in FTG to an A<sub>600</sub> of 0.4 to 0.6, was determined, the D-values at 55C were 9 and 5 min for FD-1 and Fd-1041 strains, respectively. For *C. perfringens* cells grown at 37C in FTG, Sarker *et al.* (2000) reported D-values at 55C of 12.1 min and 5.6 min for E13 and F5603 strains, respectively. In another study, Juneja and Marmer (1998)

reported D-values of 21.6, 10.2, 5.3 and 1.6 min in ground beef, and 17.5, 9.1, 4.2 and 1.3 min in ground turkey at 55, 57.5, 60, and 62.5C, respectively, for a mixture of three *C. perfringens* strains. A possible explanation for the decreased thermal resistance of *C. perfringens* in beef gravy in the present study compared to the data reported earlier in meat may be due to differences in composition (more solids in meat) between the substrates. Certainly, it would be inappropriate to predict the thermal death time values or design thermal processes in meat species from data obtained in broth or in other meat species. Roy *et al.* (1981) grew *C. perfringens* in autoclaved ground beef at 37C before thermal inactivation studies. The authors reported that *C. perfringens* vegetative cells of strains NCTC 8238 and NCTC 8798 had mean D-values at 57C of 7.33 and 11.1 min, respectively; the values were 2.3 and 3.1 min at 59C. Roy *et al.* (1981) quantified *C. perfringens* destruction in autoclaved ground beef containing 17 and 22% fat in screw-capped test tubes, whereas we studied destruction of the organism in beef gravy using a submerged coil heating apparatus. Overall, the differences in D-values may be attributed to strain variation, recovery conditions (including the composition and pH of the medium), the presence of inhibitors, time and temperature of incubation, and above all, the heating medium, i.e., the broth or the effect of different meat species and the differences in fat content between the substrates.

Labbe (1989) reported that *C. perfringens* in foods is a potential health hazard to consumers if such foods have been improperly handled at some point before consumption. The bacterial cells in foods are likely to encounter an exposure to a supraoptimal but nonlethal temperature (heat shock), i.e., temperature shifts. This is because temperature fluctuations are a common occurrence in a food processing environment as well as during transportation, distribution, storage or handling in supermarkets; also, during preparation of foods by consumers which includes low temperature-long time cooking of foods as well as the scenarios when the foods are kept on warming trays before final heating or reheating. Researchers have reported that such stressful environmental (heat-shocking) conditions experienced by pathogenic bacteria increase the resistance of foodborne pathogens, including *C. perfringens*, in foods to cooking (Juneja *et al.* 1997; Heredia *et al.* 1997). The heat-shock response and the induced thermotolerance obtained in this study were, in general, consistent with those reported in the literature. Heredia *et al.* (1997) heat-shocked vegetative cells of *C. perfringens* at 50C for 30 min and then determined the D-values at 55C. The authors reported that a sublethal heat-shock increased by at least 2 to 3-fold the heat tolerance of *C. perfringens* vegetative cells.

To determine if the heat-shock treatment used in the present study resulted in an increase in the levels of any of the major heat-shock proteins (Hsp), the protein patterns from control and heat-shocked *C. perfringens* cells were compared following electrophoresis. Following heat-shock of *C. perfringens* cells for 10 min at 48C, over-expression of heat-shock proteins was analyzed. To determine whether these proteins detected by SDS-PAGE analysis (Fig. 2A) corresponded to

any of the known Hsps, Western immunoblots were performed using polyclonal antibodies raised against *E. coli* GroEL and a *B. subtilis* SASP (Fig. 2B and C). We noticed that although denaturing gels were incapable of exhibiting differences in protein expression levels (Fig. 2A), lysates from vegetative cells exposed to 48C for 10 min clearly showed an increase in expression of proteins reacting with antiserum raised against *E. coli* GroEL (Fig. 2B) and *B. subtilis* SspC gene products (Fig. 2C). Heat-shock proteins are believed to play a role in removal of other heat-denatured proteins while enabling proper folding and activation of vital proteins for survival (Lindquist and Craig 1988). SASPs have been shown to protect DNA

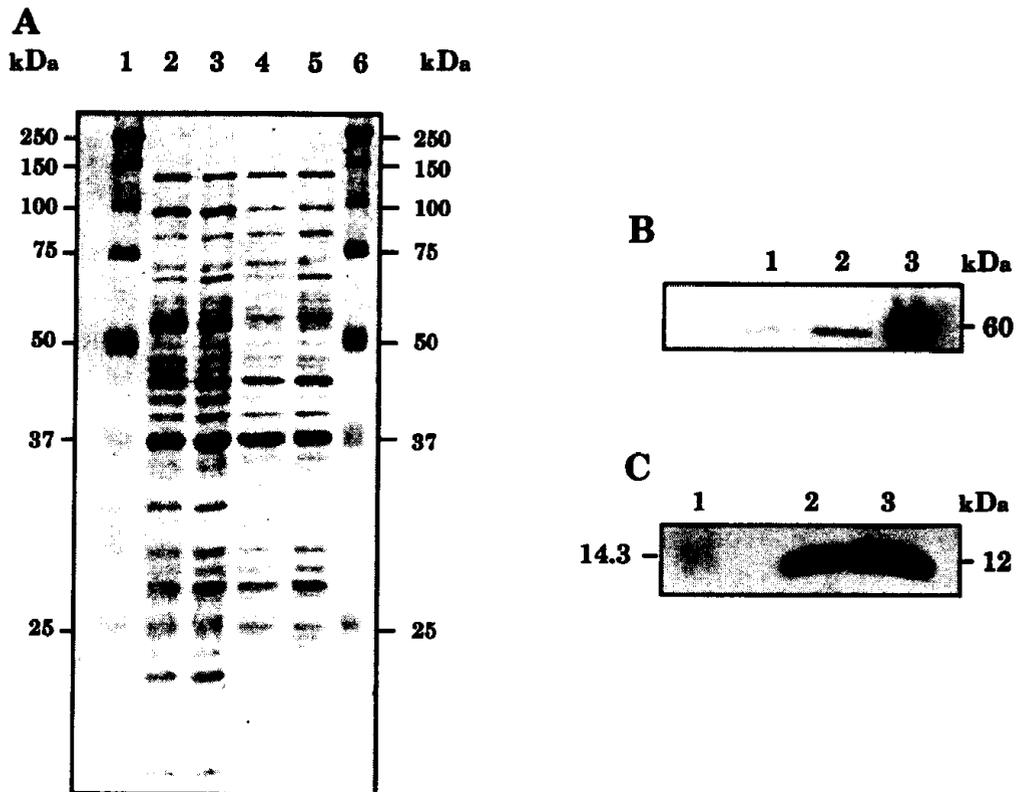


FIG. 2. (A) SDS-PAGE of *C. perfringens* vegetative cell lysates. Twenty micrograms of total protein were loaded per lane. Lanes: 1, protein molecular weight standards; 2, NCTC 10239 nonheat shocked (NHS); 3, NCTC 10239 heat shocked at 48C for 10 min (HS); 4, NB16 (NHS); 5, NB16 (HS); and 6, protein molecular weight standards. Western immunoblots of whole cell lysates from *C. perfringens* strain NB16 were reacted with antiserum raised against (B) *E. coli* GroEL and (C) *B. subtilis* SspC. Ten micrograms of total protein were loaded per lane. 2B lanes: 1, NB16 (NHS); 2, NB16 (HS); and 3, *E. coli* GroEL 60-kDa protein standard. 2C lanes: 1, 14-kDa protein molecular weight standard; 2, NB16 (NHS); and 3, NB16 (HS).

from UV irradiation damage (Fairhead *et al.* 1993) and may play a similar role here with regards to enhanced protection of DNA from heat. Following the heat-shock treatment, the levels of these proteins with epitopic and size similarity to GroEL and SASPs increased (Fig. 2B and C) and are examples of an intensively studied heat shock proteins group (Landry *et al.* 1992).

Control measures for *C. perfringens* food poisoning must ensure that large numbers of vegetative cells are not consumed. Accordingly, cooking remains a primary means of eliminating pathogens including dangerously high number of *C. perfringens* vegetative cells from ready-to-eat foods and therefore, serves to protect against food poisoning. To ensure adequate cooking, increased heat resistance due to heat shock must be considered while designing cooking/reheating regimes for precooked products; the time and temperature combinations for reheating must be sufficient to inactivate acquired enhanced thermotolerant cells. This would ensure safety of such foods contaminated with unsafe levels of *C. perfringens* vegetative cells. In the present study, we demonstrated that the safety of final cooking treatments before consumption is jeopardized following heat shock because the vegetative cells of *C. perfringens* are rendered thermotolerant. Heat-shocking *C. perfringens* vegetative cells can increase the heat resistance of the organism by as much as 1.5-fold under the conditions described in the current study. Based on the argument that reheating temperatures before consumption of the product must destroy at least 6 log<sub>10</sub> cfu/g of *C. perfringens* and the significance of heat-shock response and induced thermotolerance in foods, the present study suggests that the reheating time at 58C must be increased from 9.60 to 13.4 min to achieve a 6-D process in beef gravy. This is based on the assumption that precooked foods are improperly handled at some point before consumption. Thermal death time values from this study will assist restaurants and institutional foodservice settings in designing acceptance limits on critical control points that ensure safety against *C. perfringens* in precooked foods.

## REFERENCES

- ANON. 1989. Temperature abuses of food. Audits International Monthly, April 1989. Audits International, Highland Park, IL.
- BEAN, N.H. and GRIFFIN, P.M. 1990. Foodborne disease outbreaks in the United States, 1973-1987: pathogens, vehicles, and trends. *J. Food Prot.* 53, 804-817.
- BRYAN, F.L. 1988. Risks associated with vehicles of foodborne pathogens and toxins. *J. Food Prot.* 51, 498-508.
- COLE, M.B. and JONES, M.V. 1990. A submerged-coil heating apparatus for investigating thermal inactivation of microorganisms. *Letts. Appl. Microbiol.* 11, 233-235.

- DANIELS, R.W. 1991. Applying HACCP to new-generation refrigerated foods at retail and beyond. *Food Technol.* 45(6), 122-124.
- FAIRHEAD, H., SETLOW, B. and SETLOW, P. 1993. Prevention of DNA damage in spores and *in vitro* by small, acid-soluble proteins from *Bacillus* species. *J. Bacteriol.* 175, 1367-1374.
- HALL, H.E. and ANGELOTTI, R. 1965. *Clostridium perfringens* in meat and meat products. *Appl. Microbiol.* 13, 352-357.
- HEREDIA, N.L., GARCIA, G.A., LUEVANOS, R., LABBE, R.G. and GARCIA-ALVARADO, J.S. 1997. Elevation of the heat resistance of vegetative cells and spores of *Clostridium perfringens* type A by sublethal heat shock. *J. Food Prot.* 60, 998-1000.
- JOHNSON, E.A. 1990. *Clostridium perfringens* food poisoning. In *Foodborne Diseases*, (D.O. Cliver, ed.) pp. 229-240, Academic Press, San Diego, CA.
- JUNEJA, V.K., CALL, J.E. and MILLER, A.J. 1993. Evaluation of methylxanthines and related compounds to enhance *Clostridium perfringens* sporulation using a modified Duncan and Strong medium. *J. Rapid Methods Automation Microbiology* 2, 203-218.
- JUNEJA, V.K., KLEIN, P.G. and MARMER, B.S. 1997. Heat shock and thermotolerance of *Escherichia coli* O157:H7 in a model beef gravy system and ground beef. *J. Appl. Microbiol.* 84, 677-684.
- JUNEJA, V.K. and MARMER, B.S. 1998. Thermal inactivation of *Clostridium perfringens* vegetative cells in ground beef and turkey as affected by sodium pyrophosphate. *Food Microbiol.* 15, 281-287.
- JUNEJA, V.K., WHITING, R.C., MARKS, H.M. and SNYDER, O.P. 1999. Predictive model for growth of *Clostridium perfringens* at temperatures applicable to cooling of cooked meat. *Food Microbiol.* 16, 335-349.
- LABBE, R.G. 1989. *Clostridium perfringens*. In *Foodborne Bacterial Pathogens*, (M.P. Doyle, ed.) Marcel Dekker, New York.
- LABBE, R.G. and JUNEJA, V.K. 2001. *Clostridium perfringens*. In *Foodborne Diseases*, 2<sup>nd</sup> Ed., (D. Cliver and H. Riemann, eds.) Academic Press, San Diego, CA.
- LANDRY, S.J., JORDAN, R., MCCRACKEN, R. and GIERASCH, L.M. 1992. Different conformations for the same polypeptide bound to chaperones DnaK and GroEL. *Nature* 355, 455-457.
- LINDQUIST, S. and CRAIG, E.A. 1988. The heat-shock proteins. *Annu. Rev. Genet.* 22, 631-677.
- LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193, 265-275.
- LUGTENBERG, B., MEIJERS, J., PETERS, R., VAN DER HOEK, P. and VAN ALPHEN, L. 1975. Electrophoretic resolution of the 'major outer membrane protein' of *Escherichia coli* K12 into four bands. *FEMS Lett.* 58, 254-258.

- MARKWELL, M.A.K., HAAS, S.M., BIEBER, L.L. and TOLBERT, N.E. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* 87, 206-210.
- MILLER JR., R.G. 1981. *Simultaneous Statistical Inference*, 2nd Ed., pp. 67-70, Springer-Verlag, New York.
- NOVAK, J.S. and TABITA, F.R. 1999. Molecular approaches to probe differential NADH activation of phosphoribulokinase isozymes from *Rhodobacter sphaeroides*. *Arch. Biochem. Biophys.* 363, 273-282.
- OSTLE, B. and MENSING, R.W. 1975. *Statistics in Research*, Iowa State University Press, Ames, IA.
- PAGAN, R., CONDON, S. and SALA, F.J. 1997. Effect of several factors on heat-shock-induced thermotolerance of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 63, 3225-3232.
- ROY, R.J., BUSTA F.F. and THOMPSON D.R. 1981. Thermal inactivation of *Clostridium perfringens* after growth at several constant and linearly rising temperatures. *J. Food Sci.* 46, 1586-1591.
- SAMBROOK, J., FRITSCH, E.F. and MANIATIS, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup> Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- SARKAR, M.R., SHIVERS, R.P., SPARKS, S.G., JUNEJA, V.K. and MCCLANE, B.A. 2000. Comparative experiments to examine the effects of heating on vegetative cells and spores of *Clostridium perfringens* isolates carrying plasmid genes versus chromosomal enterotoxin genes. *Appl. Environ. Microbiol.* 66, 3234-3240.
- SAS Institute Inc. 1989. *SAS/STAT User's Guide*, Ver. 6, 4th Ed., Vol. 2, pp. 891-996, Cary, NC.
- SHOEMAKER, S.P. and PIERSON, M.D. 1976. "Phoenix phenomenon" in the growth of *Clostridium perfringens*. *Appl. Envir. Microbiol.* 32, 803-807.
- VAN GRADE, S.J. and WOODBURN, M.J. 1987. Food discard practices of householder. *J. Amer. Diet. Assoc.* 87, 322-329.
- WILLARDSSEN, R.R., BUSTA, F.F., ALLEN, C.E. and SMITH, L.B. 1978. Growth and survival of *Clostridium perfringens* during constantly rising temperatures. *J. Food Sci.* 43, 470-475.
- WYATT, L.D. and GUY, V. 1980. Relationships of microbial quality of retail meat samples and sanitary conditions. *J. Food Prot.* 43, 385-389.