Comparison of Viability and Heat Resistance of *Clostridium sporogenes* Stored at Different Temperatures

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ABSTRACT: The objective of this study was to determine the influence of storage temperature on the viability and heat resistance of *Clostridium sporogenes* spores. Spore suspension containing both spores and vegetative cells was divided into 3 groups to be stored at different temperatures of −20 (freezing), 4 (refrigerating), and 25 °C (ambient temperature). Samples stored for different times within the 2 mo were tested for viability by comparison of colony counts on plates and for heat resistance by determining *D* values at 121 °C. No significant differences were found in the viability of vegetative cells during the storage period, regardless of storage temperatures tested, while the viability of the spores stored for more than 4 wk was significantly higher at 4 °C than at −20 °C. The heat resistance of spores stored at 4 °C for more than 4 wk was remarkably higher than that at 25 °C, but similar to that at −20 °C throughout the storage period. Consequently, it turned out that a refrigerating temperature of 4 °C is satisfactory for storage of *C. sporogenes* spores in maintaining viability and heat resistance. This study suggests that storage temperature influences the viability and heat resistance of *C. sporogenes* spores.

Keywords: *Clostridium sporogenes*, heat resistance, spore, storage temperature, viability

Introduction

Clostridium sporogenes PA 3679 is a nonpathogenic, putrefactive, and spore-forming anaerobe. The ease of monitoring the presence of the organism through off-odor and gas formation has made *C. sporogenes* an excellent surrogate microorganism for modeling thermal inactivation processes of *C. botulinum* type A and B spores (Ocio and others 1994; Guan and others 2003; McGlynn and others 2003). Indeed, *C. sporogenes* is the only possible *Clostridium* surrogate for *C. botulinum* (IFT 2000, 2001). It is desirable that *C. sporogenes* spores used in validation tests are approximately 3 to 5 times as resistant to heat as *C. botulinum* spores at temperatures commonly used for commercial sterilization of canned foods (Cameron and others 1980; Brown 2000), thus reducing the amount of spores required for an experiment to validate thermal processes. In preparation for microbial validation tests, it is extremely important to ensure the viability and consistent heat resistance of the selected *C. sporogenes* spores. In general, however, *C. sporogenes* spores may have a wide range of $D_{121°C}$ values (time required for a 10-fold reduction in viable spores) ranging from 0.1 to 1.5 min (Stumbo 1973; Teixeira 2006). The variability in the $D_{121°C}$ may cause difficulties in both modeling and validating thermal processing conditions.

In the past years, researchers have proposed different ways to enhance the viability and heat resistance of *C. sporogenes* spores, especially focusing on optimizing culture conditions. However, much less attempts have been made to optimize storage conditions required to maintain the viability and heat resistance of the spores. Besides, most of the previous studies on the storage conditions have been concerned with the effect of pH of the suspending medium on heat resistance (Cameron and others 1980; Hutton and others 1991). Regarding to the storage temperature, several studies on the viability of *Clostridium* spores has been carried out with 3 different species, *C. perfringens*, *C. botulinum*, and *C. difficile* (Canada and others 1964; Odlaug and Pflug 1977; Freeman and Wilcox 2003). However, little attention has been paid to studying the influence of storage temperature on the heat resistance of the spores. Nevertheless, it is commonly accepted that freezing temperature may be favorable for the storage of spores, regardless of the genus or species of the organism. At present, however, the responses (at least in terms of changes in heat resistance) of *C. sporogenes* spores to storage temperature are largely unknown. In this study, therefore, the effect of storage temperature on both the viability and heat resistance of *C. sporogenes* spores was examined by counting viable spores and determining *D* values at 121 °C. In addition, we also investigated the influence of temperature on the suppression of vegetative cell growth and the maintenance of spore dormancy during the storage. To our knowledge, this is the 1st study addressing the responses of *C. sporogenes* spores and vegetative cells to different storage temperatures.

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Materials and Methods

Microorganism

Clostridium sporogenes PA 3679 spores were obtained from the Center for Technical Assistance of the former Natl. Food Processors Assn. (NFPA, Dublin, Calif., U.S.A.). The spore suspension was divided into cryogenic sterile vials (Fisher Scientific, Pittsburgh, Pa., U.S.A.) and kept in a freezer (−20 °C) until use. The initial concentration of the stock suspension was approximately 2×10^7 CFU/mL and was determined by the enumeration procedure described subsequently.

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Preparation and storage of spore suspension

To prepare a pure culture of vegetative cell in stationary phase, a multiple stage inoculation procedure was employed (Uehara and others 1965). The procedure utilized TPGY medium consisted of 50 g of tryptone, 20 g of yeast extract, 5 g of peptone, 4 g of dextrose and 1 g of sodium thioglycolate (all from Difco, Becton Dickinson, Sparks, Md., U.S.A.) in 1 L of distilled water (USFDA 1998). Ten milliliters of TPGY broth were inoculated with 10 μL stock spore suspension and incubated for 2 d at 32 °C in an anaerobic chamber (Coy Lab. Products, Inc., Grass Lake, Mich., U.S.A.) containing an atmosphere of 95% nitrogen and 5% hydrogen (Oxarc, Inc., Spokane, Wash., U.S.A.). Subsequently, 100 mL of TPGY broth were inoculated with 1 mL of the culture followed by incubation under the same condition. After 2 d of incubation, the culture was transferred into 1 L of TPGY broth and the flask was incubated anaerobically for 2 d. To obtain cleaned vegetative cells, the culture was washed 3 times by centrifugation in a Beckman J2-21 centrifuge (Beckman Instruments, Inc., Palo Alto, Calif., U.S.A.) with a Fiberlite F14B rotor (Fiberlite Centrifuge, Inc., Santa Clara, Calif., U.S.A.) at 15000 $\times g$ for 10 min at 4 °C, and then resuspended in 100 mL of a sporulation medium (see subsequently).

To induce sporulation of vegetative cells, the vegetative cell suspension prepared previously was added to 900 mL of a sporulation medium, giving a total volume of 1 L (final concentration of vegetative cells, approximately 10^6 CFU/mL). The sporulation medium consisted of 60 g tryptone, 1 g dextrose, 1 g sodium thioglycolate, and 5 g calcium carbonate (J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.) in 1 L of distilled water. This procedure was described by Uehara and others (1965), modified by Duncan and Foster (1968), and then further modified by adjusting initial pH to 5.0 with 1 M HCl (Lu 2006) and supplementing with 5 g calcium carbonate based on our previous observations (Mah and others 2008). After incubation for 10 d at 32 °C in an anaerobic chamber, the spore crop was washed by the same procedure as for vegetative cell and resuspended in an M/15 Sörensen's phosphate buffer (Na_2HPO_4 5.675 g, KH_2PO_4 3.63 g in 1 L of distilled water, pH 7.0). The spore suspension prepared was found to contain around 10^7 CFU/mL of both spores and vegetative cells, as determined by the enumeration technique described subsequently.

The suspension was divided into 3 groups and stored aerobically for 2 mo at different temperatures of −20 (freezing), 4 (refrigerating), and 25 °C (ambient temperature). Periodically, test samples were drawn for determination not only of viable counts of both spores and vegetative cells, but of $D_{121°C}$ values of the spores. To ensure the reproducibility of results, 3 independent experiments were conducted for each storage temperature.

Enumeration of spores and vegetative cells

The spore suspension sample was placed in a 90 °C water bath for 10 min to stimulate germination of the spores and to inactivate vegetative cells (Lu 2006). After heat treatment, the suspension was cooled in crushed ice-water and 10-fold serially diluted in sterile 0.1% peptone water. One milliliter of each dilution was pour-plated onto TPGY medium solidified with 1.5% agar. The TPGY medium was autoclaved at 121 °C for 15 min and held in a 45 °C water bath prior to use. Solidified plates were incubated anaerobically for 3 d at 32 °C. The colonies of viable spores were manually counted after both 48 and 72 h of incubation and the numbers of spores were calculated from dual plating.

The total number of vegetative cells was determined by the same procedure except for heating at 90 °C.

Measurement of *D* values

The *D* value of *C. sporogenes* spores was determined using the multiple-point method (Chung and others 2007). Test spore suspension was carefully injected into a glass capillary tube with an inner diameter of 1.8 mm and an outer diameter of 3 mm (Corning Inc., Corning, N.Y., U.S.A.) using a pipette and then the open ends of the tubes were heat sealed. The tubes were immersed completely in an oil bath (Thermo Electron Corp., Waltham, Mass., U.S.A.) at 121 °C and heated for different time intervals ranging from 15 to 120 s. After heating, the tubes were removed from the oil bath, cooled immediately in a crushed ice-water bath, and washed in 70% ethyl alcohol. Both ends of tubes were cut aseptically; suspension was flushed out with 2 mL of sterile 0.1% peptone water. The treated samples were then 10-fold serially diluted in sterile 0.1% peptone water. Each serially diluted sample was pour-plated onto TPGY medium, incubated in an anaerobic chamber, and then the colonies were manually counted. The number of survivor was calculated from duplicate determinations. Survivor curves were plotted on a semi-log chart ($\log_{10}$ survival counts compared with time) to determine *D* value. *D* values were obtained by taking the reciprocal of the slope from linear regression of the survivor curves.

Statistical analysis

All experiments were conducted in triplicate and the data were presented as means and standard deviations. The significance of differences was determined by 1-way analysis of variance (ANOVA) with Tukey's pairwise comparison module (unless mentioned specifically) of the Minitab statistical software, version 12.11 (Minitab Inc., State College, Pa., U.S.A.) and differences with *P* values of < 0.05 were considered statistically significant.

Results

Effect of storage temperature on viability of *C. sporogenes* vegetative cells

To test whether storage temperature can suppress vegetative cell growth and spore germination, and maintain spore dormancy, changes in viable numbers of vegetative cells in spore suspension during the storage at different temperatures of −20 (freezing), 4 (refrigerating), and 25 °C (ambient temperature) were determined by counting viable vegetative cells. As shown in Table 1, the viable counts of vegetative cells in the suspension stored at 25 °C were constant to be approximately 5×10^6 CFU/mL (6.73 $\log_{10}$ CFU/mL), showing little change throughout 8 wk of the storage. On the other hand, the viable counts of vegetative cells in the suspensions stored at both −20 and 4 °C were slightly but not statistically significantly lower throughout the storage than those at 25 °C (Tukey's multiple comparison tests; statistically significant differences in the viable counts were detected at 2 and 4 wk of the storage by Fisher's tests, data not shown). Meanwhile, the suspensions stored at both −20 and 4 °C exhibited an approximately 1-$\log_{10}$ reduction in viable counts after 1 wk, respectively, compared to those at 25 °C, and the viable numbers remained relatively constant thereafter.

Effect of storage temperature on viability of *C. sporogenes* spores

Changes in viable numbers of spores in spore suspension stored at 3 temperatures described previously were determined to test whether storage temperature affected spore viability. As shown in Table 2, the viable counts of spores in the suspensions were determined to be approximately 10^6 CFU/mL (6 $\log_{10}$ CFU/mL), regardless of storage period and temperature. However, although no

Table 1—Effect of storage temperature on viability of *C. sporogenes* vegetative cells.

	Storage time (wk)*				
Storage temp (°C)	0	1	2	4	8
25	$6.73 \pm 0.12^{a,A}$	$6.40 \pm 0.14^{a,A}$	$6.59 \pm 0.17^{a,A}$	$6.39 \pm 0.25^{a,A}$	$6.47 \pm 0.12^{a,A}$
4	$6.73 \pm 0.12^{a,A}$	$5.66 \pm 0.53^{a,B}$	$6.00 \pm 0.32^{a,AB}$	$5.75 \pm 0.40^{a,AB}$	$5.95 \pm 0.40^{a,AB}$
−20	$6.73 \pm 0.12^{a,A}$	$5.79 \pm 0.44^{a,B}$	$6.02 \pm 0.28^{a,AB}$	$5.65 \pm 0.36^{a,B}$	$5.97 \pm 0.37^{a,AB}$

*The values are shown as $\log_{10}$ CFU/mL. All data represent a mean ± standard deviation of 3 independent experiments. Mean values in the same column (small letters) or row (capital letters) that are not followed by the same letter are significantly different ($P < 0.05$).

Table 2—Effect of storage temperature on viability of *C. sporogenes* spores.

	Storage time (wk)*				
Storage temp (°C)	0	1	2	4	8
25	$6.01 \pm 0.12^{a,A}$	$6.55 \pm 0.10^{a,B}$	$6.51 \pm 0.17^{a,B}$	$6.50 \pm 0.17^{ab,B}$	$6.68 \pm 0.13^{b,B}$
4	$6.01 \pm 0.12^{a,A}$	$6.15 \pm 0.33^{a,AB}$	$6.25 \pm 0.20^{a,ABC}$	$6.63 \pm 0.07^{b,BC}$	$6.70 \pm 0.11^{b,C}$
−20	$6.01 \pm 0.12^{a,A}$	$6.16 \pm 0.23^{a,A}$	$6.28 \pm 0.09^{a,A}$	$6.27 \pm 0.13^{a,A}$	$6.28 \pm 0.04^{a,A}$

*The values are shown as $\log_{10}$ CFU/mL. All data represent a mean ± standard deviation of 3 independent experiments. Mean values in the same column (small letters) or row (capital letters) that are not followed by the same letter are significantly different ($P < 0.05$).

Table 3—Effect of storage temperature on heat resistance of *C. sporogenes* spores.

	Storage time (wk)*				
Storage temp (°C)	0	1	2	4	8
25	$0.88 \pm 0.09^{a,A}$	$0.70 \pm 0.11^{a,AB}$	$0.63 \pm 0.03^{a,B}$	$0.54 \pm 0.03^{a,B}$	$0.56 \pm 0.05^{a,B}$
4	$0.88 \pm 0.09^{a,A}$	$0.70 \pm 0.11^{a,A}$	$0.70 \pm 0.18^{a,A}$	$0.74 \pm 0.06^{b,A}$	$0.77 \pm 0.10^{b,A}$
−20	$0.88 \pm 0.09^{a,A}$	$0.76 \pm 0.08^{a,A}$	$0.81 \pm 0.13^{a,A}$	$0.81 \pm 0.11^{b,A}$	$0.80 \pm 0.09^{b,A}$

*The data are expressed in *D*-values in minutes determined at 121 °C. All data represent a mean ± standard deviation of 3 independent experiments. Mean values in the same column (small letters) or row (capital letters) that are not followed by the same letter are significantly different ($P < 0.05$).

significant differences were observed in the viable counts among the suspensions stored at different temperatures during the first 2 wk, the viability of spores after being stored for more than 4 wk was statistically significantly higher at 4 °C than at −20 °C. Meanwhile, spores showed somewhat differences in the pattern of change in viability during the storage at different temperatures. Particularly, the spores stored at 25 °C exhibited a half-$\log_{10}$ increment in viable counts after 1 wk, and thereafter the viable numbers remained relatively constant in the range of 3.16×10^6 CFU/mL (6.50 $\log_{10}$ CFU/mL) to 4.79×10^6 CFU/mL (6.68 $\log_{10}$ CFU/mL). The viable spores in the suspension stored at 4 °C gradually increased up to 5.01×10^6 CFU/mL (6.70 $\log_{10}$ CFU/mL) throughout the storage period. The viable numbers of spores stored at −20 °C seemed to increase slightly, but not statistically significantly, to 6.28 $\log_{10}$ CFU/mL (1.91×10^6 CFU/mL) during the first 2 wk and maintained to be constant thereafter.

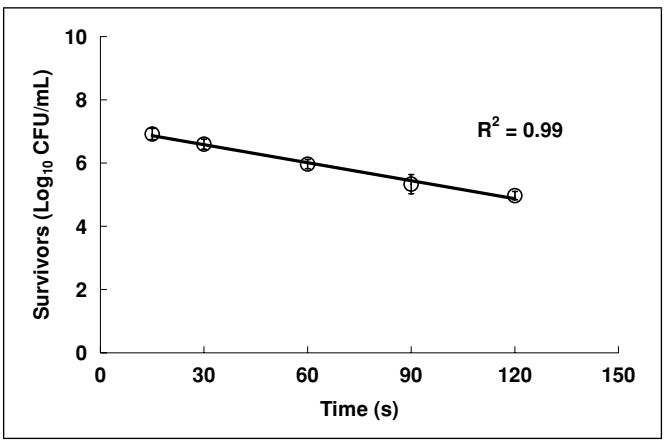

Figure 1—Thermal survivor curve for *C. sporogenes* spores. Heat treatments were performed at 121°C. Error bars indicate standard deviations calculated from triplicates.

Effect of storage temperature on heat resistance of *C. sporogenes* spores

To further investigate the responses of *C. sporogenes* spores to storage temperature, changes in heat resistance of spores stored at different temperatures were determined by measuring *D* values (time required for a 10-fold reduction in viable spores) at 121 °C. Initially, the $D_{121°C}$ value of *C. sporogenes* spores used in this study was 0.88 min, which was calculated by plotting the $\log_{10}$ survivors compared with heating time and taking the reciprocal of the slope from linear regression of the curve (Figure 1). As shown in Table 3, the spores stored at 25 °C showed a statistically significant and progressive reduction in $D_{121°C}$ values to 0.56 min during the 8 wk storage, compared to those stored at both −20 and 4 °C (Tukey's multiple comparison tests, $P < 0.05$). On the other hand, the $D_{121°C}$ values of spores stored at both −20 and 4 °C were relatively constant to be in the range of 0.70 to 0.81 min, showing no statistically significant changes throughout 8 wk of the storage. Furthermore, the $D_{121°C}$ values of spores seemed to be considerably higher at both −20 and 4 °C than those at 25 °C throughout the storage period, showing statistically significant differences, at least after 4 wk of the storage (Tukey's multiple comparison tests, $P < 0.05$). Taken together, it turned out that the heat resistance of spores stored at 4 °C for more than 4 wk was remarkably higher than that at 25 °C, but similar to that at −20 °C throughout the storage period.

Discussion

If a certain temperature is appropriate for storage of *Clostridium* spores, the temperature should suppress germination and maintain dormancy of spores, and viable counts of vegetative cells should decrease, or at least be constant, as function of time (Labbe 1989). Therefore, we tested if storage temperature can influence the

viability of vegetative cells and spores. As expected, it was found that the numbers of vegetative cells were slightly lower throughout the storage at both −20 and 4 °C than those at 25 °C. Moreover, the fact that no significant increases in viable numbers of vegetative cells were observed at both −20 and 4 °C indicates that the dormancy of *C. sporogenes* spores is probably not broken at least at temperatures lower than 4 °C. Accordingly, the viable numbers of spores stored at −20 °C were maintained to be constant during storage period, except for the first 2 wk when slight (but not significant) increases in viable counts were discernible. Interestingly, the viable numbers of spores placed at 4 °C tended to increase steadily and significantly throughout the storage period. To explain the increases in viable counts of spores, it can be speculated that the temperature tested might allow immature spores to mature during the storage, which is in agreement with our observations that while the viable counts of spores increased promptly and significantly for the 1st week at 25 °C, those at −20 °C showed less significant but detectable increases. If this is a part of the means by which the viable counts of spores increase during the storage, it indicates that the storage at a refrigerating temperature of 4 °C may be somewhat helpful to ensure sufficient amounts of *C. sporogenes* spores. At present, it remains unclear whether the increase in the viability of spores determined during the storage arises from conversion of immature spores, like forespores and endospores, to mature free spores. This issue is not discussed further herein, but it is worth noting that a refrigerating temperature of 4 °C can sustain the viability of *C. sporogenes* spores. This is in agreement with previous reports where no loss of viability of *Clostridium* spores was found at 4 °C (Odlaug and Pflug 1977; Freeman and Wilcox 2003).

Since bacterial spores including those of *Clostridium* are known to be resistant to freezing and thawing (Lund 2000; Freeman and Wilcox 2003), it is, in general, preferred to store bacterial spores at temperatures lower than −20 °C. Indeed, Wallen and Walker (1980) reported that *Bacillus subtilis* var. *niger* spores stored at −29 °C more resistant to hydrogen peroxide than those at 4 °C. However, few studies have noted whether freezing and deep-freezing conditions, like temperature, holding time and cryoprotectant, influence heat resistance of *Clostridium* spores, as briefly mentioned earlier. To protect spores from freeze–thaw damage, it is often (at least sometimes) necessary to use a cryoprotectant such as glycerol, dimethyl sulfoxide, and propylene glycol. However, Coroller and others (2001) reported that the presence of cryoprotectants causing changes in water activity of heating medium and/or recovery medium significantly affected heat resistance of *B. cereus* spores. Alpin and Hodges (1979) also observed that storage of *B. stearothermophilus* spores at a freezing temperature of −18 °C resulted in a loss of heat resistance. Furthermore, there have been no evidences that repeated freezing and thawing causes no loss of heat resistance. These subtle changes in heat resistance resulting from freezing and/or thawing are extremely important factors that can influence the efficiency and accuracy of sterilization processes because errors of over- or underestimation of heat resistance of *C. sporogenes* spores may mean a failure to optimize and validate thermal processing conditions necessary to sterilize *C. botulinum* spores. It is therefore an interesting, somewhat surprising that there were no significant differences in maintaining heat resistance of *C. sporogenes* spores between low storage temperatures of −20 and 4 °C. Thus, together with the fact that neither a cryoprotectant nor thawing step is required to protect the viability and achieve the recovery of spores stored at refrigerating temperatures, it is likely that 4 °C is more favorable for (at least short-term) storage of *C. sporogenes* spores than freezing temperatures.

C. sporogenes has been successfully used as a model microorganism for *C. botulinum* in developing thermal processing conditions as described in the Introduction. The microbial validation of thermal processes with an inoculated pack study often requires a simultaneous processing of a large number of samples to obtain statistically valid results and thereby the samples inoculated with *C. sporogenes* spores are usually stored in a cold room at 4 °C before being incubated. However, it has been unclear whether this storage causes significant changes in the viability and heat resistance of the spores. Therefore, a clear understanding of the effect of storage temperature on the viability and heat resistance of *C. sporogenes* spores is important and helpful in establishing a successful standard protocol to perform an inoculated pack study. In this study, it has been shown that both viability (and dormancy) and heat resistance of *C. sporogenes* spores can be maintained for extended periods (at least 2 mo) at 4 °C, which suggests that it would be appropriate to place the samples inoculated with the spores in a cold room maintained at 4 °C prior to being incubated at an optimal temperature. To use the information provided in this study for developing a standard protocol, however, it needs to perform a validation in target food samples.

Conclusions

This study indicates that storage temperature can influence the viability and heat resistance of *C. sporogenes* spores, and suggests that a refrigerating temperature of 4 °C would be appropriate to store not only *C. sporogenes* spores, but also the inoculated food samples with the spores.

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References

Alpin SJ, Hodges NA. 1979. Changes in heat resistance during storage of *Bacillus stearothermophilus* spores produced in chemically defined media. J Appl Bacteriol 46:623–6.

Brown KL. 2000. Control of bacterial spores. Br Med Bull 56:158–71.

Cameron MS, Leonard SJ, Barrett EL. 1980. Effect of moderately acidic pH on heat resistance of *Clostridium sporogenes* spores in phosphate buffer and in buffered pea puree. Appl Environ Microbiol 39:943–9.

Canada JC, Strong DH, Scott LG. 1964. Response of *Clostridium perfringens* spores and vegetative cells to temperature variation. Appl Microbiol 12:273–6.

Chung H-J, Wang S, Tang J. 2007. Influence of heat transfer with tube methods on measured thermal inactivation parameters for *Escherichia coli*. J Food Prot 70:851–9.

Coroller L, Leguérinel I, Mafart P. 2001. Effect of water activities of heating and recovery media on apparent heat resistance of *Bacillus cereus* spores. Appl Environ Microbiol 67:317–22.

Duncan CL, Foster EM. 1968. Role of curing agents in the preservation of shelf-stable canned meat products. Appl Microbiol 16:401–5.

Freeman J, Wilcox MH. 2003. The effects of storage conditions on viability of *Clostridium difficile* vegetative cells and spores and toxin activity in human faeces. J Clin Pathol 56:126–8.

Guan D, Gray P, Kang D-H, Tang J, Shafer B, Ito K, Younce F, Yang TCS. 2003. Microbiological validation of microwave-circulated water combination heating technology by inoculated pack studies. J Food Sci 68:1428–32.

Hutton MT, Koskinen MA, Hanlin JH. 1991. Interacting effects of pH and NaCl on heat resistance of bacterial spores. J Food Sci 56:821–2.

[IFT] Institute of Food Technologists. 2000. Overarching principles: kinetics and pathogens of concern for all technologies. In: Kinetics of microbial inactivation for alternative food processing technologies. Available from: http://www.cfsan.fda.gov/~comm/ift-toc.html. Accessed Aug 22, 2008.

[IFT] Institute of Food Technologists. 2001. Chapter 7. Comparison of NSF and ABA protocols to determine whether a food requires time/temperature for safety. In: Evaluation and definition of potentially hazardous foods. Available from: http://www.cfsan.fda.gov/čomm/ift4-toc.html. Accessed Aug 22, 2008.

Labbe R. 1989. *Clostridium perfringens*. In: Doyle MP, editor. Foodborne bacterial pathogens. New York: Marcel Dekker Inc. p 191–234.

Lu W-YW. 2006. Effects of intrinsic and extrinsic factors of sporulation conditions on heat resistance of *Clostridium sporogenes* PA 3679 spores [MSc thesis]. Pullman, Wash.: Washington State Univ.

Lund BM. 2000. Freezing. In: Lund BM, Baird-Parker TC, Gould GW, editors. The microbiological safety and quality of food. Gaithersburg, Md.: Aspen Publishers Inc. p 122–45.
Mah J-H, Kang D-H, Tang J. 2008. Effects of minerals on sporulation and heat resistance of *Clostridium sporogenes*. Int J Food Microbiol [serial online]. Doi:10.1016/j.ijfoodmicro.2008.10.002.
McGlynn WG, Davis DR, Johnson MG, Crandall PG. 2003. Modified spore inoculation method for thermal-process verification of pinto beans and green beans canned in two large reusable containers. J Food Sci 68:988–91.
Ocio MJ, Sánchez T, Fernández PS, Rodrigo M, Martínez A. 1994. Thermal resistance characteristics of PA 3679 in the temperature range of 110–121°C as affected by pH, type of acidulant and substrate. Int J Food Microbiol 22:239–47.
Odlaug TE, Pflug IJ. 1977. Effect of storage time and temperature on the survival of *Clostridium botulinum* spores in acid media. Appl Environ Microbiol 34:30–3.
Stumbo CR. 1973. Thermal resistance of bacteria. In: Stumbo CR, editor. Thermobacteriology in food processing. 2nd ed. New York: Academic Press Inc. p 93–122.
Teixeira AA. 2006. Simulating thermal food processes using deterministic models. In: Sun D-W, editor. Thermal food processing: new technologies and quality issues. Boca Raton, Fla.: CRC Press. p 73–107.
Uehara M, Fujioka RS, Frank HA. 1965. Method for obtaining cleaned putrefactive anaerobe 3679 spores. J Bacteriol 89:929–30.
[USFDA] US Food and Drug Administration. 1998. *Clostridium botulinum* (Chapter 17). In: Bacteriological analytical manual. 8th ed. Rev. A. Gaithersburg, Md.: Assn. of Official Analytical Chemists Intl.
Wallen SE, Walker HW. 1980. Effect of storage conditions on the resistance of *Bacillus subtilis* var. *niger* spores to hydrogen peroxide. J Food Sci 45:605–7.