Sporulation and Heat Resistance of Spores from a *Clostridium* sp. RKD

APARNA DIXIT, SYED IMTEYAZ ALAM, RAM KUMAR DHAKED, AND LOKENDRA SINGH

ABSTRACT: A *Clostridium* sp. RKD isolated from the intestine of decaying fish, showing 99% sequence identity with *Clostridium tetani* at a 16S rRNA level, produced a neurotoxin that was neutralized by botulinum antitoxin (A+B+E). It also showed an amplification of near-expected size when polymerase chain reaction (PCR) was performed using group- and type-specific primers for botulinum neurotoxin type B. The isolate exhibited differences with both *C. tetani* and *Clostridium botulinum* with respect to phenotypic characteristics and chemotaxonomic markers. Spore production was optimized with respect to media composition and stage of growth. Time-dependant examination of sporulation revealed 2.6% to 49.0% spores in the late stationary phase culture when grown in different broth media. A simpler method for spore production and isolation from culture grown in tryptose sulfite cycloserine (TSC)/anaerobic agar sandwich resulted in >95% sporulation, which could be purified to near homogeneity by a simple 2-step procedure. Thermal resistance of spores revealed a biphasic inactivation at lower temperatures with D values for linear inactivation varying from 26.6, 8.0, and 4.3 min at 70 °C, 80 °C, and 90 °C, respectively. The z values of 7.86 °C and 10.47 °C were obtained in the linear and tail regions, respectively. The Weibull parameter b values at 70 °C, 80 °C, and 90 °C were 27.38, 3.55, and 0.99, respectively, with a z′ value of 13.869 °C. The shape parameter n at 70 °C, 80 °C, and 90 °C were 0.63, 0.55, 0.45, respectively. Spores produced on 2 different media (cooked meat medium [CMM] and trypticase peptone yeast-extract glucose [TPYG] agar) exhibited differences in heat resistance. The addition of lysozyme (50 μg/mL) before heat treatment resulted in increased thermal resistance of spores.

Keywords: *Clostridium* sp., botulinum neurotoxin, D value, z value, b value, n value, z′ value, spore stability, thermal resistance, sporulation

Introduction

The phylogenetic analysis of genus *Clostridium* has shown that this genus of spore-forming bacteria is very heterogeneous with several deeply branching lineages, some of which also include non-clostridial species (Collins and others 1994). Among clostridia, only 2 species, namely, *Clostridium botulinum* and *Clostridium tetani*, are shown to produce neurotoxins. Botulinum neurotoxin (BoNT) is produced by 6 physiologically and phylogenetically distinct groups of clostridia (Hatheway 1992). Tetanus toxin (TeNT) is so far reported to be produced by a single serotype of *C. tetani* (Haberman and Dreyer 1986). The deadly nature of the diseases that these toxins cause has ensured them considerable attention from the scientific community.

There has been an upsurge of an entirely new branch of the food industry catering to the demands of consumers for fresh-like minimally processed highly nutrition foods. To ensure extended shelf life, a combination of minimal heat processing and a limited use of preservatives are desirable with modern packaging technologies. Unfortunately, the revolutionary processing methodologies promise convenience and health but pose serious hazards due to dangerous microorganisms possessing heat-resistant spores. Several species of bacteria produce heat-resistant spores of which members of the genera *Bacillus* and *Clostridium* are the prominent ones (Hyung and others 1983; Fernandez and others 2001). Apart from their significance in causing serious foodborne illness, clostridial neurotoxins are the most important biological and toxin warfare (BTW) agents because of their high toxicity. The negative publicity caused by a botulism outbreak can severely damage the economics of the growing food industry, especially in developing countries such as India. Moreover, the epidemiology of foodborne infections is changing with the newly emerging pathogens and well-known pathogens becoming associated with the new food vehicles.

Heat resistance of spores and their ability to germinate within minutes to form vegetative cells are of tremendous practical importance for the food-processing industry (Atrih and Foster 2001). Spores in the dormant state are not hazardous; however, their germination, outgrowth, and proliferation could result in food spoilage and/or toxin production, causing food poisoning. The spore cortex peptidoglycan is involved in maintaining spore dormancy and heat resistance (Ellaer 1978), and alteration in its usually conserved structure correlates with reduced heat resistance. Other factors affecting spore stability include core dehydration, mineralization, and dipicolinic acid content (Paidhungat and others 2000).

Heat activation of clostridial spores has remained an elusive phenomenon that can be affected by the composition of the sporulation medium, the time and temperature of the heat treatment, the length and temperature of plate incubation, and inter-strain variation (Thomas 1981). Spore yield and properties have been reported to vary with sporulation media (Hitchins and others 1972).

Heating remains the principal method of microbial inactivation, even though other inactivation methods such as high pressure and pulsed electric fields are on the rise. The now classical method of D and z values, developed by Bigelow, Ball, and Stumbo (Stumbo 1973), is widely accepted and practiced. Its usefulness is clear from a proven record in the canning industry. This method assumes 1st-

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order kinetics as a model to describe inactivation of microorganisms. The 2 parameters D_T value or Decimal reduction times (minutes of heating at T temperature for the number of survivors to drop 1 Log_{10} cycle) and z value (°C increase in the temperature of treatment for D_T value to decrease 1 Log_{10} cycle) are the thermotolerance parameters commonly used for the calculation of process times necessary to achieve sterility. Even though the use of D and z values is widely practiced, it is striking to note that many survival curves do not really seem to obey 1st-order kinetics. It has already been noted because the introduction of the D-z concept that many exceptions occur, in the form of so-called shoulders and/or tails in the survival curves (Cerf 1977), but also downward and upward concavity phenomena are frequently observed. The common explanation is that the microbial population consists of several populations, each with its own inactivation kinetics.

Several attempts have been made to cope with nonlinear survival curves. Among these are (pseudo) mechanistic models (Geerared and others 2000). Another view to microbial inactivation has recently been discussed in a series of articles (Peleg and Cole 1998, 2000; Peleg 1999, 2000a, 2000b; Peleg and Penchina 2000). It considers lethal events as probabilities, rather than as deterministic. The nonlinear curves can be perfectly explained by the Weibull model, which is a flexible, yet simple model to describe heat inactivation (Smith 1991). Several equations based on the Weibull distribution have been suggested to describe survival curves (Mafart and others 2002; Van Boekel 2002, Hassani and others 2005; Peleg and others 2005). The 2 Weibull parameters b and n, of which b is called the scale parameter and n is called as the shape parameter, are used extensively in reliability engineering to describe time to failure in electronic and mechanical systems and is also appropriate for the analysis of survival data, that is, time to failure after the application of stress.

The effect of temperature, pH, and lysozyme on the heat resistance of spores has been studied by several workers (Mazas and others 1988; Fernandez and Peck 1999). Measured heat resistance of spores of non-proteolytic *C. botulinum* has been reported to increase by 2 orders of magnitude by the presence of hen egg white lysozyme and other factors in the medium used for enumeration of survivors. Lysozyme is able to induce germination of the subpopulation (0.1% to 1%) of heat-damaged spores that posses permeable coats (Fernandez and Peck 1999). These observations have increased concerns regarding the safety of minimally heat-treated foods in the food industry because lysozyme and other lytic enzymes are present in a number of foods.

We have isolated in our laboratory a *Clostridium* species, producing a neurotoxin, which was neutralized by botulinum antitoxin (A+B+E) when tested by mouse protection bioassay (Dixit and others 2005). Although the strain exhibited a close identity with *C. tetani* at a 16S rRNA gene sequence level, it differed from both *C. tetani* and *C. botulinum* when examined for morphological, biochemical, and chemotaxonomic characteristics.

The aim of the present investigation is to study the sporulation pattern of the isolate under different nutritional state conditions. A simple method for production and purification of spores has been optimized, and the resulting spores were studied with respect to their thermal resistance.

Materials and Methods

Bacterial strain and culture conditions

A *Clostridium* sp. RKD isolated previously from decaying fish sample was used for growth and sporulation studies. Cells were grown in trypticase peptone yeast-extract glucose (TPYG) broth containing pancreatic digest of casein, 50 g; peptone, 5 g; yeast extract, 20 g; glucose, 4 g; sodium thioglycollate, 1 g; NaCl, 2 g; soluble starch, 1 g; cycloserine, 250 mg; sulphamethoxazole, 76 mg; and trimethoprim, 4 mg/L (pH 7.5) at 37 °C. The culture was grown anaerobically using 5% inoculum in serum vials containing 30 mL medium with nitrogen and hydrogen as head space gases (80:20) at 37 °C. The growth was observed by determining the absorbance at 600 nm.

Media optimization for spore production

Spore production was optimized using the following broth media (1) TPYG, (2) Duncan's Strong medium (DS) containing yeast extract, 4 g; proteose peptone, 15 g; starch, 4 g; sodium thioglycollate, 1 g; Na_2HPO_4, 10 g/L (pH 7.5), (3) cooked meat medium (CMM) containing peptone 10 g; Lab-lemco beef extract, 10 g; neutral heart tissue, 30 g; NaCl, 5 g/L (pH 7.5), (4) reinforced clostridial medium (RCM) containing yeast extract, 3 g; beef extract, 10 g; peptone, 10 g; dextrose, 5 g; soluble starch, 1 g; NaCl, 5 g; sodium acetate, 3 g; cystein HCl, 5 g/L (pH 7.5), (5) anaerobic basal medium (ABM) containing yeast extract, 1 g; NH_4Cl, 1 g; K_2HPO_4, 0.3 g; KH_2PO_4, 0.3 g; NaCl, 0.6 g; $CaCl_2$, 0.1 g; $MgCl_2$, 0.2 g; KCl, 0.1 g; trace element solution 1.5 mL; resazurin, 1 mg/L (pH 7.5), (6) peptone-yeast extract (PY) medium containing peptone, 5 g; tryptone, 5 g; yeast extract, 10 g; vitamin K, 1 μL; hemin, 0.5 g; cystein HCl, 0.5 g/L (pH 7.5); and (7) Antarctic bacterium medium (AM) containing K_2HPO_4, 2 g; KH_2PO_4, 1.5 g; yeast extract, 1 g, and tryptone, 1 g/L (pH 7.5). The cells were grown anaerobically using 5% inoculum of exponentially growing broth culture in serum vials containing 30 mL of medium with nitrogen and hydrogen as head space gases (80:20) at 37 °C. Cells were harvested every 24 h by centrifugation at 12000 × *g* for 10 min at 4 °C up to 10 d; the cell pellet was washed with saline (0.85%) and resuspended in an equal volume of phosphate buffer saline.

The sporulation was examined under light microscope (Leica Microsystems, Wetzlar, GmbH, Germany) after Gram staining. Enumeration of spores verses total cells was carried out using the image analysis software (Leica) taking 5 different fields. Percent sporulation was represented as the average value ± standard deviation. Chemicals used in the study were obtained from Sigma-Aldrich (Mumbai, India) and Hi-Media (Mumbai, India) and were of reagent grade.

Preparation of spore crops

A modification of the biphasic spore production method of Bruch and Others (1968) was used to prepare the spore crops. The agar phase consisted of 250 g of ground defatted beef heart, 5 g of peptone, 5 g of tryptone; 5 g of gelatin, 10 g of agar, and 500 mL of distilled water (pH 7.5). This was dispensed in 250-mL anaerobic bottles under constant flow of oxygen free nitrogen (N_2:H_2, 80:20) using gassing manifold and autoclaved at 121 °C for 20 min. The liquid phase of the biphasic medium consisted of 50 g of tryptone, 5 g of peptone, 2 g of dextrose, 2 g of starch, 0.5 g of cysteine HCl, and 1 L of distilled water (pH 7.5). The broth was autoclaved for 20 min at 121 °C. After cooling, the broth was placed in the anaerobic chamber, and 250 mL was aseptically added to each bottle containing agar phase. The biphasic system was placed at 37 °C for 2 d to check for possible contamination. Inoculum was prepared by growing cells anaerobically in the liquid phase at 37 °C for 2 d. The biphasic media was inoculated with freshly grown culture (5% v/v) and incubated anaerobically at 37 °C. Sporulation was examined every 24 h up to 8 d. A simple method of spore crop preparation was optimized by growing cells at the interface of TSC agar (oxoid) and anaerobic agar medium. TSC agar contained tryptose, 15 g; papaic digest of soyabean meal, 5 g; beef extract, 5 g; yeast extract, 5 g;

sodium metabisulfite, 1 g; ferric ammonium citrate, 1 g; agar, 15 g/L. Twenty 5 mL of media was dispensed anaerobically into 60-mL serum vials and after autoclaving at 121 °C for 20 min and kept in a slanting position. After solidification, the 0.1 mL of freshly grown culture was evenly spread on the agar surface, which was then overlaid with 5 mL of anaerobic agar medium containing pancreatic digest of casein, 17.5 g; glucose, 10 g; papaic digest of soyabean meal, 2.5 g; NaCl, 2.5 g; sodium thioglycollate, 2.0 g; sodium formaldehyde sulfoxylate, 1.0 g; cystein HCl, 0.4 g; methylene blue, 0.002 g; agar, 10 g/L. It was incubated at 37 °C and sporulation was examined every 24 h up to 8 d. Optimal sporulation (95%) was observed after 4 d, which was selected for spore harvesting for all subsequent spore crop preparations. The experiments for optimization of spore preparation were repeated twice, and the mean values are reflected in the results. Spores were harvested after removing the overlay in an anaerobic workstation (Don Whitley, Bradford, U.K.), washed with anaerobic saline, and finally resuspended in 10 mL of distilled water. The spore suspension was heated at 60 °C for 30 min and washed twice with anaerobic saline by centrifugation at 12000 × *g* for 20 min. Sedimented spores were finally resuspended in saline in an anaerobic cabinet at a concentration of 10^8 spores/mL and stored at –20 °C in 1-mL aliquots in 5-mL thin-walled glass ampoules (Borosil, Mumbai, India). Spores in the prepared crop were enumerated using the most probable number (MPN) technique, and the number of viable spores in the original sample was calculated (Hurley and Roscoe 1983).

Heat resistance measurements

Glass ampoules containing 1 mL spore suspension (10^8 spores/mL) was placed rapidly in a large water bath (Lauda Dr. R. Wobser, GmbH and Co. KG, Germany) set at desired temperature (70 °C, 80 °C, 90 °C, or 100 °C), and the temperature of the bath was monitored with a precision mercury-in-glass thermometer. Three uninoculated ampoules containing 1 mL of saline were used to determine the come-up-time at 70 °C, 80 °C, and 90 °C. The temperature of saline inside the ampoule was monitored using a previously calibrated copper-copper-nickel thermocouple (EC Electronics Corp.,, Hyderabad, India). The temperature was recorded to the nearest 0.1 °C at every 6 s until it reached the desired temperature. A come-up time of approximately 16 s, 22 s, and 28 s was observed for 90 °C, 80 °C, and 70 °C, respectively. The heat-treatment time for the actual experiments was recorded after the lapse of come-up times at respective temperatures. After different time intervals, 1 aliquot was removed and plunged into a deep ice bath and 0.1 mL of suspension from the aliquot was diluted in 0.9 mL ice cold sterile anaerobic saline (0.85% wt/vol). Ten-fold serial dilutions were made into sterile anaerobic saline, and enumeration of survivors was done as previously described (Fernandez and Peck 1999). In brief, 5 replicates of 30-mL serum vials containing anaerobically prepared 15 mL of TPYG medium (pH 7.5) were used per dilution, and 4 dilution series were prepared for each sample. Growth was examined by visible turbidity and production of gas at every 2-d interval. From the number of vials that showed growth, the most probable number (MPN) of viable spores in the original sample was calculated. Enumeration of viable spores in unheated controls was also carried out to establish the initial spore number per vial.

The spores were further prepared using CMM and TPYG medium containing 1.5% agar by the method described previously. After 7 d of incubation at 37 °C, the spores were purified as described previously and evaluated for their heat resistance at 80 °C. Spores (approximately 10^8/mL) prepared from the 2 media were exposed to heat in the absence and presence of lysozyme at a final concentration of 50 μg/mL. Viable spores before and after 10, 20, 30, and 60 min of heat exposure were enumerated by the MPN method using TPYG medium as described previously.

Preparation of lysozyme and addition to the medium

On the day before inoculation, sterile lysozyme (L-6876, lyophilized powder from chicken egg white, 50000 units/mg of protein, Sigma) was added to the TPYG medium for the enumeration of the samples heat-treated in the presence of lysozyme. A solution of 5 mg lysozyme/mL was made in distilled water prepared under N_2 and sterilized by filtration (0.22 μm, Millipore Millex GV filter, Millipore Pvt. Ltd., New Delhi, India) and was added to each vial to give a final concentration of 50 μg/mL.

Thermotolerance parameters

The traditional model based on the 1st-order kinetic was used to describe survival curves. D_T values were calculated from the slope of the linear as well as tailed regions of the survival curves at 70 °C, 80 °C, and 90 °C. z values were calculated from the slope of the curves obtained by plotting the $\text{Log}_{10}\ D_T$ compared with their corresponding heating temperatures (Lynt and others 1983; Chai and Liang 1992). Correlation coefficients (r^2) at 95% confidence intervals were calculated with the appropriate statistical package (Excel 5.0, Microsoft, Seattle, Wash., U.S.A.).

Survival curves were also analyzed by a model based on the Weibull distribution. For this investigation, we used the equation proposed by Mafart and others (2002):

$$\text{Log } S(t) = -(t/b)^n$$

where S(t) is the survival fraction at time t; t is the treatment time; and b and n are 2 characteristic parameters of the Weibull distribution. The b value in this case represents time to reduce the survival population in 1 Log cycle (as the traditional D_T) and is dependent on the n value. For kinetic analysis of the data, the solver tool in Excel, capable of solving the fitting of experimental data to nonlinear functions under the principle of least-squares curve fitting, was used (Harris 1998).

z′ values (°C increase in the temperature of treatment for b value to decrease 1 Log_{10} cycle) were calculated from the slope of the curves obtained by plotting the Log_{10} b versus their corresponding heating temperature.

Determination of spore magnesium content

Spore minerals were extracted for assay as previously reported (Bender and Marquuis 1985) with modifications made by Atrih and Foster (2001). Briefly, 10 mg of spores (wet wt of packed cells) were 1st autoclaved for 20 min at 120 °C. The spore suspensions were then hydrolyzed by adding an equal volume of 12 mol/L HCl. After incubation for 3 h at 60 °C, the samples were left at room temperature overnight. The spore suspensions were centrifuged at 10000 × *g* for 10 min at 4 °C, and the magnesium contents of the supernatant fluids were assayed by atomic absorption spectrometry (A Analyst 100, Perkin Elmer, Perkin-Elmer, Mass., U.S.A.).

Results and Discussion

An obligate anaerobe of *Clostridium* species (strain RKD) has been previously isolated on CBI agar from decaying fish sample by the enrichment method described earlier (Dhaked and others 2003). The phylogenetic analysis based on 16S rRNA gene sequence (EMBL accession number AJ579909) indicated that the bacterium RKD is closely related to the genus *Clostridium*. The closest relative appeared to be *C. tetani* with a similarity of 99% (Dixit and others 2005). Although the bacterium shared a high genetic

similarity with *C. tetani*, it had differences with both *C. botulinum* and *C. tetani* at morphological and biochemical levels. The neurotoxin appeared to be botulinum-like, as indicated by neutralization with botulinum antitoxin (A+B+E), which provided protection to the animal. It was further supplemented by PCR amplification using BoNT/B specific primers generating an amplicon of nearly 700 bp (Dixit and others 2005). However, when subjected to single-pass sequencing, the amplicon did not correspond to toxin gene and showed no significant match in the database when subjected to BLAST search (EMBL accession number AJ635356). It is extremely important to ensure the safety of foods, especially with respect to toxigenic, spore-forming clostridial species, because its growth results in the production of an extremely powerful neurotoxin capable of causing serious illness or death. It becomes all the more vital to do so when a novel bacterial strain producing neurotoxin is isolated. Hence, the spores produced by the strain RKD warrants investigation with respect to their thermal resistance and production under various nutritional states expected to be related to food processing and subsequent storage.

Strain RKD produced spores after the onset of stationary phase, irrespective of the growth medium. Sporulation by the bacterium on different broth media is shown in Figure 1. Maximum spores were obtained when cells were grown in CMM followed by DS showing 50% and 28% sporulation, respectively. Very little sporulation was observed when cells were grown in PY, AM, and ABM media. The morphology of vegetative cells was also affected by the media and stage of growth (Figure 2) and varied from long straight rods in chains (AM) to more stout, shorter rods (CMM). Sporulation of *C. botulinum* strains have been reported to be poor in broth media, and a biphasic method using a lower complex agar phase and upper protein-rich medium in liquid phase was reported earlier (Bruch and others 1968). Our attempts to promote sporulation using the same method did not yield sufficient sporulation. A maximum average yield of 20% was obtained after 8 d of incubation in this biphasic medium. A simpler method was optimized using commercially available TSC and anaerobic agar as described in the "Materials and Methods" section. The spores grown on the interface of the 2 media could be easily scraped from the serum vial. The spore count increased with time and reached a maximum after 4 d of incubation. The percent sporulation increased from 10% to 95% from the 1st to 4th d, after which it marginally declined.

Heat-resistance studies at different temperatures were carried out using purified spores generated on a TSC/anaerobic agar interface. Measured inactivation of high-density spore population at 80 °C and 90 °C gave rise to tailed survivor curves (Figure 3). D values were assigned to the overall inactivation at 70 °C and for the 2 distinct linear inactivation rates in the biphasic thermal destruction curves obtained for spores treated at 80 °C and 90 °C. When analyzed by using complete survivor curves, the death kinetics revealed a trend toward greater linearity as the temperature decreased. The biphasic nature of the survivor curves at higher temperature suggests heterogeneity in the thermal resistance of the spore populations. The z values for the 2 death rates were derived from plots of the $\log_{10}$ D values versus temperature. The initial linear inactivation gave a z value of 7.86 °C, and the tailing region exhibited a z value of 10.47 °C. The z values reported for non-proteolytic *C. botulinum* spores heated in various media are typically about 6 °C to 9°C, but z values of about 4 °C to 5 °C (Lynt and others 1983) to as high as 16.5 °C have also been reported (Scott and Bernard 1982). The correlation coefficients for D values in initial linear inactivation varied from 0.956 at 90 °C to 0.998 at 80 °C, whereas the values for tail regions varied from 0.884 at 90 °C to 0.921 at 80 °C (Table 1). Similar tailed thermal destruction curves for clostridial spores have also been reported earlier by other workers (Smelt and Raatjes 1980), giving a similar explanation of mere genetic heterogeneity among the spore population, with a small subpopulation being more heat-resistant than the rest of the spores. The flocculation during the course of heating has been reported as potential source of experimental artifact (Stumbo 1973). This was not a factor in this study because wet microscopic mounts of cell inocula and samples collected from heat challenges over the range of temperatures showed no aggregation.

Table 1—Calculated D values for linear and tail regions of survival curves

Temperature (°C)[a]	Linear D value (min)[b]	Linear D r^2 value	Tail D value (min)[c]	Tail D r^2 value
90	4.3	0.956	9.5	0.884
80	8.0	0.998	20.0	0.921
70	26.6	0.978	26.6	0.966

[a]Temperature ±0.05 °C.
[b]The Z value for the linear D data is 7.86 °C (r^2 = 0.970).
[c]The Z value for the tail D data is 10.48 °C (r^2 = 0.933).

The model based on the Weibull distribution also accurately described survival curves corresponding to the inactivation of the spores of *Clostridium* sp. RKD. The b and n values obtained by fitting this model to survival curves are shown in Table 2. The n values were in all cases lower than 1, indicating that all survival curves were concave upward. Experimental values were compared with the estimated values obtained by this model, indicating this model prediction was a good fit to the measured data (Figure 4a, 4b, 4c). The b and n values showed a linear relationship with temperature (Figure 5 and 6), suggesting that the temperature influenced both these parameters. From the slope of the curve drawn between Log_{10} b values and temperature, it was possible to calculate a parameter z′ (similar to the

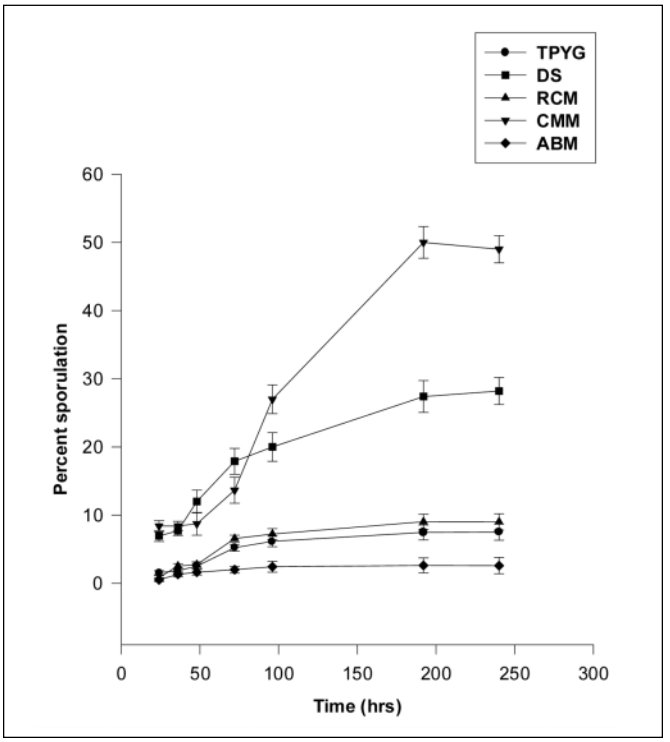

Figure 1—Optimization of sporulation media for *Clostridium* sp. RKD.

Table 2—b and n values estimated from the 1st fit of the model based on the Weibull distribution to experimental data of heat-treated spores of *Clostridium* sp. RKD at 70 °C, 80 °C, and 90 °C[a]

T (°C)[b]	b (min) (CL 95%)	n[c] (CL 95%)	r^2	RMSE
70	27.38 (26.18-28.58)	0.63 (0.58-0.68)	0.99	0.034
80	3.55 (1.70-5.40)	0.55 (0.44-0.67)	0.96	0.275
90	0.99 (0.25-1.74)	0.45 (0.36-0.54)	0.96	0.325

[a]CL = Confidence Limit; RMSE = Root Mean Square Error.
[b]Temperature, ±0.05 °C.
[c]The z′ value is 13.869 °C (r^2 = 0.9826).

z value calculated from the traditional 1st-order kinetic model) using the following equation (Van Boekel 2002):

$$\text{Log } b = a_1 - b_1 T$$

$$z' = -1/b1$$

Two quite different z values were derived from the linear and tailed region of the survival curve drawn by the 1st-order kinetic model (Table 1), whereas z′ (Table 2) takes the complete curve into account. Here the shape parameter n did depend significantly on temperature (Figure 6).

Low value of the shape parameter (n < 1) again supports the finding that the sensitive spore populations are killed almost instantaneously, whereas the remaining spore populations have less probability of dying, indicating that the remaining spores are the sturdy ones, perhaps adapting to stress or due to heat shock–induced thermotolerance (Baranyi and others 1996; Marechal and others 1999). In an investigation carried out by Van Boekel (2002) analyzing the survival curves for heat treatment of vegetative cells of various microorganisms, it was concluded that the shape parameter was larger than 1 in most cases, which indicates that the microorganisms become increasingly susceptible to heat damage with prolonged heating times. However, there were few notable exceptions in which the shape parameter is <1, suggesting that growth conditions and the composition of the heating menstruum can have a large effect on the actual behavior of a microorganism. Peleg and others (2005) have reported the n and k values for *C. botulinum* spores at 102 °C heat treatment has been 0.35 °C and 0.31 °C $^{-1}$, and no other reports of such kind are available for either *C. tetani* or other Clostridial strains.

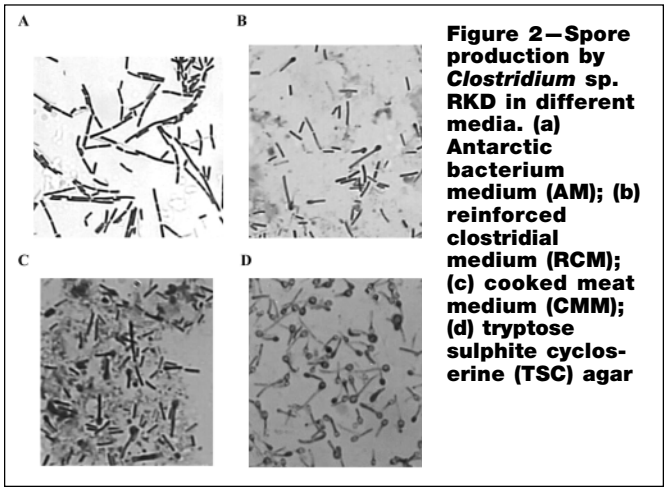

Figure 2—Spore production by *Clostridium* sp. RKD in different media. (a) Antarctic bacterium medium (AM); (b) reinforced clostridial medium (RCM); (c) cooked meat medium (CMM); (d) tryptose sulphite cycloserine (TSC) agar

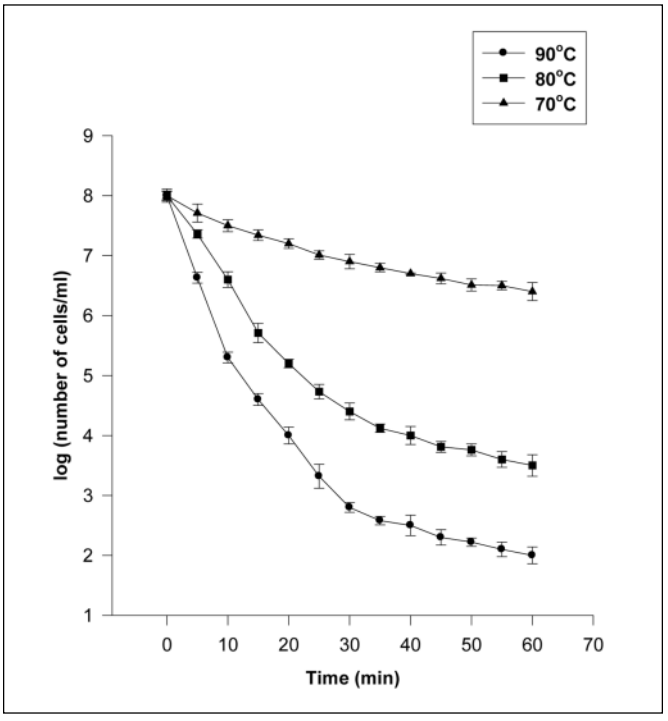

Figure 3—Thermal destruction of spores of *Clostridium* sp. RKD heated at 70 °C, 80 °C, and 90 °C

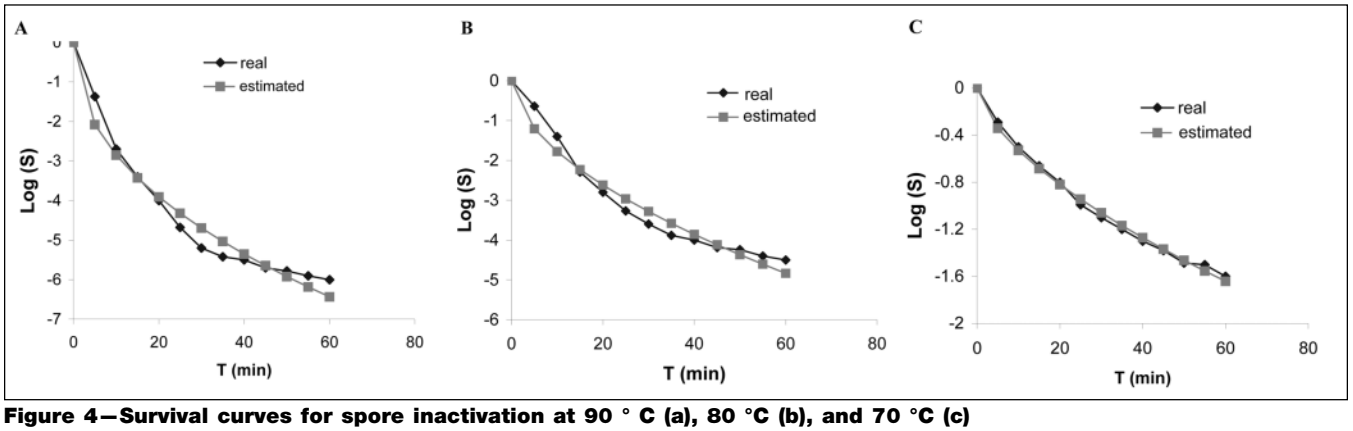

Figure 4—Survival curves for spore inactivation at 90 ° C (a), 80 °C (b), and 70 °C (c)

The variations in the morphology of cells grown on different media prompted us to examine whether the spores generated on them varied in their thermal resistance. Two media showing distinct cell morphology (TPYG and CMM) were selected for this purpose. Heat challenge at 80 °C revealed a D value of 9.2 min for spores produced on TPYG agar and 7.8 min for those from CMM agar. The overall inactivation was more linear when heat treatment was given and survivors were enumerated in the presence of lysozyme. There was apparent increase in thermal resistance in the presence of lysozyme in both the cases with D values of 15.2 min and 13.9 min for spores prepared from TPYG and CMM agar, respectively. The role of lysozyme in promoting germination of heat-damaged spores has been well documented, and hydrolysis of peptidoglycan in the lysozyme-permeable spore cortex has been proposed for this effect (Peck and others 1993). An increase in the thermal resistance for *C. botulinum* type E spores in the presence of lysozyme in the recovery medium has been reported with D values of 48.3, 12.6, and 3.17 min at 85 °C, 90 °C, and 95 °C, respectively (Lindstorm and others 2003). The wide spread occurrence of lysozyme or other lytic enzymes in food of all types (Proctor and Cunningham 1988; Stringer and Peck 1996) makes it essential from a safety point of view to consider the effect of lysozyme on the thermal resistance of spores. Environmental conditions during sporulation have been shown to have a dramatic effect on the final heat resistance properties of dormant spores (Slepecky and Foster 1959). Varying media composition for spore production of *Bacillus subtilis* resulted in alteration in the structure of spore cortex, spore composition, and resistance properties (Atrih and Foster 2001). Bacterial spores accumulate minerals, which contribute to spore stability and heat resistance. The combination of peptidoglycan defects with low protoplast density has been proposed to explain the instability of spores after their release. Furthermore, spores prepared in the 2 different media differed in their magnesium content. Spores formed on TPYG medium accumulated a lower level of magnesium (1.3 ± 0.11 μg / mg of spores) when compared with those from CMM medium (3.8 ± 0.14 μg /mg). The presence of salts at high concentrations in the sporulation medium has been suggested to contribute to spore resistance when media supplemented with (CCY) salts were used (Atrih and Foster 2001). The CCY salts contain the following: $MgCl_2.6H_2O$ (0.5 m*M*), $MnCl_2.4H_2O$ (0.01 m*M*), $FeCl_3.6H_2O$ (0.05 m*M*), $ZnCl_2$ (0.05 m*M*), and $CaCl_2.6H_2O$ (0.2 m*M*). Our observation of preferential accumulation of magnesium in spores grown in CMM and TPYG without any salt supplement is interesting.

A number of guidelines and recommendations for the safe production of minimally processed foods have been made. Processing, formulation, and storage of refrigerated processed foods of extended durability (REPFEDs), including vacuum-packaged dairy and meat products, takes into consideration the D and z values for toxigenic clostridia. Such data with respect to spores of proteolytic and non-proteolytic *C. botulinum* strains are available through several investigations (Lindstrom and others 2003) in which the effect of heating media and the presence of lysozyme in the recovery medium have been studied. But data in terms of b, n, and z′ values for different clostridial strains are still scanty. Thermo stability data of the spores produced by *Clostridium* sp. RKD provide the necessary time-temperature treatment to pasteurize or sterilize foods with respect to this particular strain of neurotoxigenic *Clostridium* sp.

Conclusions

The neurotoxigenic *Clostridium* sp. RKD exhibited differences in the morphology of cells and extent of sporulation when grown in different broth media. Sporulation efficiency dramatically increased when culture was grown on the interface of the 2 anaerobic agar media. The survival curves showed upward concavity, indicating the presence of 2 spore populations with different thermal resistance. The heat-resistance parameters such as D values, z values, and the Weibull parameters b, n, and z′ indicated that the strain produces heat-resistant spores and remains at the margin of safety. There is a need for analyzing spores from other clostridial species using the Weibull model to make meaningful comparisons of their resistance properties. The studies indicate that accumulation of minerals, without addition of extra salts in the media, can take place and perhaps modulate heat-resistance properties of the spores. The heterogeneity of spores of this extremely neurotoxigenic strain, with respect to thermal resistance within the species, warrants reevaluation of the food-safety parameters, keeping in mind the emerging newer strains producing potent toxins.

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References

[ACMSF] Advisory Committee on the Microbiological Safety of Foods. 1992. Report on vacuum packaging and associated processes. London, U.K.: Her Majesty's Stationary Office.

Atrih A, Foster SJ. 2001. Analysis of the role of bacterial endospores cortex structure in resistance properties and demonstration of its conservation among species. J Appl Microbiol 91:364–72.

Baranyi J, Jones A, Walker C, Kaloti A, Robinson TP, Mackey BM. 1996. A combined model for growth and subsequent thermal inactivation of *Brchrothrix*

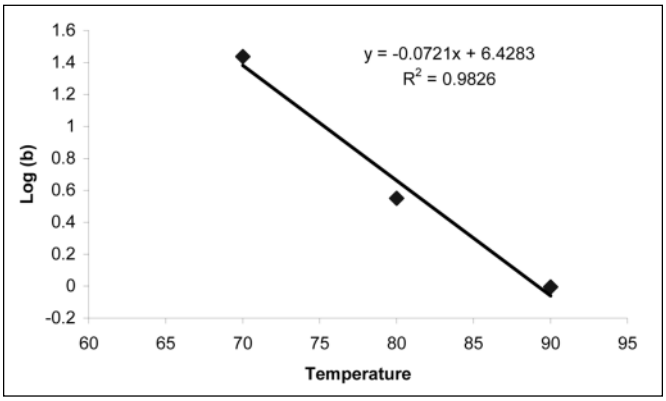

Figure 5—Relationship between b value and temperature

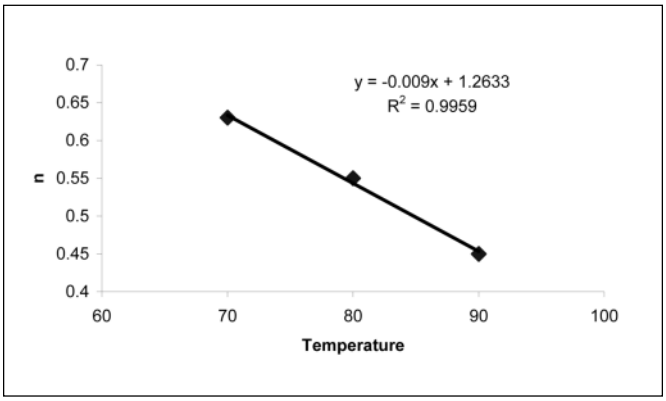

Figure 6—Relationship between n value and temperature

thermosphacta. Appl Environ Microbiol 62:1029–35.
Bender GR, Marquis RE. 1985. Spore heat resistance and specific mineralization. J Bacteriol 50:1414–21.
Bruch MK, Bohrer SW, Denny CB. 1968. Adaptation of biphasic culture technique to the sporulation of *Clostridium botulinum* types E. J Food Sci 33:108–9.
Cerf O. 1977. Tailing of survival curves of bacterial spores. J Appl Bacteriol 42:1–19.
Chai TJ, Liang KT. 1992. Thermal resistance of spores from five type E *Clostridium botulinum* strains in eastern oyster homogenates. J Food Prot 55:18–22.
Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandez-Garayzabl J, Garcia P, Cai J, Hippe H, Farrow JAE. 1994. The phylogeny of the genus *Clostridium*: proposal of five new Genera and eleven new species combinations. Int J Syst Bacteriol 44:812–26.
Dhaked RK, Sharma SK, Parida MM, Singh L. 2003. Isolation and characterization of *Clostridium botulinum* type E from soil of Gwalior, India. J Nat Toxins 11:49–56.
Dixit A, Dhaked RK, Alam SI, Singh L. 2005. Characterization of *Clostridium* sp. RKD producing botulinum like neurotoxin. Syst Appl Microbiol (Forthcoming).
Ellaer DJ. 1978. Spore specific structures and their function. Symp Soc Gen Microbiol 28:295–334.
[ECFF] European Chilled Food Federation. 1996. Guidelines for the hygienic manufacture of chilled foods. London: ECFF.
Fach P, Gilbert M, Griffais R, Guillou JP, Popoff MR. 1995. PCR and gene probe identification of botulinum neurotoxin A, B, E, F, and G producing *Clostridium sp*. and evaluation in food samples. Appl Environ Microbiol 61:389–92.
Fernandez A, Ocio MJ, Fernandez PS, Martinez A. 2001. Effect of heat activation and inactivation conditions on germination and thermal resistance parameters of Bacillus cereus spores. Int J Food Microbiol 63:257–64.
Fernandez PS, Peck MW. 1999. A predictive model that describes the effect of prolonged heating at 70 to 90°C and subsequent incubation at refrigeration temperatures on growth from spores and toxigenesis by non-proteolytic *Clostridium botulinum* in the presence of lysozyme. Appl Environ Microbiol 65:3449–57.
Geeraerd AH, Herremans CH, Van Impe JF. 2000. Structural model requirements to describe microbial inactivation during a mild heat treatment. Int J Food Microbiol 59:185–209.
Habermann E, Dreyer F. 1986. Clostridial neurotoxins: handling and action at the cellular level. Curr Topics Microbiol Immunol 129:93–179.
Harris DC. 1998. Non linear least-squares curve fitting with Microsoft Excel solver. J Chem Edu 75:118–21.
Hassani M, Alvarez I, Raso J, Condon S, Pagan R. 2005. Comparing predicting models for heat inactivation of *Listeria monocytogenes* and *Pseudomonas aeruginosa* at different pH. Int J Food Microbiology 100:213-22.
Hatheway CL. 1992. *Clostridium botulinum* and other clostridia that produce botulinum neurotoxin. In: Hauschild AHW, Dodds KL, editors. *Clostridium botulinum*—ecology and control in foods. New York: Marcel Dekker. p 3–20.
Hitchins AD, Greene RA, Slepecky RA. 1972. Effect of carbon source on size and associated properties of *Bacillus megaterium* spores. J Bacteriol 110:392–401.
Hurley MA, Roscoe ME. 1983. Automated statistical analysis of microbial enumeration by dilution series. J Appl Bacteriol 55:159–64.
Hyung HH, Zeikus JG, Longin R, Millet J, Ryter A. 1983. Ultrastructure and extreme heat resistance of spores from thermophilic *Clostridium* species. J Bacteriol 156:1332–7.
Lindstorm M, Nevas M, Hielm S, Koreala H. 2003. Thermal inactivation of non-proteolytic *Clostridium botulinum* type E in model fish media and in vacuum-packaged hot-smoked fish products. Appl Environ Microbiol 69(7):4029–36.
Lynt RK, Kautter DA, Solomon HM. 1983. Effect of delayed germination by heat damaged spores on estimates of heat resistance of *Clostridium botulinum* types E and F. J Food Sci 48:226–9.
Mafart P, Couvert O, Gaillard S, Leguerinel I. 2002. On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model. Int J Food Microbiol 72:107–13.
Marechal PA, Martinez de Marnanon I, Poirier I, Gervais P. 1999. The importance of the kinetics of application of physical stresses on the viability of microorganisms: significance for minimal food processing. Trends Food Sci Technol 10:15–20.
Mazas M, Lopez M, Gonzalez I, Gonzalez A, Barnardo A, Martin R. 1988. Effects of the heating medium pH on the heat resistance of *Bacillus cereus* spores. J Food Safety 18:25–36.
Paidhungat M, Setlow B, Driks A, Setlow P. 2000. Characterization of spores of *Bacillus subtilis* which lack dipicolinic acid. J Bacteriol 182:5505–12.
Peck MW, Fairbairn DA, Lund BM. 1993. Heat resistance of spores on non-proteolytic *Clostridium botulinum* estimated on medium containing lysozyme. Lett Appl Microbiol 16:126–31.
Peleg M. 1999. On calculating sterility in thermal and non-thermal preservation methods. Food Res Int 32:271–8.
Peleg M. 2000a. Microbial survival curves- the reality of flat "shoulders" and absolute thermal death times. Food Res Int 33:531–8.
Peleg M. 2000b. Modeling and simulating microbial survival in foods subjected to a combination of preservation methods. In: Barbosa-Canovas GV, Gould GW, editors. Innovations in food processing. Lancaster, Pa.: Technomic. p 163–81.
Peleg M, Cole MB. 1998. Reinterpretation of microbial aurvival curves. Crit Rev Food Sci Nutr 38:353–80.
Peleg M, Cole MB. 2000. Estimating the survival of Clostridium botulinum spores during heat treatments. J Food Prot 63:190-5.
Peleg M, Normand MD, Corradini MG. 2005. Generating microbial survival curves during thermal processing in real time. J Appl Microbiol 98:406–17.
Peleg M, Penchina CM. 2000. Modeling microbial survival during exposure to a lethal agent with varying intensity. Crit Rev Food Sci Nutr 40:159–72.
Proctor VA, Cunningham FE. 1988. The chemistry of lysozyme and its use as a food preservative and a pharmaceutical. Crit Rev Food Sci Nutr 26:359–95.
Scott VN, Bernard DT. 1982. Heat resistance of spores from non-proteolytic type B *Clostridium botulinu*m. J Food Protect 45:909–12.
Slepecky R, Foster JW. 1959. Alteration in metal content of spores of Bacillus megaterium and the effect on some spore properties. J Bacteriol 78:117–23.
Smelt JP, Raatjes PM. 1980. Heat resistance of *Clostridium botulinum* in acid ingredients and its signification for the safety of chilled foods [Acad. dissertation]. Univ. of Utrecht, The Netherlands. p 80–6.
Smith RL. 1991. Weibull regression models for reliability data. Reliab Eng Syst Safety 34:55–77.
Stringer SC, Peck MW. 1996. Vegetable juice aids the recovery of heated spores of non-proteolytic *Clostridium botulinum*. Lett Appl Microbiol 23:407–11.
Stumbo CR. 1973. Thermobacteriology in food processing. 2nd ed. New York: Academic Press.
Thomas JM. 1981. Effect of plating medium on heat activation requirement of *Clostridium botulinum* spores. Appl Environ Microbiol 42:734–6.
Van Boekel MAJS. 2002. On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. Int J Food Microbiol 74:139–59.