Journal of Food Protection, Vol. 67, No. 1, 2004, Pages 83–89
Copyright ©, International Association for Food Protection

Growth of Heat-Treated Enterotoxin-Positive *Clostridium perfringens* and the Implications for Safe Cooling Rates

KARIN G. ANDERSEN, TINA B. HANSEN, AND SUSANNE KNØCHEL*

Department of Dairy and Food Science, Centre for Advanced Food Studies, LMC; The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

03-176: Received 22 April 2003/Accepted 7 August 2003

ABSTRACT

Clostridium perfringens 790-94 and 44071.C05 carrying a chromosomal and a plasmid *cpe* gene, respectively, were used to determine differences in heat resistance and growth characteristics between the genotypes. Heat inactivation experiments were conducted using an immersed coil apparatus. Spore germination, outgrowth, and lag phase, together named GOL time, as well as generation times were determined during constant temperatures in fluid thioglycollate (FTG) medium as well as in vacuum-packed, heat-treated minced turkey. GOL time and growth were also monitored during cooling scenarios from 65 to 10°C for 3, 4, 5, 6, and 7 h in vacuum-packed, heat-treated minced turkey. Spores of strain 790-94 were approximately 10-fold more heat resistant at 85°C than those of strain 44071.C05, and strain 790-94 also had a higher temperature growth range in FTG. The higher growth range for a chromosomal enterotoxin-producing CPE$^+$ strain was confirmed using two other strains carrying a chromosomal (NCTC8239) and plasmid (945P) *cpe* gene. Moreover, strain 790-94 had shorter GOL times at 50°C in turkey and approximately half the generation time compared with strain 44071.C05 at temperatures ≥45°C in both FTG and turkey. Strain 790-94 increased with 0.3, 1.0, 1.7, and 2.0 logs, respectively, during cooling from 65 to 10°C in 4, 5, 6, and 7 h, which was significantly higher than for strain 44071.C05. A maximum acceptable cooling time of 5 h between 65 and 10°C is suggested.

Clostridium perfringens type A is one of the most commonly reported bacterial agents in foodborne disease in many western countries *(3, 20, 27, 28).* The organism is widely distributed in a variety of foods, but the vehicle of transmission is often a cooked meat or poultry product in which the *C. perfringens* spores have survived the cooking process. The heat treatment may activate spore germination, and the subsequent slow/insufficient cooling or hot holding of food at temperatures too low may then allow germination, outgrowth, and multiplication. As a result of the rapid growth, high levels of vegetative cells can be reached within a short time. If foods containing a large number of vegetative cells ($>10^5$ g^{-1}) are consumed, some cells may survive the passage through the stomach and, subsequently, sporulate in the intestine. In association with sporulation, some *C. perfringens* produce an enterotoxin (CPE), which is the responsible agent in food poisoning *(17).* It has been estimated that 2 to 8% of the global *C. perfringens* population harbor the enterotoxin gene (*cpe*) *(10, 18, 23, 31).* Moreover, not all CPE-producing (CPE$^+$) strains seem to have the same potential to cause food poisoning in humans. Cornillot et al. *(8)* showed that the *cpe* gene can be located either on the chromosome or a plasmid, and several recent studies *(6–8, 24)* have shown that all human food poisoning isolates genotyped to date carry a chromosomal *cpe* gene. The basis for this association between isolates carrying a chromosomal *cpe* gene and food poisoning is not yet fully understood. Sarker et al. *(21)* showed that both vegetative cells and spores of strains carrying a chromosomal *cpe* gene were far more heat resistant in a laboratory medium than spores of strains carrying a plasmid *cpe* gene, which would favor the survival of these strains during heat treatment of foods. Juneja et al. *(15),* however, could not confirm this difference in heat resistance in a beef gravy system. If spores and cells are more heat resistant, it could be hypothesized that the growth temperature range would also be higher. However, to our knowledge, nothing is known of the impact of the two carrier systems on the subsequent growth potential. The objective of the present study was to determine the growth potential after heat treatment of CPE$^+$ isolates carrying a chromosomal or plasmid *cpe* gene at different constant temperatures between 20 and 50°C and different cooling profiles.

Slow cooling is considered one of the main reasons for food poisoning caused by *C. perfringens,* and regulations on cooling have been made in most countries, although limited scientific data have been available regarding growth of the organism after heat treatment. Considerable variations in the regulations are found, and even within the European Union, there are no uniform guidelines. Our objective was therefore also to provide better decision support for determining safe cooling rates for heat-treated foods.

MATERIALS AND METHODS

Test organisms and working stocks. *C. perfringens* 790-94 and NCTC8239 carrying a chromosomal *cpe* gene *(4)* and *C. perfringens* 44071.C05 and 945P carrying a plasmid *cpe* gene *(8)*

* Author for correspondence. Tel: +45 35283258; Fax; +45 35283231; E-mail: skn@kvl.dk.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/67/1/83/1672468/0362-028x-67_1_83.pdf by guest on 12 January 2023

were kindly supplied by Sigrid Brynestad and Per Einar Granum, Norwegian School of Veterinary Science, Oslo. The strains 790-94 and NCTC8239 are human food poisoning isolates *(4),* while *C. perfringens* 44071.C05 and 945P were isolated from goat intestines and a sheep lung, respectively *(8).* Working stocks were prepared as described by McDonel and McClane *(19)* and maintained at −20°C in cooked meat medium (Difco Laboratories, Detroit, Mich.).

Preparation of vegetative cells for inoculum. From the working stock of *C. perfringens* 790-94 and NCTC8239, 0.13 ml was inoculated into 13 ml of freshly prepared fluid thioglycollate medium (FTG; Oxoid, Basingstoke, UK) in screw-cap tubes and heat shocked at 75°C for 20 min. The strains 44071.C05 and 945P had a lower concentration of spores; the whole working stock (10 ml) was therefore heat shocked, and 1.3 ml was subsequently inoculated into 13 ml FTG. All tubes were incubated at 37°C for 18 h, hereafter referred to as 18-h-FTG cultures.

Sporulation. Active cultures of strains 790-94 and 44071.C05 were produced by adding 10% of the 18-h-FTG culture into FTG and incubating at 37°C for 4 h. We inoculated 13 ml of freshly prepared, modified (pH raised to pH 7.8 by adding filter-sterilized 0.66 M sodium carbonate to the autoclaved media) Duncan and Strong sporulation media (DS) *(11)* in screw-cap tubes with 1% of the active culture and incubated them at 37 or 42°C for 22 to 24 h. The percentage of sporulation was determined after 8 h at 37°C or after 6 h at 42°C using phase-contrast microscopy by counting the number of sporulated cells out of approximately 200 cells. Five to six DS tubes were examined for each combination of strain and temperature. Spore suspensions were made by centrifugation of the 22- to 24-h DS cultures at 8,000 × *g* for 15 min (Sorvall RC5B Plus, Buch and Holm A/S, Herlev, Denmark), and pellets were washed in sterile deionized water. Centrifugation and washing were repeated twice, and the spores were finally resuspended in sterile deionized water and stored at 4°C until use.

Enumeration. Serial dilutions were made in physiological saline (0.9% NaCl; Merck, Darmstadt, Germany) with 0.1% peptone (Bacto Peptone, Difco) (PSP), and 0.1 ml was spread plated on tryptose sulfite cycloserine agar (Merck) without added cycloserine. Tryptose sulfite cycloserine agar plates were overlaid with an additional 10 ml of tryptose sulfite cycloserine agar and allowed to solidify before incubation in anaerobic jars with an Anaerocult A (Merck) kit. The total *C. perfringens* population was determined after 2 days at 37°C.

Heat inactivation. Heat inactivation trials were conducted for spores of strains 790-94 and 44071.C05 produced at both 37 and 42°C. Twenty milliliters was centrifuged at 1,300 × *g* for 20 min at 4°C (International Equipment Co., Centra MP4R, Gibco BRL, Life Technologies A/S, Roskilde, Denmark), and pellets were resuspended in 20 ml of freshly prepared DS. Fifteen milliliters was immediately heated at 85°C in an immersed coil heating apparatus *(5),* and samples (0.1 to 0.15 ml) were taken at predetermined time intervals. These samples were dispensed directly into 4 ml PSP (20°C), ensuring instantaneous cooling of the sample. Simultaneously, the remainder of the DS spore culture (5 ml) was heat shocked to kill vegetative cells in order to determine the number of spores present in the culture at the start of the heating. Two to three replicates of each heat inactivation trial were carried out.

Growth of vegetative cells at constant temperatures. The growth of vegetative cells was examined in screw-cap tubes with FTG that had been preincubated at the desired growth temperature overnight. These cells were inoculated with an appropriate dilution of an 18-h-FTG culture to obtain a final concentration of approximately 3 log CFU ml^{-1}. For the strains 790-94 and 44071.C05, tubes were incubated at 30, 37, 45, or 50°C, and duplicate tubes were removed after 0, 1, 2, 4, 6, 8, and 10 h for enumeration. For the strains NCTC8239 and 945P, growth at 50°C was examined after 0, 1, 2, 3, and 4 h.

Growth of spore inoculum at constant temperatures. Growth of spores of *C. perfringens* 790-94 and 44071.C05 produced at 37°C was examined in both FTG and heat-treated minced turkey. The inoculum for FTG was prepared as an appropriate dilution of the spore suspensions in 4°C sterile deionized water, and these suspensions were heated at 70°C for 10 min to simulate a cooking procedure and activate the spore germination system. Subsequently, the suspensions were cooled to 37°C before the inoculation of approximately 3 log spores ml^{-1} to the FTG tubes that were preincubated at the desired growth temperature. Minced turkey (9% fat), obtained from a local retail market, was placed in a 5- to 10-mm layer in autoclave bags (about 10 by 20 cm) and autoclaved at 105°C for 30 min. The heat-treated minced turkey (pH 6.3) was cooled at room temperature overnight and manually "rubbed" to ensure an even distribution of meat and meat juice in the samples. For inocula, appropriate dilutions of the spore suspensions were made in 4°C sterile deionized water. The heat-treated minced turkey, 10 ± 0.1 g, was aseptically weighed into bags (Cryovac cook-in CN300, Sealed Air Denmark A/S, Herlev, Denmark) and inoculated with 20 μl of the *C. perfringens* inoculum to obtain a final concentration of approximately 3 log spores g^{-1}. The bags were vacuum packed (99% vacuum, Komet X200, Maschinenfabrik, Stuttgart, Germany) and manually massaged to distribute the spores throughout the meat sample, which was approximately 0.4 by 5 by 5 cm. Negative controls consisted of bags containing uninoculated heat-treated minced turkey. The samples were heated at 70°C for 10 min and afterward quickly cooled to the growth temperature. Growth in FTG and heat-treated minced turkey was examined at 20, 30, 37, 45, or 50°C, where 5 to 12 samples in duplicate were removed after predetermined times. The meat was mixed with 90 ml PSP with 1% peptone (Bacto Peptone, Difco) and homogenized at medium speed for 1 min in a Seward Stomacher 400 Lab Blender (London, UK) before serial dilution and enumeration.

Growth during cooling from 65 to 10°C. Growth during the cooling of spores produced at 37°C of strains 790-94 and 44071.C05 was examined in heat-treated minced turkey prepared, inoculated, packed, and heated as described above. The cooling from 65 to 10°C in 3, 4, 5, 6, or 7 h was carried out in a stepwise manner with predetermined time intervals at 60, 50, 45, 37, 30, 20, and 10°C (water baths). The times were determined in order for the cooling profile to resemble equation 1, which has previously been shown by Blankenship et al. *(2)* to mimic the temperature curve during cooling.

$$T = T_0 e^{-at} \quad (1)$$

where T_0 is the initial temperature, T is the temperature during cooling after a time t, and a is the exponential cooling rate constant. During heating and cooling, the temperature in two meat samples was continuously monitored using thermocouples and a datalogger (Grant 1200 series Squirrel meter/logger, Islef + Hagen AS, Søborg, Denmark). Duplicate samples were removed at appropriate time intervals, and enumeration was performed as described above.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/67/1/83/1672468/0362-028x-67_1_83.pdf by guest on 12 January 2023

TABLE 1. *Sporulation percentage for* Clostridium perfringens *790-94 and 44071.C05 at 37 and 42°C and the* $D_{85°C}$*-values of the spores*

Strain	Sporulation			$D_{85°C}$ (min)[a]		
	Temp (°C)	%, average	Standard deviation	Average	Standard deviation	No. of trials
790-94	37	88	5.4	19	4.4	2
	42	48	8.8	17	6.8	3
44071.C05	37	14	7.9	2.1	1.9	2
	42	6	1.3	1.0	0.9	2

[a] The survivor curves from each trial had between 6 and 11 data points and correlation coefficients (r^2) between 0.88 and 0.98.

Data analysis. Estimates of heat resistance were expressed as $D_{85°C}$-values (defined as the time in minutes at 85°C required to reduce the viable cell population by 1 log) and calculated as the absolute value of the inverse slope of the least-squares regression lines fitted to the survivor curves (log of surviving cell numbers [CFU ml^{-1}] plotted against time at 85°C). Each survivor curve had between 6 and 11 data points, and correlation coefficients (r^2) were between 0.88 and 0.98.

Growth curves were generated from the experimental data using the Gompertz equation *(12),* and the Gompertz parameter values were subsequently used to calculate GOL (lag) and generation times as described by Gibson et al. *(12).*

The increases in log CFU g^{-1} during cooling were estimated as the difference between the inoculation level, which was determined as the mean value of four samples within the first hour, and the level of the last four samples removed before and after the interval at 10°C when no growth was observed.

Statistics. The effects of growth temperature, strain, and growth media on the GOL and generation time were tested by analysis of variance. The analyses of variance were conducted using the Statistical Analysis System program package (version 8.02, SAS, Cary, N.C.) and the General Linear Models procedure with effects of growth temperature, strain, and growth media as class variables. The mean values presented are least-squares estimates of marginal means, computed by the statement LSMEANS and pairwise tested by the option PDIFF in SAS.

For each cooling experiment, the effect of strain and cooling time on the log CFU g^{-1} was tested by analysis of variance conducted in SAS by use of the General Linear Models procedure with the effect of strain and cooling time as class variables. A statistically significant increase in log CFU g^{-1} during a cooling sequence was determined by pairwise *t* tests against the log CFU g^{-1} at 0 h.

RESULTS

Sporulation and heat resistance. The percentage of sporulating cells for *C. perfringens* 790-94 and 44071.C05 at 37°C was 88 and 14%, respectively, which was approximately twofold higher than at 42°C (Table 1). The sporulation temperature had no effect on the heat resistance of the spores (Table 1). Spores of strain 790-94, which has a chromosomal *cpe* gene, were the most heat resistant and had a $D_{85°C}$-value almost 10-fold higher than spores of strain 44071.C05 (plasmid *cpe* gene) (Table 1).

Growth at constant temperatures. The comparisons of growth kinetics at constant temperatures between the strains carrying a chromosomal and plasmid *cpe* gene, *C. perfringens* 790-94 and 44071.C05, respectively, were performed with vegetative cells and spores in FTG as well as spores in heat-treated minced turkey. *C. perfringens* was not detected in the negative controls of heat-treated minced turkey. The two strains had very similar growth kinetics at 20, 30, and 37°C, and there were no statistically significant differences between their GOL ($P \geq 0.78$) or generation times ($P \geq 0.28$) in FTG or heat-treated minced turkey (Tables 2 and 3). In contrast, a clear difference was observed between the growth kinetics of the strains at 45 and 50°C (Fig. 1). In FTG at 45°C, the vegetative cells of strain 790-94 reached a higher maximum viable cell level than those of strain 44071.C05 (Fig. 1A), and at 50°C, the viable count increased for strain 790-94, while it decreased below the detectable level after 3 h for strain 44071.C05 (Fig. 1B). A similar pattern was detected for vegetative cells of the strains NCTC8239 and 945P carrying a chromosomal and

TABLE 2. *Estimated GOL (germination, outgrowth, and lag) times (hours) of* Clostridium perfringens *790-94 and 44071.C05 in fluid thioglycollate medium (FTG) and heat-treated minced turkey*[a]

Strain	Medium	GOL times (h) at temp (°C):				
		20	30	37	45	50
790-94	FTG	10.9	3.8	2.8	1.5	1.0
	Heat-treated minced turkey	11.7	2.3	1.8	1.2	1.1
44071.C05	FTG	12.0	2.8	1.1	1.5	>7
	Heat-treated minced turkey	9.5	2.5	2.1	1.3	2.0

[a] The experimental data used to generate the growth curves and calculate the GOL times consisted of duplicate samples removed 5 to 12 times during the experiments.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/67/1/83/1672468/0362-028x-67_1_83.pdf by guest on 12 January 2023

TABLE 3. *Estimated generation times (minutes) of* Clostridium perfringens *790-94 and 44071.C05 in fluid thioglycollate medium (FTG) or heat-treated minced turkey*[a]

Strain	Medium	Generation times (min) at temp (°C): 20	30	37	45	50
790-94	FTG[b]	113	20.4	14.8	14.0	15.9
	Heat-treated minced turkey	79.0	14.6	12.2	8.2	8.2
44071.C05	FTG[b]	100	28.1	26.8	37.2	NG[c]
	Heat-treated minced turkey	84.4	12.9	11.8	17.8	17.0

[a] The experimental data used to generate the growth curves and calculate the generation times consisted of duplicate samples removed 5 to 12 times during the experiments.

[b] The generation time in FTG is the average of estimates from experiments with vegetative cells and spores, as there was no statistically significant difference ($P = 0.998$) between these.

[c] NG, no growth.

plasmid *cpe* gene, respectively, at 50°C (Fig. 1B). When spores of *C. perfringens* 790-94 and 44071.C05 were inoculated into FTG, no outgrowth was observed for spores of strain 44071.C05 at 50°C during the 7-h experiment, whereas strain 790-94 initiated exponential growth after a GOL time of 1.0 h (Fig. 1D and Table 2). Likewise, in heat-treated minced turkey at 45 and 50°C, strain 790-94 reached the highest maximum viable cell level. Unlike the results in FTG, strain 44071.C05 was able to grow in turkey meat at 50°C, but both GOL and generation times were approximately twofold longer than for 790-94 (Fig. 1F and Table 2).

The overall shortest GOL times were around 1 h and were detected at a higher temperature for strain 790-94 than for strain 44071.C05 (Table 2). At 20°C, the GOL times were approximately three- to fivefold longer than at 30°C. There was no statistically significant difference ($P = 0.23$) between the GOL times in FTG versus heat-treated minced turkey, but the generation times in FTG were statistically significantly ($P = 0.005$) longer than in turkey. For strain 790-94, the shortest generation time observed was 8.2 min in heat-treated minced turkey at 45 and 50°C. For strain 44071.C05, the shortest generation time, 11.8 min, was observed at 37°C (Table 3).

Growth during cooling from 65 to 10°C. No statistically significant ($P \geq 0.07$) outgrowth of spores of either *C. perfringens* 790-94 or 44071.C05 in heat-treated minced turkey was observed during the 3-h cooling from 65 to 10°C. Using the 4-h cooling profile, only growth of 790-94 was observed (Fig. 2). Growth was detected for both strains using 5-, 6-, and 7-h cooling profiles (Figs. 2 and 3), where the GOL times were approximately 2 and 2.5 h for strains 790-94 and 44071.C05, respectively. *C. perfrin-*

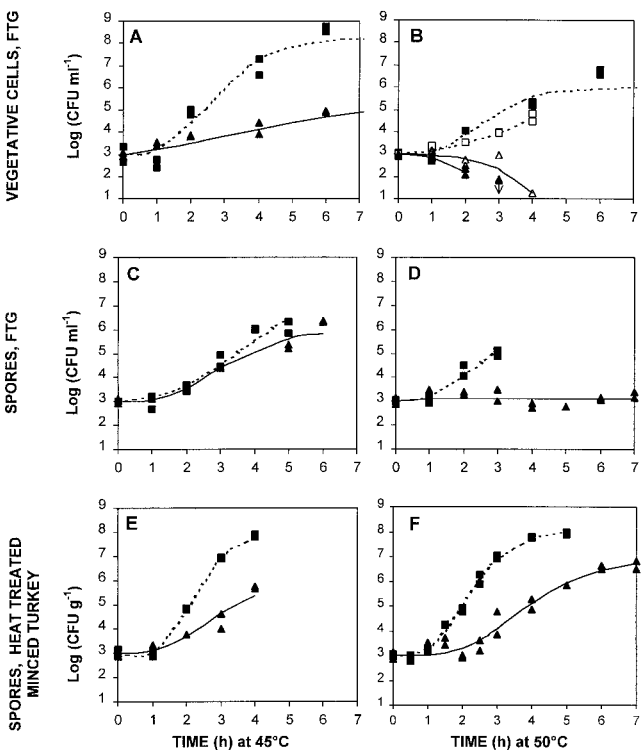

FIGURE 1. *Growth of vegetative cells of* Clostridium perfringens *790-94 (■) and 44071.C05 (▲) at 45°C in fluid thioglycollate medium (FTG) (A). Growth of vegetative cells of* C. perfringens *790-94 (■), 44071.C05 (▲), NCTC8239 (□), and 945P (△) at 50°C in FTG (B). Outgrowth of spores of* C. perfringens *790-94 (■) and 44071.C05 (▲) in FTG at 45°C (C) and 50°C (D) and in heat-treated minced turkey at 45°C (E) and 50°C (F). The dotted and solid lines represent the fitted growth curves.*

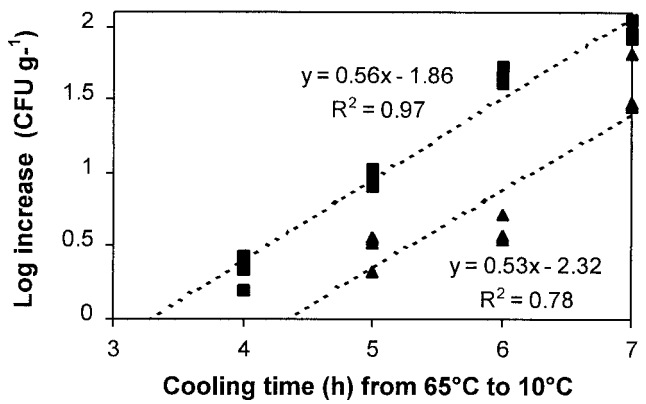

FIGURE 2. *Log increase of* Clostridium perfringens *790-94 (■) and 44071.C05 (▲) in heat-treated minced turkey during cooling from 65 to 10°C in 4, 5, 6, and 7 h. The dotted lines represent the linear regression lines, of which the formula and correlation coefficient* (R^2) *are also shown.*

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/67/1/83/1672468/0362-028x-67_1_83.pdf by guest on 12 January 2023

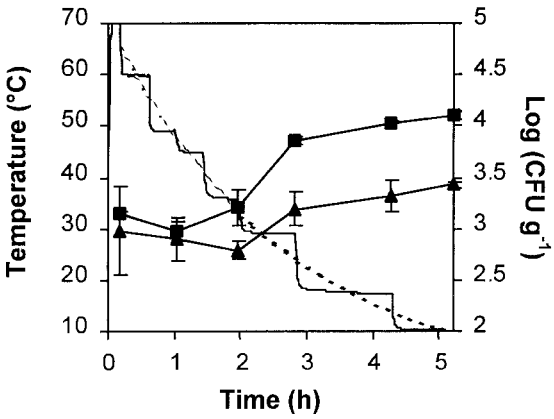

FIGURE 3. *Outgrowth of spores of* Clostridium perfringens *790-94 (■) and 44071.C05 (▲) in heat-treated minced turkey during cooling from 65 to 10°C in 5 h. The dotted line (·····) represents the cooling profile modeled by equation 1, and the solid line (——) represents the actual temperature measured during heating (70°C, 10 min) and cooling. The error bars shown are the standard deviation of the replicates.*

gens 790-94 grew to statistically significantly ($P \leq 0.005$) higher levels than *C. perfringens* 44071.C05 (Fig. 2). An almost linear relationship between the cooling time and the log increase was observed for both strains in the examined cooling time range from 4 to 7 h (Fig. 2).

DISCUSSION

Spores of strain 790-94 carrying a chromosomal *cpe* gene were more heat resistant than spores of strain 44071.C05 carrying a plasmid *cpe* gene, which is in agreement with observations by Sarker et al. *(21).* This difference in heat resistance was observed for both sporulation temperatures used. Sarker et al. *(21)* suggested that the higher heat resistance of the chromosomal *cpe* isolates was part of the reason for the strong association between these isolates and food poisoning outbreaks. For thoroughly heated foods, e.g., casseroles, this could be the case. Hansen and Knøchel *(13)* measured the minimum pasteurization value of the turkey meat in a commercially produced hot-filled turkey casserole to be 22 min at 100°C (z = 10°C). According to Sarker et al. *(21),* this heat treatment would inactivate spores of strains carrying a plasmid *cpe* gene. It is therefore likely that production of thoroughly heated foods will only allow spores of highly heat-resistant isolates to survive and, subsequently, germinate and multiply post-processing. However, in foods that are only mildly heated, e.g., roasts, spores of the low heat resistance strains may also survive. In a study by Hansen et al. *(14),* commercially produced sous vide roast beef was cooked to a pasteurization value of approximately 10 min at 70°C (z = 10°C). The lowest reported D-value for spores of strains carrying a plasmid *cpe* gene (1.0 min at 85°C, present study) corresponds to 32 min at 70°C (if z = 10°C), which means that the heat treatment of the roast beef will cause less than a 1-log reduction of these spores. In very marginally heat-treated foods, spores of strains with both high and low heat resistance may therefore survive, and their growth kinetics would therefore determine the final level in the food. We showed that both strains carrying a chromosomal *cpe* gene had a higher temperature growth range than the two strains with the plasmid *cpe* gene. Furthermore, strain 790-94 grew faster at the upper end of the temperature growth range than strain 44071.C05. If the hot-holding temperature is too low, the chromosomal *cpe* isolates examined in this paper would outgrow the plasmid *cpe* isolates and, as a result, only chromosomal *cpe* isolates would be detected, and hot-holding temperatures at around 50°C would only allow the growth of the latter genotype. We also observed that during the cooling from 65 to 10°C, strain 790-94 grew to a significantly higher level than strain 44071.C05; strain 790-94 is therefore more likely to reach a level that can cause food poisoning. Further investigations with a larger panel of strains carrying the chromosomal versus the plasmid *cpe* gene should be performed to validate these differences in growth range and kinetics. It is also possible that other differences, such as the prevalence in raw foods, add to the dominance of chromosomal *cpe* isolates in foodborne disease.

In the present study, germination, outgrowth, and growth of *C. perfringens* in minced turkey were observed when the cooling time from 65 to 10°C was ≥4 h, which corresponds to about ≥2 h in the growth range (50 to 15°C) of *C. perfringens.* Our 5-h cooling period corresponds roughly to U.S. Department of Agriculture stabilization guidelines for the cooling of meat products *(30),* which require that the temperature interval from 54.4 to 26.6°C be passed in 1.5 h and that the interval from 26.6 to 4.4°C be passed in 5 h; these intervals were passed in 1.9 and 4.7 h in our experiments, respectively. Within that period, the observed growth was less than 1 log. Danler et al. *(9)* recently simulated a cooling profile close to U.S. Department of Agriculture guidelines (except the first interval was passed in 2 h instead of 1.5) using beef and pork homogenate and also observed limited growth of up to 0.7 log. Compared with the studies by Juneja et al. *(16),* Steele and Wright *(25),* and Thippareddi et al. *(29)* (Table 4), we observed a more rapid growth. Part of this could be due to different inoculation levels, heat treatments, growth conditions (pH of meat, added ingredients, degree of treatment before inoculation and packaging), or strains. However, the model system in our study reflects a situation in which meat containing spores of *C. perfringens* is given a mild heat treatment corresponding to sous vide cooked roast beef and is subsequently exposed to an exponential cooling. If the results are considered in relation to a safe cooling rate of heat-treated food, the initial count of *C. perfringens* spores is of relevance. According to the U.S. Department of Agriculture-Food Safety and Inspection Service *(30),* 4 log *C. perfringens* g^{-1} has been found in chicken. However, Taormina et al. *(26),* who examined 99 raw uncured meat products, found the maximum total level of *C. perfringens* to be 2.9 log g^{-1}, of which 2.1 log g^{-1} were spores. Spices and herbs are reported to contain up to 4 log g^{-1} *(17).* A level of more than 4 log g^{-1} is therefore highly unlikely in a product prior to heat treatment, and only a small percentage of these are likely to be CPE+. If foods containing more than 5 log g^{-1} are considered hazardous *(30),* the maximum accept-

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/67/1/83/1672468/0362-028x-67_1_83.pdf by guest on 12 January 2023

TABLE 4. *Published data on growth of* Clostridium perfringens *during cooling*

Growth medium	Strains	Inital count (log CFU g^{-1} or ml^{-1})	Heat treatment	Temp range (°C)	Cooling time (h)	GOL time (h)	Increase (log CFU ml^{-1} or g^{-1})	Reference
FTG	H-3	4	80°C, 15 min	60 → 10[a]	≤3.3	—[b]	0	*22*[c]
					5	3	1	
					6.7	5	1.5	
					10	6	3	
Autoclaved ground beef (pH 6), 15 g, tubes	H-3	4	80°C, 15 min	60 → 15[a]	4.5	3.5	0.7	
					9	4	3	
				60 → 25[a]	4.7	2.3	3	
Chili, 20 g, tubes	NCTC8679, 8238, 8239, R42, PS44	4	75°C, 20 min	50 → 25	4	2	2.6	*2*[c]
					6	2	3	
Autoclaved ground beef (pH 6.2), 3 g, bags (15 by 22.9 cm), vacuum	NCTC8238, 3239, ATCC10288	1.5	75°C, 20 min[d] + 4.4°C → 60°C, 1 h	54.4 → 7.2	<12	—	0	*16*[c]
					15	3	1	
					18	2	4.5	
Turkey roast, 4 kg	NCTC8238, 8239, 10388	1	To 72°C (internal)	48.9 → 12.8	6	—	0	*25*[c]
					8	4	0.7	
					10	3	1.3	
Cured[e] ground beef (pH 5.1), 10 g, bags (5 by 7.6 cm), vacuum	NCTC8238, 8239, 10240	2.5	75.5°C, 20 min	54.5 → 7.2	18	NR[f]	1.5	*20*
					21	NR	5.3	
Cured[e] ground pork (pH 6.1), 10 g, bags (5 by 7.6 cm), vacuum	NCTC8238, 8239, 10240	2.5	75.5°C, 20 min	54.5 → 7.2	18	NR	3.7	
					21	NR	4.4	
Cooked beef homogenate (pH 5.9), 25 g, vacuum	NCTC8238, 8239, 10240	3.0	75°C, 10 min	54.4 → 26.6	2			*9*
				26.6 → 4.4	5	NR	0.5	
Cooked pork homogenate (pH 6.3), 25 g, vacuum	NCTC8238, 8239, 10240	3.4	75°C, 10 min	54.4 → 26.6	2			
				26.6 → 4.4	5	NR	0.7	

[a] Linear cooling profile.
[b] —, no growth.
[c] Estimated from growth curves by authors.
[d] The spore suspension was heat shocked before inoculation.
[e] Injected with 0.85% NaCl, 0.25% potato starch, and 0.2% potassium tetrapyrophosphate.
[f] NR, not reported.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/67/1/83/1672468/0362-028x-67_1_83.pdf by guest on 12 January 2023

able increase during the cooling of foods containing very high initial levels is 1 log g^{-1} corresponding to a maximum acceptable cooling time from 65 to 10°C of 5 h. As an example of the cooling legislation in a European Union member state, heat-treated foods in Denmark must be cooled from 65 to 10°C within 3 h *(1).* This is, according to the present results, an overly strict guideline. The most important temperature interval to pass in order to restrict the growth of *C. perfringens* is between 50 and 15°C, and, according to our results, this interval should be passed within 3 h in order not to reach levels of *C. perfringens* that can cause food poisoning.

ACKNOWLEDGMENT

This project was financially supported by Danish Directorate for Food, Fisheries, and Agro Business.

REFERENCES

1. Anonymous. 2001. Bekendtgørelse nr. 198 af 22/03/2001 om fødevarehygiejne. (Order no. 198 of 22/03/2001 on Food Hygiene.)
2. Blankenship, L. C., S. E. Craven, R. G. Leffler, and C. Custer. 1988. Growth of *Clostridium perfringens* in cooked chili during cooling. *Appl. Environ. Microbiol.* 54:1104–1108.
3. Brynestad, S., and P. E. Granum. 2002. *Clostridium perfringens* and food borne infections. *Int. J. Food Microbiol.* 74:195–202.
4. Brynestad, S., B. Synstad, and P. E. Granum. 1997. The *Clostridium perfringens* enterotoxin gene is on a transposable element in type A human food poisoning strains. *Microbiology* 143:2109–2115.
5. Cole, M. B., and M. V. Jones. 1990. A submerged-coil heating apparatus for investigating thermal inactivation of microorganisms. *Lett. Appl. Microbiol.* 11:233–235.
6. Collie, R. E., J. F. Kokai-Kun, and B. A. McClane. 1998. Phenotypic characterization of enterotoxigenic *Clostridium perfringens* isolates from non-food-borne human gastrointestinal diseases. *Anaerobe* 4: 69–79.
7. Collie, R. E., and B. A. McClane. 1998. Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastrointestinal diseases. *J. Clin. Microbiol.* 36:30–36.
8. Cornillot, E., B. Saint-Joanis, G. Daube, S. Katayama, P. E. Granum, B. Canard, and S. T. Cole. 1995. The enterotoxin gene (*cpe*) of *Clostridium perfringens* can be chromosomal or plasmid-borne. *Mol. Microbiol.* 15:639–647.
9. Danler, R. J., E. A. E. Boyle, C. L. Kastner, H. V. Thippareddi, D. Y. C. Fung, and R. K. Phesus. 2003. Effect of chilling rate on outgrowth of *Clostridium perfringens* spores in vacuum-packaged cooked beef and pork. *J. Food Prot.* 66:501–503.
10. Daube, G., P. Simon, B. Limbourg, C. Manteca, J. Mainil, and A. Kaeckenbeeck. 1996. Hybridization of 2659 *Clostridium perfringens* isolates with gene probes for seven toxins (α, β, ϵ, ι, θ, μ, and enterotoxin) and for sialidase. *Am. J. Vet. Res.* 57:496–501.
11. Duncan, C. L., and D. H. Strong. 1968. Improved medium for sporulation of *Clostridium perfringens. Appl. Microbiol.* 16:82–89.
12. Gibson, A. M., N. Bratchell, and T. A. Roberts. 1987. The effect of sodium chloride and temperature on the rate and extent of growth of *Clostridium botulinum* type A in pasteurised pork slurry. *J. Appl. Bacteriol.* 62:479–490.
13. Hansen, T. B., and S. Knøchel. 1999. Quantitative consideration used in HACCP applied for hot-fill production line. *Food Control* 10:149–159.
14. Hansen, T. B., S. Knøchel, D. Juncher, and G. Bertelsen. 1995. Storage characteristics of sous vide cooked roast beef. *Int. J. Food Sci. Technol.* 30:365–378.
15. Juneja, V. K., J. S. Novak, L. Huang, and B. S. Eblen. 2003. Increased thermotolerance of *Clostridium perfringens* following sublethal heat shock. *Food Control* 14:163–168.
16. Juneja, V. K., O. P. Snyder, and M. Cygnarowicz-Provost. 1994. Influence of cooling rate on outgrowth of *Clostridium perfringens* spores in cooked ground beef. *J. Food Prot.* 57:1063–1067.
17. Labbé, R. G. 2000. *Clostridium perfringens,* p. 1110–1135. *In* B. M. Lund, T. Baird-Parker, and G. W. Gould (ed.), The microbiological safety and quality of food, vol. II. Aspen Publishers, Inc., Gaithersburg, Md.
18. Kokai-Kun, J. F., J. G. Songer, J. R. Czeczulin, F. Chen, and B. A. McClane. 1994. Comparison of western immunoblots and gene detection assays for identification of potentially enterotoxigenic isolates of *Clostridium perfringens. J. Clin. Microbiol.* 32:2533–2539.
19. McDonel, J. L., and B. A. McClane. 1988. Production, purification, and assay of *Clostridium perfringens* enterotoxin. *Methods Enzymol.* 165:94–103.
20. Olsen, S. J., L. C. MacKinnon, J. S. Goulding, N. H. Bean, and L. Slutsker. 2000. Surveillance for foodborne-disease outbreaks—United States, 1993–1997. *Morb. Mortal. Wkly. Rep.* 49:1–51.
21. Sarker, M. R., R. P. Shivers, S. G. Sparks, V. K. Juneja, and B. A. McClane. 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.
22. Shigesa, T., T. Nakagami, and S. Taji. 1985. Influence of heating and cooling rates on spore germination and growth of *Clostridium perfringens* in media and in roastbeef. *Jpn. J. Vet. Sci.* 47:259–267.
23. Songer, J. G., and R. P. Meer. 1996. Genotyping of *Clostridium perfringens* by polymerase chain reaction is a useful adjunct to diagnosis of clostridial enteric disease in animals. *Anaerobe* 2:197–203.
24. Sparks, S. G., R. J. Carman, M. R. Sarker, and B. A. McClane. 2001. Genotyping of enterotoxigenic *Clostridium perfringens* fecal isolates associated with antibiotic-associated diarrhea and food poisoning in North America. *J. Clin. Microbiol.* 39:883–888.
25. Steele, F. M., and K. H. Wright. 2001. Cooling rate effect on outgrowth of *Clostridium perfringens* in cooked, ready-to-eat turkey breast roasts. *Poult. Sci.* 80:813–816.
26. Taormina, P. J., G. W. Bartholomew, and W. J. Dorsa. 2003. Incidence of *Clostridium perfringens* in commercially produced cured raw meat product mixtures and behavior in cooked products during chilling and refrigerated storage. *J. Food Prot.* 66:72–81.
27. The Danish Veterinary and Food Administration. 2000. Reported cases of food borne diseases 1999. Available (in Danish) at: http://www.foedevaredirektoratet.dk/kontrolinfo/uft/heltxt.html. Accessed 24 November 2001.
28. The Danish Veterinary and Food Administration. 2001. Reported cases of food borne diseases 2000. Available (in Danish) at: http://www.vfd.dk/publikationer/publikationer/publikationer/fodbaarsyg2000/indberettet/txt.html. Accessed 24 November 2001.
29. Thippareddi, H., V. K. Juneja, R. K. Phebus, J. L. Marsden, and C. L. Kastner. 2003. Control of *Clostridium perfringens* germination and outgrowth by buffered sodium citrate during chilling of roast beef and injected pork. *J. Food Prot.* 66:376–381.
30. U.S. Department of Agriculture, Food Safety and Inspection Service. 1999. Performance standards for the production of certain meat and poultry products. *Fed. Regist.* 64:732–749.
31. van Damme-Jongsten, M., K. Wernars, and S. Notermans. 1989. Cloning and sequencing of the *Clostridium perfringens* enterotoxin gene. *Antonie Leeuwenhoek* 56:181–190.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/67/1/83/1672468/0362-028x-67_1_83.pdf by guest on 12 January 2023