Further Comparison of Temperature Effects on Growth and Survival of *Clostridium perfringens* Type A Isolates Carrying a Chromosomal or Plasmid-Borne Enterotoxin Gene

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Clostridium perfringens type A isolates can carry the enterotoxin gene (*cpe*) on either their chromosome or a plasmid, but food poisoning isolates usually have a chromosomal *cpe* gene. This linkage between chromosomal *cpe* isolates and food poisoning has previously been attributed, at least in part, to better high-temperature survival of chromosomal *cpe* isolates than of plasmid *cpe* isolates. In the current study we assessed whether vegetative cells and spores of chromosomal *cpe* isolates also survive better than vegetative cells and spores of plasmid *cpe* isolates survive when the vegetative cells and spores are subjected to low temperatures. Vegetative cells of chromosomal *cpe* isolates exhibited about eightfold-higher decimal reduction values (D values) at 4°C and threefold-higher D values at –20°C than vegetative cells of plasmid *cpe* isolates exhibited. After 6 months of incubation at 4°C and –20°C, the average log reductions in viability for spores of plasmid *cpe* isolates were about fourfold and about threefold greater, respectively, than the average log reductions in viability for spores from chromosomal *cpe* isolates. *C. perfringens* type A isolates carrying a chromosomal *cpe* gene also grew significantly faster than plasmid *cpe* isolates grew at 25°C, 37°C, or 43°C. In addition, chromosomal *cpe* isolates grew at higher maximum and lower minimum temperatures than plasmid *cpe* isolates grew. Collectively, these results suggest that chromosomal *cpe* isolates are commonly involved in food poisoning because of their greater resistance to low (as well as high) temperatures for both survival and growth. They also indicate the importance of proper low-temperature storage conditions, as well as heating, for prevention of *C. perfringens* type A food poisoning.

Clostridium perfringens isolates are commonly classified into five types (types A to E) based on the production of four typing toxins (alpha, beta, epsilon, and iota toxins) (24). Type A isolates, the most abundant toxinotype, produce alpha toxin, but not beta, epsilon, or iota toxin (11, 14, 19). Some type A isolates also produce another toxin, *C. perfringens* enterotoxin (CPE). These enterotoxigenic type A strains cause several human enteric diseases, including *C. perfringens* type A food poisoning, which is among the three most common food-borne illnesses in the United States, and some cases of non-food-borne human gastrointestinal disease, including antibiotic-associated diarrhea and sporadic diarrhea (5, 6).

In *C. perfringens* type A isolates, the enterotoxin gene (*cpe*) can be located on either the chromosome or a large plasmid (6, 7). Interestingly, in food poisoning isolates the *cpe* gene is typically on the chromosome, perhaps due to integration of a 6.3-kb *cpe*-carrying transposon that is flanked by IS1470 sequences (3). In contrast, the *cpe* gene is usually plasmid borne in non-food-borne human gastrointestinal disease isolates, and it typically is present on either a 75.3-kb plasmid that also encodes beta2 toxin or a 70.5-kb plasmid that lacks the beta2 toxin gene (10, 16, 17). Both *cpe* plasmids found in type A isolates may be capable of conjugative transfer due to the presence of Tn916-related open reading frames (4, 17).

To date, there are at least two explanations for the strong

association between type A isolates carrying a chromosomal *cpe* gene and food poisoning. First, a recent study (26) that evaluated the presence of *cpe*-positive isolates in American retail foods showed that all 13 *cpe*-positive type A isolates recovered from the foods surveyed had a chromosomal *cpe* gene. The findings indicated that, at least in part, chromosomal *cpe* isolates are the predominant cause of food poisoning because they are the *cpe*-positive type A isolates that are most often present in food.

However, other evidence suggests that the strong association between chromosomal *cpe* isolates and food poisoning may involve additional factors. Several labs (1, 2, 22) have determined that the vegetative cells or spores of type A chromosomal *cpe* isolates obtained from food poisoning outbreaks survive heating much better than the cells or spores of type A plasmid *cpe* isolates survive heating. For example, one study (22) showed that the average decimal reduction value (D value) at 55°C for vegetative cells of chromosomal *cpe* food poisoning isolates was twofold higher than the average D value at 55°C for vegetative cells of plasmid *cpe* isolates. Furthermore, the average D value at 100°C for spores of chromosomal *cpe* food poisoning isolates was nearly 60-fold higher than the average D value at 100°C for spores of plasmid *cpe* isolates. These *cpe* genotype-related heat resistance differences appear to be important since survival at elevated temperatures should be helpful for a food-borne pathogen like *C. perfringens* that is primarily transmitted via temperature-abused foods.

When spores of the chromosomal *cpe* isolates recovered from retail foods during a recent survey (26) were tested for

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heat sensitivity, they also exhibited considerably greater heat resistance than the spores of type A plasmid *cpe* isolates exhibited. Since the chromosomal *cpe* isolates were recovered from raw meat that was never cooked, heat resistance appears to be an intrinsic trait of chromosomal *cpe* isolates rather than merely a trait selected for in food poisoning isolates that survive cooking. Little is known about the mechanisms responsible for the specific heat resistance of chromosomal *cpe* isolates, although a recent study (20) showed that (i) the heat resistance of chromosomal *cpe* isolates is not dependent on the presence of a functional *cpe* gene and (ii) the heat sensitivity of plasmid *cpe* isolates is not dependent on the presence of a *cpe* plasmid.

In the food environment, pathogens often encounter many lethal or inhibitory environmental stresses in addition to heat. One of the most important such food stresses facing a pathogen is low temperature, which is universally used for food preservation in the form of either refrigeration or freezing (18). Since understanding the strong association between type A isolates carrying a chromosomal *cpe* gene and food poisoning has implications for controlling *C. perfringens* type A food poisoning, in the current study we compared the effects of temperature, especially refrigeration and freezer temperatures, on both the survival and the growth of type A isolates carrying a chromosomal *cpe* gene and type A isolates carrying a plasmid-borne *cpe* gene.

MATERIALS AND METHODS

Bacteria. The *C. perfringens* type A isolates used in this study are listed and described in Table 1. All isolates were maintained as stock cultures in cooked meat medium (Oxoid), which were stored at -20°C .

Evaluation of survival of *C. perfringens* vegetative cells at low temperatures. To determine the survival of *C. perfringens* vegetative cells at low temperatures (4°C and -20°C), a starter vegetative culture of each *C. perfringens* isolate surveyed was prepared by inoculating 0.1 ml of the cooked meat medium stock culture into 6 ml of fluid thioglycolate (FTG) medium (Difco). After overnight growth of the starter culture at 37°C , a 0.2-ml aliquot was inoculated into 6 ml of fresh FTG medium, which was then incubated for 2 h at 37°C . Following mixing, a 0.1-ml aliquot of each 2-h FTG medium culture was serially diluted (dilution range, 10^{-2} to 10^{-7}) with sterile FTG medium. The diluted samples were plated onto brain heart infusion (BHI) (BD Biosciences) agar plates, which were incubated overnight in an anaerobe jar to determine the total number of vegetative cells that were present in each FTG medium culture at the start (day 0) of the cold treatment. The remainder of each 2-h FTG medium culture was then mixed well and divided into small tubes, half of which were incubated in a cold room kept at $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and half of which were incubated in a commercial freezer kept at $-20^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Aliquots were removed from these small tubes every 2 to 7 days for up to 4 weeks. After an aliquot was removed, the tube was sterilized and discarded. Each aliquot removed was diluted (dilution range, from 10^{-2} to 10^{-7}) with sterile FTG medium, and each dilution was immediately plated onto BHI agar plates. After overnight anaerobic incubation, colonies on the BHI agar plates were counted to determine the number of viable CFU that was present per ml of culture at each time for both low-temperature conditions. The CFU values were then graphed to determine a D value, which is the time that a culture must be kept at a given temperature until there is a 90% reduction in the number of viable vegetative cells for the *C. perfringens* isolate examined (22).

Evaluation of survival of *C. perfringens* spores at low temperatures. To assess the survival of *C. perfringens* spores at low temperatures (4°C and -20°C), sporulating cultures were prepared for each *C. perfringens* isolate. For all isolates surveyed except 458 and C1841 this involved inoculating 0.2 ml of a starter FTG medium culture (prepared as described above) into 10 ml of Duncan-Strong (DS) sporulation medium (12). Because isolates 458 and C1841 sporulated better in modified Duncan-Strong (MDS) sporulation medium (12), sporulating cultures of these two isolates were prepared by inoculating 0.2 ml of an FTG medium starter culture into 10 ml of MDS medium. After overnight incubation at 37°C , the presence of sporulating cells in each DS or MDS medium culture was confirmed by phase-contrast microscopy. Following mixing, 1-ml aliquots

TABLE 1. Isolates used in this study

Isolate	Source	Genotype
NB16	Human antibiotic-associated diarrhea, Europe, 1980s	Plasmid <i>cpe</i> , IS1151
T34058	Human antibiotic-associated diarrhea, North America, late 1990s	Plasmid <i>cpe</i> , IS1470-like
F4969	Human sporadic diarrhea, Europe, 1990s	Plasmid <i>cpe</i> , IS1470-like
F5603	Human sporadic diarrhea, Europe, 1990s	Plasmid <i>cpe</i> , IS1151
153	Pig enteritis, Scotland, 1990s	Plasmid <i>cpe</i> , IS1470-like
222	Dog diarrhea, United States, 1990s	Plasmid <i>cpe</i> , IS1470-like
458	Dog diarrhea, North America, early 1990s	Plasmid <i>cpe</i> , IS1470-like
NCTC8239	Food poisoning outbreak, Europe, 1950s	Chromosomal <i>cpe</i>
NCTC10239	Food poisoning outbreak, Europe, 1950s	Chromosomal <i>cpe</i>
C-1841	Vermont food poisoning outbreak, 1980s	Chromosomal <i>cpe</i>
FD1041	North American food poisoning outbreak, 1980s	Chromosomal <i>cpe</i>
E13	Food poisoning outbreak, North America, 1960s	Chromosomal <i>cpe</i>
191-10	Food poisoning outbreak, Hawaii, 1980s	Chromosomal <i>cpe</i>
01E809	Food poisoning outbreak, Oklahoma, 1999	Chromosomal <i>cpe</i>
Pork-1	American retail food, 2003	Chromosomal <i>cpe</i>
Sausage-1	American retail food, 2003	<i>cpe</i> negative
Fish-2	American retail food, 2003	<i>cpe</i> negative
Steak	American retail food, 2003	<i>cpe</i> negative
ATCC 3624	Control	<i>cpe</i> negative

were removed from each sporulating culture and heat shocked at 75°C for 20 min to kill any remaining vegetative cells and to promote spore germination. A 0.1-ml aliquot of each heat-shocked DS or MDS medium culture was then serially diluted (dilution range, 10^{-2} to 10^{-7}) with sterile FTG medium. The diluted samples were plated onto BHI agar plates, which were incubated overnight in anaerobe jars at 37°C to determine the total number of spores that were present in each DS medium culture at the start (day 0) of each cold treatment. The remainder of each DS or MDS medium culture was divided into small tubes, half of which were incubated at 4°C and half of which were incubated at -20°C . Aliquots were removed from these small tubes every 2 months for up to 6 months. After the aliquots were removed, the tubes were sterilized and discarded. The aliquots that were removed were heat shocked at 75°C for 20 min and then diluted (dilution range, 10^{-2} to 10^{-7}) with sterile FTG medium. Each dilution was immediately plated onto BHI agar plates. After overnight anaerobic incubation at 37°C , the colonies on each BHI agar plate were counted to determine the number of viable spores that were present per ml of culture at each time for each low-temperature treatment. The values were then graphed to determine the log reduction in the number of viable spores over 6 months for each *C. perfringens* isolate (1, 15).

Temperature effects on the growth rate and mean generation time of *C. perfringens* isolates carrying a chromosomal *cpe* gene or a plasmid-borne *cpe* gene. After inoculation from an FTG medium starter culture (prepared as described above), a 10-ml FTG medium culture was grown at 37°C until the stationary phase was reached (~ 8 to 10 h), based on the optical density at 600 nm (OD_{600}) of the culture. A 0.1-ml aliquot was removed from each FTG medium stationary-phase culture and transferred into 10 ml of fresh FTG medium, which was then incubated at 4°C , 25°C , 37°C , 43°C , 50°C , or 55°C . Every 1 to 2 h, an aliquot of each culture was removed, and the OD_{600} of the aliquot was determined. From the OD_{600} values, a growth curve was plotted, which allowed calculation of a growth rate constant and a mean generation time for each isolate (25).

Determination of maximum and minimum growth temperatures for *C. perfringens* isolates carrying a chromosomal *cpe* gene or a plasmid-borne *cpe* gene. To determine the minimum and maximum growth temperature for each *C. perfringens* isolate surveyed, 0.1 ml of an FTG medium stationary-phase culture was transferred into 10 ml of fresh FTG medium. The cultures were then incubated at temperatures between 10°C and 20°C (for 1 week) or between 49°C

FIG. 1. Thermal death curves at low temperatures for vegetative cells of NCTC10239, a type A isolate carrying a chromosomal *cpe* gene, and isolate 458, a type A isolate carrying a plasmid *cpe* gene. Vegetative cultures of NCTC10239 or isolate 458 were incubated at 4°C (A) or -20°C (B) for different times, and then aliquots were removed and plated onto BHI agar to determine the number of viable bacteria per milliliter of culture. The data are the results of a representative experiment; similar results were obtained when the experiment was repeated.

and 54°C (for 12 h). At defined intervals, the OD₆₀₀ of each culture was determined to evaluate isolate growth.

Statistical analyses. Statistical analyses were performed using the Student *t* test.

RESULTS

Comparison of the low-temperature survival of vegetative cells of *C. perfringens* type A isolates carrying a chromosomal *cpe* gene and the low-temperature survival of vegetative cells of *C. perfringens* type A isolates carrying a plasmid-borne *cpe* gene. The survival of vegetative cells of 15 *C. perfringens* type A isolates carrying either a chromosomal *cpe* gene or a plasmid *cpe* gene was determined at 4°C (refrigeration temperature) and -20°C (commercial freezer temperature), and the results were compared. Figure 1 shows representative death curves obtained at 4°C and -20°C for NCTC10239, a type A food poisoning isolate carrying a chromosomal *cpe* gene, and for type A isolate 458, which carries a plasmid *cpe* gene. From the results shown in Fig. 1, the D value at 4°C (D₄ value) for NCTC10239 was calculated to be 11 days, while the D₄ value for isolate 458 was determined to be 1.8 days. The D values

FIG. 2. Survival of *cpe*-positive *C. perfringens* type A vegetative cells at low temperatures: average D values for vegetative cells incubated at 4°C or -20°C for eight type A chromosomal *cpe* isolates and seven type A plasmid *cpe* isolates. At each temperature, D values were determined in two independent experiments for each isolate, as described in Materials and Methods. The error bars indicate standard deviations, and two asterisks indicate that the difference is statistically significant at a *P* value of <0.01.

at -20°C (D₋₂₀) were significantly lower than the D₄ values for vegetative cells of both isolates; for NCTC10239 vegetative cells the D₋₂₀ value was 1.5 days, and for vegetative cells of isolate 458 the D₋₂₀ value was 0.6 day. To ensure that spore contamination did not affect these results, duplicate aliquots taken from each FTG medium culture were subjected to a heat shock (75°C, 20 min) prior to plating on BHI medium; no colonies grew on the plates, indicating that the FTG medium cultures did not contain spores.

Figure 2 summarizes results for similar low-temperature survival experiments performed with vegetative cultures of seven additional food poisoning isolates carrying a chromosomal *cpe* gene and six additional non-food-borne human gastrointestinal disease or veterinary isolates carrying a plasmid *cpe* gene. The average D₄ value determined for all eight *C. perfringens* chromosomal *cpe* isolates surveyed (Fig. 2) was nearly eightfold higher than the average D₄ value for the seven *C. perfringens* plasmid *cpe* isolates surveyed; the difference is statistically significant at a *P* value of <0.01. Similarly, Fig. 2 shows that the average D₋₂₀ value for the eight *C. perfringens* chromosomal *cpe* isolates was more than threefold higher than the average D₋₂₀ value for the seven *C. perfringens* plasmid *cpe* isolates surveyed, and the difference is also statistically significant at a *P* value of <0.01. Notably, at either 4°C or -20°C, there was no overlap between the vegetative cell survival of chromosomal *cpe* isolates and the vegetative cell survival of plasmid *cpe* isolates; i.e., the D₄ and D₋₂₀ values for the vegetative cells of the least cold-resistant chromosomal *cpe* isolates were higher than the D₄ and D₋₂₀ values for the vegetative cells of the most cold-resistant plasmid *cpe* isolates.

For comparison, D₄ and D₋₂₀ values were also calculated for vegetative cells of four *cpe*-negative type A isolates. The D values determined for these *cpe*-negative isolates were between the D values shown in Fig. 2 for the type A chromosomal *cpe*

FIG. 3. Thermal death curves at low temperatures for spores from NCTC10239, a type A isolate carrying a chromosomal *cpe* gene, and isolate 458, a type A isolate carrying a plasmid *cpe* gene. Sporulating cultures of NCTC10239 or isolate 458 were incubated at 4°C (A) and -20°C (B) for different times, and then aliquots were removed, heat shocked, and plated onto BHI agar to determine the number of viable bacteria per milliliter of culture. The data are the results of a representative experiment; similar results were obtained when the experiment was repeated.

isolates surveyed and the D values shown in Fig. 2 for the type A plasmid *cpe* isolates surveyed (data not shown).

Comparison of the low-temperature survival of spores of *C. perfringens* type A isolates carrying a chromosomal *cpe* gene and the low-temperature survival of spores of *C. perfringens* type A isolates carrying a plasmid *cpe* gene. Microscopic spore counts (6) revealed no consistent differences in spore formation at 37°C between the chromosomal and plasmid *cpe* isolates surveyed (data not shown). When the spores formed at 37°C were subsequently incubated for up to 6 months at 4°C or -20°C, there was still not a 1-log reduction in the viability of the spores produced by some *C. perfringens* isolates. Therefore, it was necessary to express the effects of low temperature on spore viability in terms of the log reduction in the number of viable spores, a method used by other workers to study the sensitivity of *C. perfringens* spores to temperature (1, 15), rather than as formal D values.

FIG. 4. Survival of *cpe*-positive *C. perfringens* type A spores at low temperatures: average log reductions in the number of viable spores after incubation at 4°C or -20°C for eight type A chromosomal *cpe* isolates and seven type A plasmid *cpe* isolates. At each temperature, log reduction values were determined in two independent experiments for each isolate, as described in Materials and Methods. The error bars indicate standard deviations, and two asterisks indicate that the difference is statistically significant at a *P* value of <0.01.

Representative death curves obtained at 4°C and -20°C for isolates NCTC10239 and 458 are shown in Fig. 3. Figure 3 clearly shows that only 0.32- and 0.42-log reductions in spore viability occurred when chromosomal *cpe* isolate NCTC10239 was incubated for 6 months at 4°C and -20°C, respectively. In contrast, 1.34- and 1.64-log reductions in spore viability occurred when plasmid *cpe* isolate 458 was incubated for 6 months at 4°C and -20°C, respectively.

When similar analyses were performed with our 13 other *cpe*-positive isolates (Fig. 4), the average log reductions in viability for spores produced by plasmid *cpe* isolates were nearly fourfold higher than the average log reductions in viability for spores produced by chromosomal *cpe* isolates after 6 months of incubation at 4°C. Similarly, the average log reductions in viability for spores produced by plasmid *cpe* isolates were nearly threefold higher than the average log reductions in viability for spores produced by chromosomal *cpe* isolates after 6 months of incubation at -20°C. The differences between chromosomal and plasmid *cpe* isolates for both spore survival at 4°C and spore survival at -20°C were statistically significant at a *P* value of < 0.01. It is also notable that at either 4°C or -20°C there was no overlap between the spore survival of the chromosomal *cpe* isolates and the spore survival of the plasmid *cpe* isolates; i.e., spores of the most sensitive chromosomal *cpe* isolates were more cold resistant than spores of the most resistant plasmid *cpe* isolates.

For comparison, the effects of incubation at 4°C and -20°C on the viability of spores of four *cpe*-negative type A isolates were also determined. This analysis revealed (data not shown) that spores of these four CPE-negative isolates have cold sensitivity similar to that of the type A plasmid *cpe* isolates surveyed.

Temperature effects on the growth rates and mean generation times of *C. perfringens* type A isolates carrying a chromosomal *cpe* gene and *C. perfringens* type A isolates carrying a

A

B

FIG. 5. Average growth rate constants (A) and mean generation times (B) for *cpe*-positive *C. perfringens* type A isolates at various temperatures. Eight type A chromosomal *cpe* isolates and seven type A plasmid *cpe* isolates were grown at different temperatures, aliquots of each culture were removed periodically, and growth was measured spectrophotometrically, as described in Materials and Methods. At each temperature, the growth of each isolate was determined for two independent cultures. The error bars indicate standard deviations; two asterisks indicate that the difference is statistically significant at a *P* value of <0.01 , and one asterisk indicates that the difference is statistically significant at a *P* value of <0.05 .

plasmid-borne *cpe* gene. The growth rate constants and mean generation times were determined at six temperatures for eight isolates carrying a chromosomal *cpe* gene and for seven isolates carrying a plasmid *cpe* gene (Fig. 5). In this initial survey, no isolates grew at 4°C, but all 13 isolates surveyed grew at 25°C. For all isolates, the growth rate constant increased (Fig. 5A) and the mean generation time decreased (Fig. 5B) with higher temperatures up to an optimum temperature of 43°C. At temperatures above 43°C, the growth rates rapidly decreased and the generation times sharply increased, until all isolates stopped growing at 55°C.

At 25°C, the average growth rate constant for the eight chromosomal *cpe* isolates surveyed (range, 1 to 1.2) was nearly twice the average growth rate constant for the seven plasmid *cpe* isolates (range, 0.4 to 0.8); the difference is statistically significant at a *P* value of <0.01 . At 37°C, the average growth rate constant for the chromosomal *cpe* isolates (range, 2.5 to 3.4) was 1.4-fold higher than the average growth rate constant for the plasmid *cpe* isolates surveyed (range, 0.8 to 2.7). Despite a slight overlap between the growth rate constants at 37°C for the two isolate groups, the difference was still significant at a *P* value of <0.02 . Similarly, the average growth rate constant at 43°C for the chromosomal isolates surveyed (range, 3.5 to 5)

was ~1.3-fold higher than the average growth rate constant at 43°C for the plasmid *cpe* isolates (range, 1.4 to 3.8). Again, despite a slight overlap between the growth rate constants at 43°C for these two isolate groups, the growth rate differences were still significant at a *P* value of <0.02 . While the average growth rate constant at 50°C for isolates carrying a chromosomal *cpe* gene was also slightly higher than the average growth rate constant at 50°C for plasmid *cpe* isolates, the difference was not statistically significant ($P > 0.1$).

Similarly, the mean generation times at 25°C for the eight chromosomal *cpe* isolates surveyed ranged from 34 to 42 min, and the average value was approximately one-half of the value for the seven plasmid *cpe* isolates (whose generation times ranged from 54 to 101 min); the difference is statistically significant at a *P* value of <0.01 . At 37°C, the mean generation time for the chromosomal *cpe* isolates (range, 12 to 17 min) was ~60% of the mean generation time for the plasmid *cpe* isolates surveyed (range, 15 to 54 min). Despite a slight overlap between the generation times for the two isolate groups at 37°C, the difference was still significant at a *P* value of <0.03 . Similarly, the mean generation time at 43°C for the chromosomal isolates surveyed (range, 8 to 12 min) was ~65% less than the mean generation time at 43°C for the plasmid *cpe* isolates (range, 11 to 30 min). Again, despite a slight overlap between the generation times for the two isolate groups, the difference between the mean generation times at 43°C was still significant at a *P* value of <0.02 . At 50°C the mean generation time for the isolates carrying a chromosomal *cpe* gene (range, 47 to 154 min) was ~90% of the mean generation time for the plasmid *cpe* isolates (range, 82 to 149 min), but the difference was not statistically significant ($P > 0.1$).

For comparison, growth rate constants and mean generation times were also determined for vegetative cultures of four *cpe*-negative type A isolates. At 25°C, 37°C, and 50°C, the CPE-negative isolates surveyed (data not shown) had growth rates and generation times between those of the plasmid *cpe* isolates and those of the chromosomal *cpe* isolates surveyed. At 43°C, the CPE-negative isolates surveyed had an average growth rate and generation time similar to the average growth rate and generation time of chromosomal *cpe* isolates.

Comparison of the minimum and maximum growth temperatures for *C. perfringens* type A isolates carrying a chromosomal *cpe* gene and *C. perfringens* type A isolates carrying a plasmid-borne *cpe* gene. More precise experiments were then conducted to determine the exact minimum and maximum growth temperatures for the chromosomal and plasmid *cpe* isolates surveyed (Fig. 6). These studies showed that the average minimum growth temperature for the eight isolates carrying a chromosomal *cpe* gene was $12^{\circ}\text{C} \pm 1.9^{\circ}\text{C}$. In contrast, the average minimum growth temperature for the seven plasmid *cpe* isolates surveyed was $18.9^{\circ}\text{C} \pm 1.6^{\circ}\text{C}$. The minimum growth temperatures for the chromosomal *cpe* isolates surveyed ranged from 10 to 14°C, but the minimum growth temperatures for the plasmid *cpe* isolates ranged from 16 to 20°C; i.e., no overlap between the minimum growth temperatures for the two *cpe* genotypes was detected. The differences were statistically significant at a *P* value of <0.01 .

Similar conclusions were reached regarding maximum growth temperatures. The average maximum growth temperatures for the eight isolates carrying a chromosomal *cpe* gene

FIG. 6. Maximum and minimum growth temperatures for *cpe*-positive *C. perfringens* type A isolates. Eight type A chromosomal *cpe* isolates and seven type A plasmid *cpe* isolates were inoculated into cultures that were then incubated at 10 to 20°C and at 49 to 54°C. Aliquots of each culture were removed periodically, and growth was measured spectrophotometrically, as described in Materials and Methods. At each temperature, the growth of each isolate was measured for two independent cultures. The error bars indicate standard deviations, and two asterisks indicate that the difference is statistically significant at a *P* value of <0.01.

surveyed were $53.3^{\circ}\text{C} \pm 0.7^{\circ}\text{C}$, while the average maximum growth temperatures for the seven isolates carrying a plasmid *cpe* gene were $50.4^{\circ}\text{C} \pm 1.1^{\circ}\text{C}$. The differences were statistically significant at a *P* value of <0.01. The maximum growth temperatures for the chromosomal *cpe* isolates ranged from 52 to 54°C, but the maximum growth temperatures for the plasmid *cpe* isolates ranged from 48 to 51°C; i.e., there was no overlap between the maximum growth temperatures for the two *cpe* genotypes.

For comparison, the minimum and maximum growth temperatures were determined for four *cpe*-negative type A isolates, and the average minimum and maximum growth temperatures were found to be $17^{\circ}\text{C} \pm 3.5^{\circ}\text{C}$ (range, 12 to 20°C) and $51 \pm 0^{\circ}\text{C}$ (all four *cpe*-negative isolates grew at temperatures up to only 51°C), respectively.

DISCUSSION

With the ultimate goal of better controlling *C. perfringens* type A food poisoning, workers have begun investigating why this food-borne illness is so strongly associated with type A isolates carrying a chromosomal *cpe* gene. CPE is clearly responsible for the symptoms of *C. perfringens* type A food poisoning (21), but the relationship between food poisoning and chromosomal *cpe* isolates does not appear to involve isolates that produce either a more potent CPE or larger amounts of CPE than plasmid *cpe* isolates produce (6, 14). Since temperature abuse is the leading factor responsible for *C. perfringens* type A food poisoning outbreaks (14), in two studies (1, 22) workers recently compared the heat resistance of type A isolates carrying a chromosomal *cpe* gene and the heat resistance of type A isolates carrying a plasmid-borne *cpe* gene. Both studies showed that the vegetative cells and spores of chromosomal *cpe* isolates are more heat resistant than the cells and

spores of plasmid *cpe* isolates, which should favor survival of chromosomal *cpe* isolates in the inadequately heated foods often involved in food poisoning.

However, heat is not the only important physical factor commonly used for food preservation. Both cool storage (i.e., storage at temperatures from ~16°C down to 2°C) and freezing (storage at temperatures of ~-20°C) are extensively employed by the food industry and consumers to prolong the safety of cooked and raw food (9, 18). Therefore, in this study we compared the survival of chromosomal *cpe* isolates and the survival of plasmid *cpe* isolates at refrigeration (4°C) and commercial freezer (-20°C) temperatures. The results of these analyses clearly indicate that the vegetative cells and spores of chromosomal *cpe* isolates survive refrigeration and freezing significantly better than the cells and spores of plasmid *cpe* isolates survive refrigeration and freezing. Our observation that a chromosomal *cpe* isolate obtained from raw pork has resistance to refrigeration and freezing similar to that of food poisoning isolates suggests that cold resistance is an intrinsic trait of chromosomal *cpe* isolates rather than a trait selected for in isolates during food poisoning (26). Collectively, our findings strongly suggest that chromosomal *cpe* isolates are strongly associated with food poisoning not only because of their exceptional heat resistance but also because of their unusual tolerance of low temperature storage.

The determination that most or all chromosomal *cpe* isolates are unusually cold tolerant also provides one potential explanation for why in a recent survey (26) workers detected both spores and vegetative cells of chromosomal *cpe* isolates but not spores and vegetative cells of plasmid *cpe* isolates in retail foods. The foods surveyed in this study (26) included the most common vehicles for *C. perfringens* type A food poisoning, which are meats or meat-containing products normally stored at refrigeration or freezing temperatures. The greater ability of chromosomal *cpe* isolates to survive at low temperatures should increase the presence, and the probability of detection, of these isolates in foods.

In this regard, the spores of chromosomal *cpe* isolates showed a particularly remarkable ability to survive under refrigeration conditions compared to spores of either plasmid *cpe* or *cpe*-negative *C. perfringens* isolates. Even after 6 months of storage at 4°C, there was a <1-log reduction in the viability of chromosomal *cpe* spores under the experimental conditions used in our study. While there have been several reports (9, 13–15) that *C. perfringens* spores survive cold treatment better than vegetative cells survive cold treatment, an enhanced cold survival phenotype for spores (and to a lesser extent vegetative cells) of chromosomal *cpe* food poisoning isolates has not been reported previously to our knowledge. The physiologic basis of the enhanced cold and heat resistance phenotypes exhibited by the spores and vegetative cells of chromosomal *cpe* isolates is not clear, but we plan to study this question using proteomic approaches.

In addition to adaptation for surviving exposure to extreme high and low temperatures, the ability to grow rapidly should be important for *C. perfringens* food poisoning isolates since (i) *C. perfringens* food poisoning develops only after ingestion of heavily contaminated foods (1) and (ii) *C. perfringens* likely encounters growth-permissive temperatures only transiently in foods. Consistent with previous studies of *C. perfringens* growth

(8, 9, 13, 14), which involved mainly *cpe*-negative isolates, we observed optimal growth at 43°C for all *C. perfringens* isolates, whether they were *cpe* positive or *cpe* negative. As noted previously for *C. perfringens*, the mean generation time at 43°C for our *C. perfringens* isolates was extremely short; many isolates had generation times of ~10 min at this temperature. For all isolates surveyed, the growth rate decreased quickly at temperatures higher than 43°C and decreased more slowly at temperatures less than 43°C.

Specifically comparing temperature effects on the growth rates and generation times of chromosomal *cpe* and plasmid *cpe* isolates revealed that chromosomal *cpe* isolates grow faster than plasmid *cpe* isolates at 25°, 37°C, 43°C, and 50°C, and the differences are statistically significant at all temperatures except 50°C. Again, the chromosomal *cpe* isolate obtained from raw retail pork (26) had a short generation time and high growth rate constant typical of chromosomal *cpe* food poisoning isolates, suggesting that rapid growth is an intrinsic property of all chromosomal *cpe* isolates (rather than a trait selected for during food poisoning). The results for eight type A chromosomal *cpe* isolates and seven type A plasmid *cpe* isolates are in agreement with the preliminary results of another study which showed that the growth rates at 45°C and 50°C were higher for two type A chromosomal *cpe* isolates than for one type A plasmid *cpe* isolate (1). Collectively, the results of these two studies suggest that rapid growth could be another inherent characteristic that favors the involvement of chromosomal *cpe* isolates in food poisoning. Proteomic approaches may also provide insight into the faster growth of chromosomal *cpe* isolates than of plasmid *cpe* isolates.

Pathogens often encounter a broad range of temperatures in the food environment, so it should be possible for food poisoning strains to grow at both relatively high and low temperatures. The data for *C. perfringens*, which were largely derived using isolates whose *cpe* position was not determined, indicate that the minimum growth temperature ranges from 10 to 18.5°C (8, 9, 13). In our study we obtained an average minimum growth temperature of 12°C for chromosomal *cpe* isolates, which is at the low end of the minimum-temperature range for growth previously reported, while our *cpe*-negative and plasmid *cpe* isolates grew at minimum temperatures of 17°C and 19°C, respectively, which are near the high end of the previously reported minimum-temperature range for growth of *C. perfringens*. Similarly, the previously reported data for *C. perfringens* (13, 23), primarily based on isolates whose *cpe* position is unknown, generally indicate that this bacterium grows at maximum temperatures of 50 to 52°C, which is similar to the maximum temperature determined for growth of our plasmid *cpe* isolates (50°C) and *cpe*-negative isolates. However, our chromosomal *cpe* isolates grew at temperatures greater than those reported previously (13, 23), and on average, growth continued at temperatures up to >53°C. The newly described ability of chromosomal *cpe* isolates to continue growing at temperatures greater than those tolerated by plasmid *cpe* isolates could also be important for the development of food poisoning in foods kept under inadequate conditions. Again, it is not clear why chromosomal *cpe* isolates are more heat and cold tolerant, but it is hoped that proteomic approaches may shed light on this question.

In summary, the results of this study strongly suggest that

spores and vegetative cells of chromosomal *cpe* isolates are better able to survive refrigeration and freezing than spores and cells of plasmid *cpe* isolates. This survival advantage should allow a prolonged presence of chromosomal *cpe* isolates in foods, so that when a contaminated food is warmed to growth-permissive temperatures, the bacteria can grow rapidly until the concentration needed to cause *C. perfringens* type A food poisoning is reached (10^6 to 10^7 vegetative cells/g of food) (14). Similarly, the ability to grow at higher maximum temperatures and lower minimum temperatures probably also helps chromosomal *cpe* isolates reach pathogenic levels in foods more easily than plasmid *cpe* isolates reach pathogenic levels. Combined with the previously described enhanced ability to survive heating, our findings indicate that the strong association of chromosomal *cpe* isolates with food poisoning may be multifactorial. These findings also emphasize the importance of proper high- and low-temperature food preparation and storage and holding conditions for preventing *C. perfringens* food poisoning and, if confirmed by additional surveys, would require some revision in values (and perhaps public health guidelines) for high- and low-temperature survival and growth characteristics for the *C. perfringens* isolates most relevant in food poisoning. Moreover, these findings do not preclude the possibility that chromosomal *cpe* isolates are also more resistant to other non-temperature-related food stresses, a possibility that we are currently investigating. Finally, while the emerging environmental resistance characteristics of chromosomal *cpe* isolates provide explanations for the strong association of these isolates with food poisoning, the observations do not preclude the possibility that plasmid *cpe* isolates could occasionally cause unusual food poisoning outbreaks, particularly in foods prepared or stored under less stressful conditions.

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