Production of Phospholipase C by Nine Strains of 
*Clostridium perfringens* at 37 C and at a 
Constantly Rising Temperature

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ABSTRACT

Nine strains of *Clostridium perfringens* were compared for phospholipase C production in autoclaved ground beef (AGB) at 37 C. Enzyme production is reported as units per colony forming unit (CFU). Phospholipase C concentrations were determined by the hemolysin indicator method and colonies were observed on Tryptose Sulfite Cycloserine agar. Enzyme production by each of the nine strains was significantly different. The increase and decrease of viable cells of *C. perfringens* in AGB exposed to temperatures rising constantly at a rate of 4.1 C/h was accompanied by the appearance and disappearance of phospholipase C. This demonstrated that *C. perfringens* can produce detectable levels of phospholipase C when grown during dynamic increasing temperatures.

*Clostridium perfringens* foodborne illness occurs upon ingestion of a large number of viable organisms. To establish this organism as the causative agent, it is necessary to estimate the number of viable *C. perfringens* in a suspect food by using quantitative cultural methods. When frozen or held under prolonged refrigeration, *C. perfringens* cells rapidly lose viability, making it difficult or impossible to obtain reliable counts. Harmon and Kautter (6.7.8) have suggested the hemolysin indicator plate test as an index of growth of this organism in foods which have been refrigerated or frozen. This method involves quantifying the extracellular phospholipase C (alpha toxin) produced by *C. perfringens*. The quantity of phospholipase C detected may then be used to estimate the extent of previous growth of the organism (7).

Long-time low-temperature (LTLT) cookery is used extensively in industry, foodservice and the home. *C. perfringens* can grow to high population levels under LTLT conditions (19). One objective of this research was to determine if phospholipase C would be produced at detectable levels in simulated LTLT environments. Since many foodborne disease strains of *C. perfringens* have been reported to produce phospholipase C at low levels (9,10,14), the second objective was to compare phospholipase C production by nine strains of *C. perfringens*, eight of which are associated with foodborne illness.

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MATERIALS AND METHODS

Test organisms and culture preparation

*C. perfringens* strains NCTC 8238, NCTC 10240 and ATCC 3624 were obtained from D. A. Adams, North Carolina State University, Raleigh NC; NCTC 8798 was obtained from R. G. Labbe, University of Wisconsin, Madison, WI; NCTC 8239, S-40, S-45, and NCTC 9851 were obtained from the culture collection, University of Minnesota, St. Paul, MN and ATCC 13124 was obtained from American Type Culture Collection, Rockville, MD. The method of Willardson et al. (19) was used with slight modifications in preparation of stock cultures and test cultures. Test cultures were prepared by transferring 0.1 ml of a freshly thawed stock culture to 12 ml of laboratory-prepared Fluid Thioglycollate medium (FTG) and incubating at 37 C for 20 h. FTG was no more than 1 week old and, if not freshly prepared, was steamed 10 min and cooled immediately before use. After incubation in FTG, a composite of eight strains (all listed strains except ATCC 13124) was prepared as detailed by Willardson et al. (19). Single strain studies were conducted on each strain by using the appropriate dilution of the 20 h FTG culture. Dilutions were made with 0.1% peptone water.

Growth and enumeration media

The autoclaved ground beef (AGB) test medium was prepared according to the method of Willardson et al. (19). The ground beef was frozen for a maximum of 6 months at -30 C. Four batches (fat content 17.0, 20.5, 23.9 and 28.5%) were used during the duration of this research. Before use, the tubes were tempered to the desired initial temperature (37 C for constant temperature strain comparisons, or 25 C for constantly rising temperatures studies), and inoculated with 0.1 ml of inoculum diluted in 0.1% peptone water to the desired cell population. The diluted inoculum was carefully injected into the bottom of the AGB tubes with a 13-cm long 15 gauge needle.

The enumeration medium was laboratory prepared Tryptose Sulfite Cycloserine (TSC) agar (17) prepared as detailed by Willardson et al. (19). The TSC agar was made no more than one day before use and stored at room temperature.

Enumeration procedure

At each appropriate sampling time, the ground beef sample was aseptically transferred to an 18 x 30 cm, 3/8-mil polyethylene bag using a sterile 23-cm chrome letter opener. The sample was diluted to 100 ml with sterile distilled water (85 ml), and macerated for 60 sec in a Colworth 400 Stomacher (16). Serial dilutions were made into 9.0, 9.0 ml of 0.1% peptone (Difco), and a 0.1-ml portion of the appropriate dilution was spread over the surface of TSC agar and overlaid with TSC agar without egg yolk. The inoculated plates were incubated at 37 C overnight in anaerobic jars (BBL Gaspak System) which were evacuated to 15 psi vacuum and flushed to 5 psi, three times, with a 90% N2-10% CO2 mixture or with 100% N2.

Extraction and quantification of phospholipase C

The method described by Harmon and Kautter (8) was employed, with a slight modification for extraction of the enzyme. Phospholipase C was extracted after homogenization of a 25-g portion of AGB culture, with 100 ml of 0.4 M NaCl, buffered at pH 8 with 0.05 M

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N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), for 1 min in a Colworth 400 Stomacher (16). Portions of two AGB cultures (15 g each) were used to obtain 25 g. Samples were removed from the test tube with a sterile 23-cm chrome letter opener. The layer of fat which developed on the surface of the AGB was removed and discarded before sample removal. After centrifuging the homogenate at 20,000 x g for 20 min at 4 C, the supernatant fluid was decanted through Whatman No. 1 filter paper to remove the remaining fat and meat solids. The supernatant fluid was sterilized by filtration through a Seitz Filter (No. Lb, Hercules Division, Republic Filter Corp., Milford, CT) and the sterile extract was concentrated to less than 10 ml by dialysis overnight against polyethylene glycol (PEG; molecular weight 20,000) at 4 C. The dialysis tubing (No. 3377-D32, A. H. Thomas Co., Philadelphia, PA; molecular weight cutoff 12,000) was autoclaved in distilled water for 15 min at 121 C and rinsed once with sterile physiological saline solution. The sterile extract was concentrated to less than 10 ml by distilled water for 15 min at 121 C and rinsed once with sterile physiological saline solution. and the washings and extract were distilled water for 15 min at 121 C and rinsed once with sterile physiological saline. Seitz Ioyes procedures to recover all the AGB extract and washings. The supernatant fluid was sterilized by filtration through a thin-walled stainless steel tube (3 mm O.D., No. 9 surgical tubing) plunged into the agar (17). Eight two-fold dilutions of the concentrated extract were made with sterile physiological saline solution, using a fresh serological pipette for each dilution. The undiluted extract and eight dilutions were placed in the wells of duplicate HI plates, along with anti-alpha toxin and diluent controls (17). C. perfringens (wclclcl) Type A diagnostic sera (anti-alpha) was purchased from Wellcome Reagents, Ltd., Beckenham, England. Aseptic procedures were used in handling the extract. The plates were incubated for 24 h at 37 C and examined for hemolytic zones around the wells. All hemolytic zones were measured in mm (width from edge of well) with calipers. The hemolytic zones produced by phospholipase C on HI plates were clear and easily differentiated from the unreacted red blood cell region.

Standard curve

A lipophilized standardized preparation of Type A test enzyme (alpha toxin, E.C.3.1.4.3., phospholipase C, lecithinase C) from C. perfringens was purchased from Sigma Chemical Co., St. Louis, MO. The Sigma commercial enzyme preparation was used as a standard. An initial 1:10 dilution was made with filter-sterilized HEPES, followed by a 1:10 dilution in physiological saline solution. Two-fold dilutions of the commercial enzyme-HEPES-saline dilution were made with sterile physiological saline solution. These dilutions were added to HI plates and incubated in a manner identical to the unknowns. Standard curves were constructed for use with each experimental trial by plotting the log activity of the standard phospholipase C dilutions in units/ml versus the reaction zone size in mm. Regression analysis was employed to construct the line of best fit.

Experimental apparatus and design

Water baths and temperature maintenance and control apparatuses for 37 C constant temperature as well as 4.1 C/h-constantly rising temperatures have been described by Willardsen et al. (15).

AGB was inoculated with either a single strain or the eight-strain composite of C. perfringens. The AGB inoculated with the single strains was incubated at 37 C for 4.5-5 h to make comparisons among strains. AGB inoculated with the eight strain composite or with strain ATCC 13124 was exposed to a linear temperature increase of 4.1 C/h. Colony forming units on TSC were determined on samples taken every 10-20 min. Samples were plated immediately to determine the viable count. Samples expected to be greater than 10^6 CFU/g (for strain comparisons, the target population was 10^6 CFU/g) were assayed for phospholipase C. Samples monitored for phospholipase C activity were placed immediately into ice water to prevent further growth of the organisms. The samples were held at 4 C overnight, with extraction of the enzyme the following day. Quantification was by comparison of unknown sample dilutions resulting in 1-mm reaction zones with the standard curve. If a 1-mm zone was not present, the dilution which would have resulted in a 1-mm zone was calculated from the zone sizes of the two closest dilutions. The calculated dilution was then compared to the standard curve. For one trial of the three with ATCC 13124 at 37 C, the phospholipase C was quantified with an estimated value for the Sigma standard, enzyme activity which produced a 1-mm reaction zone (reference activity), rather than a freshly prepared standard curve. The estimated enzyme activity was the average of reference activities of Sigma phospholipase C for 15 standard curves constructed in previous experiments. The arithmetic average of the 15 reference activities was 7.8 x 10^-3 units/ml with a standard deviation of 4.4 x 10^-3 unit/ml. Reference activities ranged from 1.7 x 10^-3 to 1.6 x 10^-3 unit/ml. The phospholipase C unit per CFU calculated with the estimated reference activity, 7.8 x 10^-3 unit/ml, was between the results of the other two ATCC 13124 trials. Subsequent experiments using the same average, rather than a standard curve, also yielded results in agreement with duplicate and triplicate trials which were analysed with standard curves (data not presented).

Statistical analyses

Analysis of variance was used to detect differences in the phospholipase C production of the nine strains examined. A clustering analysis method described by Scott and Knott (13) as appropriate for grouping means in the analysis of variance was employed in an attempt to divide the strains into groups based upon their level of phospholipase C production.

RESULTS AND DISCUSSION

A comparison of the production of phospholipase C by each of the 9 strains of C. perfringens in AGB at 37 C is presented in Fig. 1. The data are presented as units of phospholipase C per colony forming unit to make the comparison on a per cell basis. In each instance the populations were between 5 x 10^7 and 3 x 10^8 CFU/g. By direct microscopic observation, an average colony forming unit consisted of 2-3 cells.

Analysis of variance revealed that there was no significant difference from the first to second replication of the experiment, while there were significant differences between units/CFU for each of the nine strains (p < 0.05). An attempt was made to group the nine strains by ability to produce phospholipase C according to a clustering of means procedure (15). The mean enzyme units/CFU of the nine strains could not be separated into two or more groups (p < 0.05); therefore, they were considered as one group. This reveals that although each of the strains can be considered different from the other eight strains in its ability to produce phospholipase C in AGB at 37 C, the differences between any two or more groups formed were not clear enough to merit a division with 95% confidence with this limited amount of data.

Many investigators have reported differences in phospholipase C production between strains (1,4,5,12).
PHOSPHOLIPASE PRODUCED AT RISING TEMPERATURE

Figure 1. Phospholipase C production per colony forming unit for nine strains of Clostridium perfringens grown in autoclaved ground beef at 37 C. Strains examined were: S-40 (A); S-45 (B); ATCC 3624 (C); NCTC 8238 (D); NCTC 8239 (E); NCTC 8798 (F); NCTC 9851 (G); NCTC 10240 (H); and ATCC 13124. Data presented are the averages and ranges of 2-3 determinations.

Figure 2. Growth and phospholipase C production by ATCC 13124 and an eight-strain composite of Clostridium perfringens in autoclaved ground beef exposed to a 4.1 C/h temperature increase. Data presented are averages and ranges of duplicate and quadruplicate trials with ATCC 13124 (○) and the composite (●) respectively. The phospholipase C activity of the composite at 40 C is an estimated value.

NCTC 8239 (strain E) has been reported (1) to produce small amounts, and ATCC 13124 was recognized (12) as producing large amounts of phospholipase C. However, the data presented here describe the strain comparison of phospholipase C production by C. perfringens on a units/CFU basis. From our observation on a cellular basis, NCTC 8238 (strain D) and NCTC 8798 (strain F) produced small amounts of phospholipase C. ATCC 13124 produced a large amount of phospholipase C on a cellular basis.

C. perfringens has been observed to grow to high populations during dynamic temperature conditions (19). Production of phospholipase C by C. perfringens during growth at constantly rising temperatures has not been reported. Strain ATCC 13124 was studied singly, and the remaining eight strains were studied as a composite during exposure to dynamic temperatures increasing at linear rates of 4.1 C/h. The eight-strain composite is estimated to be 0.013 unit/ml at 40 C. Harmon and Kautter (7) indicate that populations of ca. 10^6/g are needed to attain detectable phospholipase C levels. Our experiments with the eight-strain composite of C. perfringens also indicate a requirement for about 10^6 CFU/g to attain measurable phospholipase C. The estimated 0.013 unit/ml activity at 40 C is just above the detection limit under the test conditions employed, making the estimate of enzyme activity at 40 C valid.

Growth of the single strain was initiated at about 32 C. Rapid growth was observed to about 50 C with inactivation beginning at temperatures above approximately 52 C. Phospholipase C activity showed an increase, leveling and decline which appeared to correspond closely to the population level. Growth and phospholipase C production by the eight-strain composite were similar to strain ATCC 13124. With the eight-strain composite, the phospholipase C was again observed to parallel the growth pattern, increasing as CFU's increased, then leveling and declining at high
temperatures. This comparison accentuates the ability of ATCC 13124 to produce phospholipase C at high levels which is in agreement with Möllby et al. (12). For example, from 45 to 58°C, populations of ATCC 13124 nearly identical to the eight-strain composite yielded phospholipase C activities 0.5 to 1.5 logs higher than did the composite. High phospholipase C levels were expected from ATCC 13124, based upon the comparison of the nine C. perfringens strains.

In conclusion, all nine strains studied produced detectable levels of phospholipase C in AGB at 37°C, although comparable populations resulted in a wide range of enzyme activity. There was a large difference in the ability of various strains to produce phospholipase C on a per cell basis. This work demonstrates that C. perfringens can produce detectable levels of phospholipase C during exposure to dynamic temperatures, which are representative of LTLT cooking conditions for beef and other products. These data do not indicate, nor were they intended to show, that all high and low phospholipase C producing strains of C. perfringens would yield detectable phospholipase C when grown in AGB at simulated LTLT conditions. Rather, the study demonstrates that detectable phospholipase C can and may be produced under these conditions. During a constantly rising temperature rate of 4.1°C/h in autoclaved ground beef, the appearance and disappearance of phospholipase C parallels the increase and decrease of C. perfringens populations. The decrease in phospholipase C activity upon exposure to high temperatures indicates that heat has a detrimental effect upon either synthesis or stability of phospholipase C. Further studies are underway in this laboratory to ascertain the effect of high temperature on phospholipase C.

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