"Phoenix Phenomenon" in the Growth of *Clostridium perfringens*

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The "Phoenix phenomenon" was observed with *Clostridium perfringens* Hobbs' serological type 9 (HT₉) in a cooked-meat medium at 51.7°C by a decrease in plate count (phase I), followed by an increase in count to the initial level (phase II) and a continued increase above the initial count (phase III). The effects of sporation, age of inoculum, assay medium, anaerobiosis, diluent, and growth inhibitor were studied. This phenomenon was reproduced in experiments with sporation-negative mutants derived from HT₉, inocula of various cell ages, and different assay media (sulfite-iron agar, tryptose-soytone-yeast extract agar, prereduced peptone-yeast extract agar, prereduced veal agar, and veal agar). When strict anaerobic conditions were employed, it was necessary to increase the heating temperature to 52.3°C to observe the phenomenon. The phenomenon was eliminated at 52.3°C when a combination of strict anaerobic conditions, prereduced media, and prereduced veal diluent was employed. The addition of nalidixic acid at the minimum point of the growth curve (end of phase I) had no effect on the appearance of phase II; however, phase III was completely inhibited. This indicated that phases I and II were an injury-recovery process.

An unusual growth pattern at 50°C was reported in several food poisoning strains of *Clostridium perfringens* by Collee et al. (3). After inoculation of a broth with *C. perfringens*, they observed an apparent cell number decrease within the first few hours at 50°C, followed by an increase to a maximum count within 6 h. They termed this curious pattern the "Phoenix phenomenon," probably after the Phoenix, which in ancient times symbolized immortality through "death and resurrection." The "Phoenix phenomenon" was reproduced by using an inoculum of spores only, vegetative cells only, and cells that had been grown at 50°C prior to inoculation. They suggested that this sublethal heat treatment might have a mutagenic effect since filamentous forms of the organism were observed after heating at 50°C. Mead (7) reproduced the "Phoenix phenomenon" with a mixture of spores and vegetative cells of *C. perfringens* Hobbs' serological type 1 (HT₁) in cooked-meat medium (CMM; pH 7.2) at 50°C. In a chicken leg medium (pH 6.8), he observed an initial increase followed by a decrease in viable cells before a rapid increase in cell numbers. In chicken breast medium (pH 5.8), there was only a logarithmic increase in viable cells. From these results he concluded that the growth pattern at 50°C was influenced considerably by the nature of the medium.

The purpose of this study was to explain the "Phoenix phenomenon" and to further add to the knowledge of the behavior of *C. perfringens* at sublethal temperatures.

**MATERIALS AND METHODS**

Aerobic media and diluents. Cooked-meat broth (CM), CMM, Hedley Wright's broth, and Ringer diluent were prepared as described by Collee et al. (3), with a final pH of 7.0. The meat used in all media was fresh veal. Sulfite-iron agar (SIA) was prepared as described by Mossel et al. (8), but it was modified to include 0.05% ferric citrate and to give a final concentration of 2.5% agar. Veal agar (VA) and veal diluents (CM) consisted of CM plus 2.5% agar and CM, respectively, and were autoclaved for 15 min at 121°C.

Prereduced media and diluents. All prereduced media were prepared following the procedures outlined by Holdeman and Moore (5). Prereduced cooked-meat broth (PCM), cooked-meat medium (PCMM), peptone-yeast extract agar (PYA; 2.5% agar), Hedley Wright's broth, tryptose-soytone-yeast extract (TSY), veal agar (PVA; 2.5% agar), and veal diluents (PCM) were prepared by boiling the hydrated medium for 10 min, cooling under oxygen-free carbon dioxide, dispensing into rubber-stoppered Erlenmeyer flasks or roll tubes under oxygen-
free nitrogen, and autoclaving for 15 min at 121°C. Cysteine hydrochloride (0.05%) and resazurin (0.001%) were added to prereduced media (5). PYA was prepared from peptone, 15 g (Difco Laboratories); yeast extract, 7.5 g; cysteine hydrochloride, 0.37 g; resazurin, 0.75 mg; salts solution, 30 ml (5); distilled water, 750 ml; and agar, 2.5% (Difco). Prereduced TSY consisted of tryptose, 15 g (Difco); soytone, 3.75 g (Difco); yeast extract, 3.75 g; ferric ammonium citrate, 0.75 g; resazurin, 0.75 mg; cysteine hydrochloride, 0.37 g; distilled water, 750 ml; and agar, 2.5% (Difco). The chopped-meat medium as described by Holdeman and Moore (5) was used as the maintenance medium for all cultures. Prereduced peptone dilution blanks consisted of peptone (0.1%) and resazurin (0.0001%).

Test organism. Cultures of C. perfringens type A including Hobbs' serological type 9 (HT9) and Hobbs' serological type 10 (HT10) were obtained from the Virginia Polytechnic Institute and State University (VP&SU) Anaerobe Laboratory. Sporulation-negative mutant strains 8-1, 8-5, 8-7, 8-16, and 8-17 of HT9 (4) were kindly furnished by C. L. Duncan (University of Wisconsin). The cultures were maintained in a chopped-meat medium. Stock cultures were transferred monthly, incubated for 4 h at 35°C, and stored at room temperature.

Aerobic culturing techniques. To reproduce the "Phoenix phenomenon" the procedures of Collee et al. (3) were followed as closely as possible. Their transfer techniques, inoculations, samplings, and dilutions were made aerobically, but the presence of oxygen was minimized by placing media and diluents in boiling water for 15 min and cooling them slowly before use. The assay media, SIA and VA, were prepared the day of use. Instead of using Astell roll tubes, the samples were inoculated into rubber-stoppered tubes containing SIA and spun on spinners for 5 min. The stoppers were replaced with sterile aluminum foil.

Anaerobic culturing techniques. In the experiments following the procedures of Collee et al. (3), aluminum foil-covered roll tubes were incubated in a cold catalyst anaerobe jar for 18 to 24 h at 37°C. Anaerobiosis was achieved in the jar by the use of GasPaks (Baltimore Biological Laboratory).

Experiments employing prereduced media and strict anaerobic techniques were performed to ensure quantitative enumeration of C. perfringens (9). The procedures of Collee et al. (3) were modified by using PCM, prereduced Hedley Wright's broth, PCMM, and prereduced 0.1% peptone diluent. Assay media were PYA, TSY, and PVA. Where prereduced media or diluents were used, all culture transfers, inoculations, samplings, and dilutions were conducted as described by Ades (G. L. Ades, Ph.D. thesis, VP&SU, Blacksburg, 1973). Roll tubes were incubated for 18 to 24 h at 37°C.

Temperature control. Temperatures were controlled (±0.05°C) with a Haake model E52 constant-temperature circulator contained in an insulated water bath.

Direct microscopic counts. Direct microscopic counts (DMC) were obtained by taking a 0.5-ml portion of the heating menstruum, adding it to the 0.5 ml of 4% (wt/vol) formalin and 0.5 ml of 40% (wt/vol) glycerin, and mixing them together. DMCs were performed using a Petroff-Hauser counting chamber and phase-contrast microscopy.

Spor counts. Spore counts were conducted by taking 2-ml samples from the heating menstruum at hourly intervals for 10 h. The samples were heated in a water bath for 10 min at 80°C (2, 4), and 0.5 ml was transferred to each of two tubes of SIA or PYA.

Nalidixic acid (NA) experiments. The effect of the addition of the minimum inhibitory concentration (MIC; 35 µg/ml) of NA on the growth of C. perfringens HT9 and HT10 was examined under aerobic and anaerobic conditions. Duplicate CMM growth flasks were inoculated. One flask served as the control to which no inhibitor was added, and to the other flask, an MIC of NA was added when minimum counts in the "Phoenix phenomenon" were observed.

Dilution study. The temperature and the type of diluent were investigated for C. perfringens HT9 and HT10 under aerobic and anaerobic conditions. In the aerobic studies, Ringer solution was compared to CM diluent tempered at both 25 and 47°C, whereas in the anaerobic studies, prereduced 0.1% peptone was compared to PCM diluent tempered at both 25 and 47°C. In each study, samples were taken from the same growth vessel and were diluted and assayed on VA or PVA within 4 to 6 min after sampling.

RESULTS

C. perfringens HT9 and HT10 were studied. For the purpose of this paper only the results with HT9 will be given since HT10 exhibited a similar response.

Reproduction of "Phoenix phenomenon." The growth curves of C. perfringens HT9 in CMM between 50 and 52°C are shown in Fig. 1. The typical "Phoenix phenomenon" was observed at 51.7°C. This phenomenon consisted of three phases: phase I was characterized by a decrease in plate count; phase II was characterized by an increase to the initial level; and phase III was characterized further increase above the initial level of plate count. The observation of this phenomenon at a higher temperature than reported previously (3, 7) probably reflects small deviations in the culture procedure, in the quality of veal and other ingredients used in the media, and in the method of temperature control used in our experiments. In addition, all media and diluents were adjusted to a pH of 7.0 (±0.1) as compared to a pH of 7.2 to 7.4 used by Collee et al. (3) and Mead (7). Spore counts were performed throughout this study and, in all cases, counts of no greater than 101 were obtained.

Sporulation-negative mutants. Collee et al. (3) suggested that spores might contribute to the observation of the phenomenon. According to their theory, spores might survive the initial
heat shock when transferred as inoculum to the heating menstruum at 51.7°C, whereas vegetative cells would die, causing an initial decrease in counts (phase I). Selective germination and outgrowth of heat-adapted variants would occur, causing a final increase in counts (phases II and III). This theory was tested using sporulation-negative mutant strains 8-1, 8-5, 8-16, and 8-17 (4).

At 51.5°C, strains 8-5 and 8-17 exhibited an initial decrease in counts (phase I) followed by an increase to give maximum counts within 5 h (phases II and III). However, after 8 h, counts decreased again. At 51.5°C strains 8-1, 8-7, and 8-16 did not survive. The characteristic phenomenon occurred at 51.0°C for strain 8-16 and at 50.5°C for strains 8-1 and 8-7. Spore counts were performed throughout this study and in no case were spores detected. Thus, the survival of organisms was not dependent on spores surviving the heat treatment with subsequent germination and outgrowth.

**Age of inoculum.** As the age of inoculum for HT₉ was increased from 4 to 8 to 12 or 18 h, the phenomenon at 51.7°C became more pronounced, although maximum counts (phase III) decreased as the age of the inoculum increased (Fig. 2). In addition, the time taken to reach minimum counts (end of phase I) increased in most cases with the age of the inoculum. The "Phoenix phenomenon" was observed in the 2-h inoculum, although the initial count was nearly 3 log cycles less than the initial counts for the other ages of inocula. Spore counts were performed on all samples, and in all cases spores could not be detected.

**Anaerobiosis.** Since the work of Collee et al. (3) was performed with media prepared under aerobic conditions, oxygen or oxidized media in addition to temperature might have served as an initial stress factor accounting for phase I. The vegetative cells might die until they reduce the oxidation-reduction potential sufficiently for growth. This theory was tested with preduced media and dilutions and by performing all transfers, inoculations, dilutions, and samplings under an atmosphere of oxygen-free CO₂.

Results from this study on an 18-h inoculum of HT₉ are given in Fig. 3. The phenomenon was not observed at 51.7°C in PCMM as under aerobic conditions, but instead the organism grew readily with a generation time of 16 min. When the temperature was increased the "Phoenix phenomenon" was observed, giving
the most typical results at 52.3°C.

**Assay medium.** The effect of the type of assay medium on the phenomenon was examined. When the procedure of Collee et al. (3) was followed at 51.7°C and the cells were enumerated on either SIA, VA, PYA, or TSY, there were no differences in observations. Similarly, no difference in counts was found when the procedure included prereduced growth and heating media and assaying with PYA and PVA (data not presented).

**Diluent.** Gray (R. J. H. Gray, Ph.D. thesis, University of Illinois, Urbana, 1972) studied the effects of temperature shifts on the growth of *Pseudomonas aeruginosa*. He found an apparent decrease in viable cell numbers followed by an unusually rapid increase in cell numbers when the temperature of the growth medium was shifted from 25 to 36°C and back to 25°C. This was not observed when a diluent of the same composition as the growth medium was used. Since Collee et al. (3) used a diluent that differed from the heating menstruum or growth medium, this might have been a source of stress to *C. perfringens*. The temperature of the diluent could have been significant in that some stress was possibly exerted on the cells when they were sampled from a heating menstruum held at 51.7°C into a diluent tempered at 25°C. The effects of diluent composition and temperature were investigated by using the procedure of Collee et al. (3) and under prereduced conditions. Under Collee’s conditions, CM diluent relieved some of the stress to the organism by giving a less pronounced "Phoenix phenomenon" than that with Ringer diluent; however, the temperature of the diluent had no effect (data not presented).

A combination of a prereduced growth medium and PCM diluent (at 47°C) eliminated the so-called "Phoenix phenomenon" (Fig. 4). When diluents were at 47°C instead of at 25°C, there was a slightly less apparent phenomenon.

NA. The possibility that the "Phoenix phenomenon" involves an injury-recovery process was investigated. In this case, viable cell numbers would remain constant, but there would be an increase in sensitivity to the assay conditions, resulting in an apparent decrease in cell numbers (injury) followed by a recovery process giving an apparent increase in cell numbers. This theory was tested with NA, a deoxyribonucleic acid synthesis inhibitor, that eliminated the possibility of cell division. Similar results were obtained under both aerobic and anaerobic conditions, and the results for anaerobic conditions are shown in Fig. 5. The addition of an MIC of NA at the end of phase I of the "Phoenix phenomenon" had no effect on the increase in cell numbers (phase II) to the initial inoculum level. Phase III was not observed.

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**Fig. 3.** Growth curves for *C. perfringens HTₐ* at various temperatures in PCMM.

**Fig. 4.** Growth curves for *C. perfringens HTₐ* at 52.3°C in PCMM, using a 0.1% prereduced peptone diluent at 25 and 47°C and a prereduced veal diluent at 25 and 47°C, and assayed on PVA.
This apparent increase in cell numbers was not due to growth. The "Phoenix phenomenon" appears to be an injury-recovery process.

DISCUSSION

Injury of bacteria has been interpreted as the increased sensitivity to otherwise uninhibitory compounds in the assay media (1). Normally, bacterial cells are injured at a sublethal temperature in a buffer and harvested and recovered in a fresh medium in which the cells can grow. Heat-injured bacteria are normally detected by comparing the counts obtained on a nonstress medium with counts obtained on a stress medium. The difference in counts on the two media represents the degree of injury (6).

Ades (G. L. Ades, Ph.D. thesis, VPI&SU, Blacksburg, 1973) demonstrated both heat injury and recovery of cells in C. perfringens HT10 in prereduced peptone-yeast extract broth at 51.0°C. The extent of injury was detected by comparing the counts obtained on peptone-yeast extract agar to counts obtained on peptone-yeast extract agar plus 1.5% NaCl. We observed that in a CMM, C. perfringens HT9 and HT10 exhibited heat injury and recovery. It is concluded that the "Phoenix phenomenon" represents three phases: heat injury (phase I), recovery (phase II), and growth (phase III). Phases I and II represent an injury-recovery phenomenon in which there is no change in cell numbers, as evidenced by the experiments with NA. As a result of the initial temperature shock at 51.7°C, the viable cells became sensitive to assay conditions (phase I), but after a period of time these cells apparently recovered their tolerance to assay conditions (phase II) and finally grew (phase III). Apparently, phase III was very sensitive because it was not observed consistently.

A nonstress assay medium that would alone eliminate phases I and II was not found. As the age of inoculum was decreased, phases I and II were less pronounced. Under anaerobic conditions using only prereduced media and diluents, the temperature had to be raised to 52.3°C to observe the phenomenon. The cells were made less sensitive to assay conditions by eliminating the effects of oxygen or oxidized media. A combination of prereduced heating menstrua, assay media, and veal diluent and strict anaerobic conditions eliminated the "Phoenix phenomenon" at 52.3°C. Attempts to correlate direct microscopic counts (Petroff-Hauser method) failed because the level of cells in most cases was less than 107/ml. Radiotracer techniques were attempted to further support our findings, but they failed because of the complexity of the heating menstruum.

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LITERATURE CITED


