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Effect of Temperature on the Cannibalistic Behavior of *Bacillus subtilis*▽

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Bacillus subtilis **resorts to cannibalism to delay sporulation under severe nutritional limitation. We report the effect of temperature on the dynamics of cannibalism demonstrated by *B. subtilis*. A model consisting of a delay differential equation may explain the effect of temperature on the dynamics of cannibalism.**

Cannibalism as a strategy offers a valuable resource for microorganisms that are limited by nutrients. *Bacillus subtilis*, a soil microorganism, has been shown to resort to cannibalism and predation under conditions of limited nutritional availability (5). *B. subtilis* normally forms spores under stress conditions. However, sporulation is an energy-intensive process and requires a minimal amount of nutrients to be present. The cells commit to sporulation only when they are certain about the impending stress. *B. subtilis* has therefore evolved strategies to delay sporulation (2). Cannibalism by *B. subtilis* is shown to be initiated by an extracellular killing factor and an intracellular signaling protein that acts cooperatively to cause death among sister cells in a population and impede sporulation. Environmental changes are known to have an effect on the cannibalistic behavior of species. Abiotic factors like temperature alter the interaction between species in ecology and affect the population dynamics. Here, we study the effect of temperature on the cannibalistic behavior of *B. subtilis*. We demonstrate that the cannibalistic property of *B. subtilis* under extreme nutrient deficiency is dependent on the medium temperature. We analyzed the data by proposing a model using a delay differential equation.

The wild-type *B. subtilis* strain 168trpC2 was used in this work (5). The mutant strain *B. subtilis* IRN 235/238 was obtained from the laboratory of A. D. Grossman, Department of Biology, MIT, Cambridge, MA (3). The mutant strain *B. subtilis* ybcO, lacking *skfA* (168 *skfA*::pMutin1), was given by J. M. Van Dijl and Lidia Westers of Groningen University (8). *B. subtilis* was grown in Luria broth (LB) at 37°C and 240 rpm. LB and phosphate-buffered saline (PBS) were used as the seed and the experimental medium, respectively. One liter of PBS contained 9 g NaCl, 1.15 g Na_2HPO_4, 0.2 g KCl, and 0.2 g KH_2PO_4. The pH was adjusted to 7 for experiments by using its own salts. *B. subtilis* was grown in LB at 240 rpm and 37°C. The cells were grown for 8 h to reach an optical density of 6 during mid-exponential stage. The broth of *B. subtilis* was centrifuged aseptically, and the supernatant was discarded. The concentrated pellet was resuspended into PBS, resulting in initial cell concentrations of approximately 10^{15}, 10^{12}, 10^{10}, 10^8, 10^6, 10^4, and 10^2 CFU/ml. An initial concentration of 10^{15} CFU/ml was suspended in 20 ml of PBS in a shake flask and incubated at different temperatures, namely, 20°C, 32.5°C, 37°C, 40°C, and 45°C. Samples were drawn every 2 h and analyzed for viable cells. Numbers of viable cells were determined using the methylene blue reduction test (1). The experiments were also conducted using initial cell concentrations in the range of 10^{15} to 10^2 CFU/ml at 37°C. To quantify the killing factor produced during cannibalism, the supernatants from different experiments using PBS were taken to determine the extent of killing of *B. subtilis* grown in a rich medium. The cells were uniformly spread on an agar plate, and disks of blotting paper, which were dipped in the supernatant, were placed on the agar plate to determine the zone of inhibition. Blotting paper disks of the same size, dipped in either alcohol or distilled water, were used as positive and negative controls, respectively.

The viable *B. subtilis* cells demonstrated oscillatory behavior when introduced into PBS at 37°C (Fig. 1a). Since PBS does not contain any nutrients, the growth observed in PBS was due to the cannibalistic behavior of *B. subtilis*. Further, no spores were identified in the PBS medium, which may be due to the nonavailability of nutrients for initiation of spore formation (5). A model was developed to capture the dynamics of cannibalism in PBS for *B. subtilis*. It is known that the cannibalistic behavior is due to the synthesis of a killing factor. There is a time lag between the synthesis of the killing factor and the availability of the nutrients for growth. Accounting for the above-mentioned mechanism, the following dynamic equation was proposed for the viable cell count in PBS:

$$\frac{dN_B}{dt} = -K_d \times N_B(t) + \mu^{\max} \times [N_B(t - \tau)]^n \quad (1)$$

where N_B is the number of *B. subtilis* cells that are viable, K_d is the first-order death rate constant, $\mu^{\max}$ is the maximum growth rate on the nutrients obtained from the lysed cells, τ is the delay time that is essential for the availability of nutrients for growth at any given time (t), and n is a parameter that captures the nonlinearity of the synthesis of the killing factor and lyses of the sister cells of *B. subtilis*. The first term in the equation captures the death rate of *B. subtilis* in PBS, and the second term accounts for the growth of the cells on its sister cells. It should be noted that the

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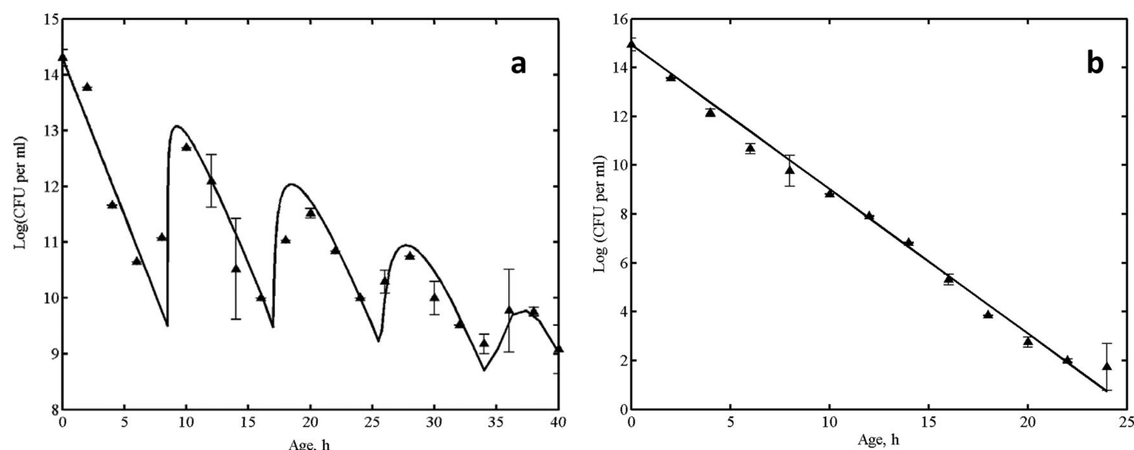

FIG. 1. Variation in viability of *B. subtilis* cells in PBS. (a) Wild-type *B. subtilis* in PBS at 37°C. Oscillatory behavior was observed, indicating cannibalistic behavior of *B. subtilis*. (b) Mutant strain lacking Spo0A in PBS at 37°C. Oscillatory behavior was not observed, indicating an absence of cannibalistic behavior. Spo0A is known to regulate the synthesis of killing factor essential for cannibalism. The linear profile shows normal death of *B. subtilis* in PBS. The solid line (a) and the dashed line (b) were obtained using simulations of the model equation (equation 1).

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value of K_d, the death rate constant, incorporates both the normal death of cells due to nonavailability of nutrients and the death rate due to cannibalism. The model equation (equation 1) is thus a delay differential equation, where the value of N_B at any instant t depends on the value of N_B in the past (i.e., at time $t - \tau$). The data of viable count of *B. subtilis* obtained from experiments were fitted to equation 1 to obtain the parameter values (Table 1). The model equation was able to capture the oscillatory dynamics (Fig. 1a), and the delay differential equation was essential to capture the dynamics, as no oscillations were observed when the value of τ was set to 0.

Experiments with a mutant strain of *B. subtilis* lacking *spo0A* demonstrated a log-linear profile without any oscillations (Fig. 1b). This indicated that the killing factor was under Spo0A regulation, as reported earlier (7, 10). In this case, the rate constant for death was estimated to be 0.8 h^{-1}, which was lower than that observed for the wild-type, indicating that cannibalism enhanced the death rate about 1.6-fold.

Experiments were conducted at various temperatures to evaluate the effect of temperature on the cannibalism of *B. subtilis* (Fig. 2). The profiles at 32.5°C and 40°C (Fig. 2b and c) were similar to that observed at 37°C, with the parameter values for K_d, μ, and τ being similar to that obtained at 37°C (Table 1). This demonstrated that the synthesis of the killing factor and the mechanism of cannibalism were operational in the temperature range of 30 to 40°C. However, on lowering the temperature to 20°C, the amplitudes of the oscillations decreased drastically (Fig. 2a). The values for the model parameters also indicated that the rate constants for death and growth on the siblings by *B. subtilis* decreased two- and five-fold, respectively, compared to that observed in the temperature range of 30 to 40°C. The lowering of death and growth rates resulted in the observed low-amplitude oscillations. However, the final cell count after 40 h was slightly lower (about 10^8 cells) than that observed in the case of 37°C (about 10^9 cells). When the temperature was increased to 45°C, no oscillations were observed and the cell count decreased linearly in the log scale (Fig. 2d). The model parameter suggested that there was no growth observed, due to lyses of sister cells induced by *B. subtilis*, at 40°C (Table 1).

To further check the presence of the killing factor at 20°C and 45°C, experiments were conducted using the supernatant obtained from PBS containing *B. subtilis* cells at these temperatures. The supernatant was used to check its potency to kill freshly grown cells of *B. subtilis*. The supernatant was used to obtain antibiotic zones on agar plates having colonies of *B. subtilis*. An antibiotic zone was observed for supernatant obtained at 20°C, while no such zone was observed for supernatant obtained from PBS at 45°C (Fig. 3). This further proved that while killing factor responsible for cannibalism was synthesized at 20°C, no such factor was released at 45°C. Thus, the high death rate observed at 40°C was mainly due to the effect of temperature and not due to cannibalism.

To study the effect of initial concentration, *B. subtilis* was introduced at different concentrations into PBS at 37°C. The model was used to predict the oscillatory behavior. The parameter values obtained with an initial cell concentration of 10^{15} CFU/ml in PBS were able to predict the behavior for initial cell concentrations of $\sim10^{10}$ to $\sim10^4$ CFU/ml (Fig. 4). However, upon a further decrease the cell concentration to 10^2 CFU/ml, no oscillations were observed and a linear profile in the log scale was observed. This indicated that a sufficient

TABLE 1. Parameter values obtained by fitting the delay differential equation to experimental data[a]

Temp (°C)	K_d (h^{-1})	μ (h^{-1})	τ (h)
20	0.7	0.04	8
32.5	1.28	0.2	9.7
37	1.3	0.22	8.5
40	1.35	0.19	8.7
45	1.41	0	0

[a] The parameters are as follows: K_d, first-order kinetic constant for the death of *B. subtilis*; μ, specific growth rate of *B. subtilis* on the debris obtained through cannibalism; and τ, the delay time necessary to produce the debris.

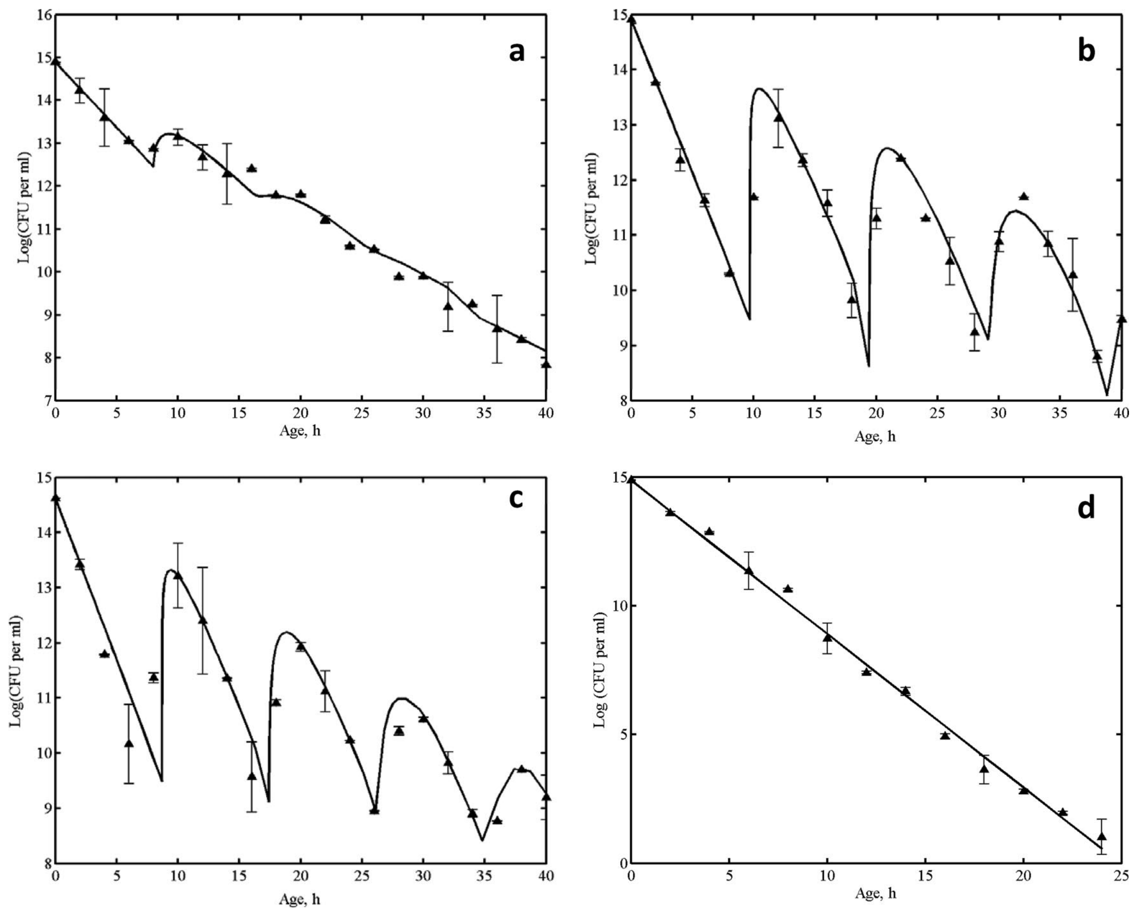

FIG. 2. Effect of temperature on the cannibalistic behavior of *B. subtilis*. The solid line represents the simulation results obtained using the model, and ▲ represents the experimental values for CFU of *B. subtilis* in PBS. The temperatures used were 20°C (a), 32.5°C (b), 40°C (c), and 45°C (d). It can be observed that no oscillations were observed at 45°C, while small peaks were observed at 20°C.

amount of the killing factor was not produced at such low cell concentrations. The study with the agar plate did not demonstrate any antibiotic zones, indicating negligible synthesis of the killing factor (results not shown).

In summary, the current study has demonstrated that cannibalism in *B. subtilis* depends on the rates of death and growth of *B. subtilis* on the sister cells. These in turn are dependent on the synthesis of the killing factor, which is a function of tem-

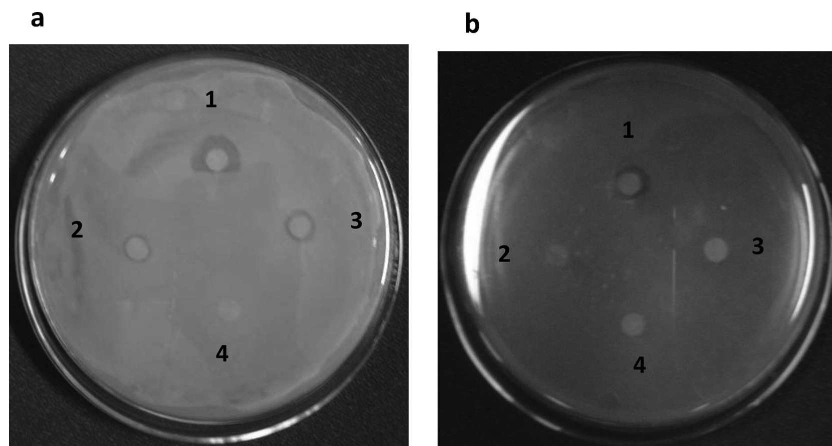

FIG. 3. Effect of the killing factor with supernatants of cannibalistic culture of *B. subtilis* in PBS at 20°C (a) and 45°C (b). Numbers 1 and 4 on the agar plates indicate controls with alcohol and distilled water, respectively. Numbers 2 and 3 indicate the effects of the supernatant. It can be seen that disk number 1 shows a clear zone of death due to the effect of alcohol, while around disk 4 no such zone was observed at either temperature. Around disks 2 and 3, while a zone can be seen at 20°C, no zone was observed at 45°C. This indicated that the killing factor was produced at 20°C and was not produced at 45°C.

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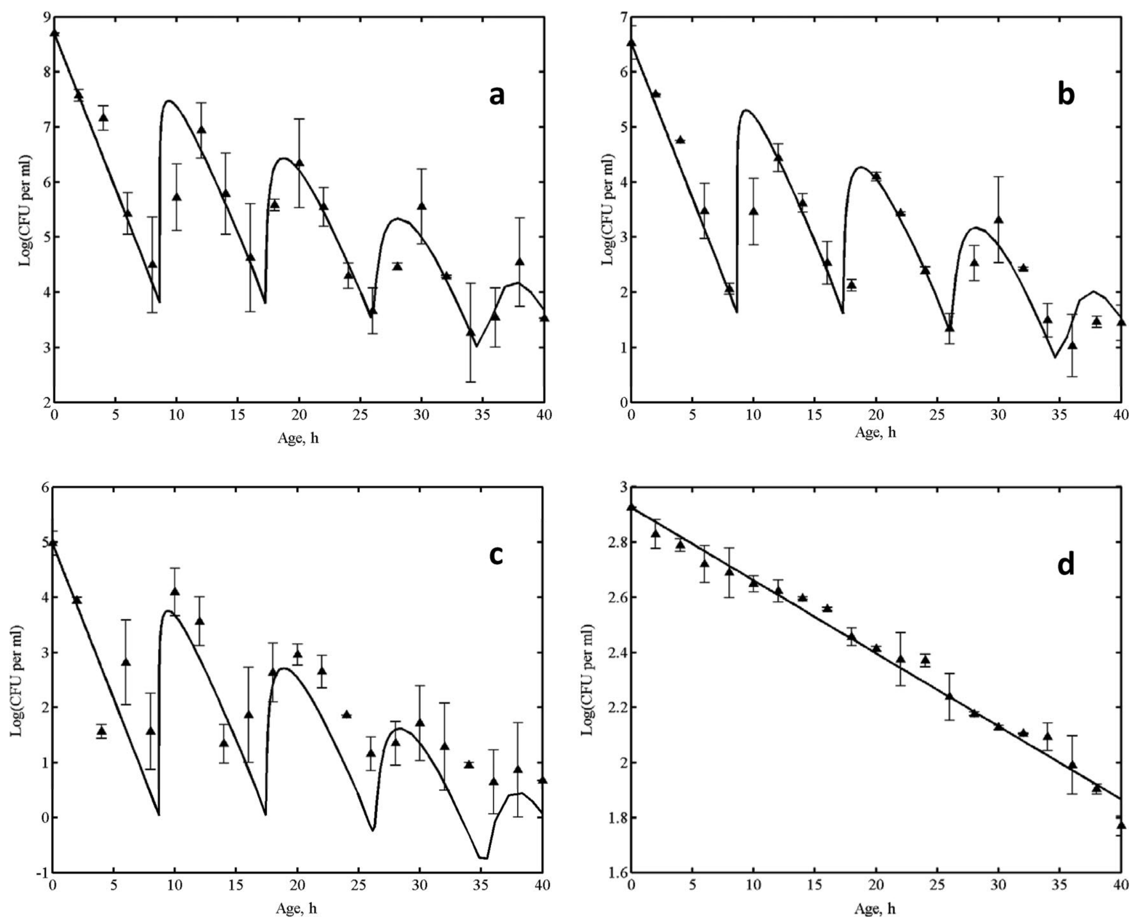

FIG. 4. Effect of initial concentration of viable cells on the cannibalistic tendency of *B. subtilis*. The different concentrations used were 10^8 (a), 10^6 (b), 10^4 (c), and 10^2 (d) CFU/ml.

perature and initial cell concentration in PBS. Cannibalism has been shown to be dependent on environmental factors and also on the initial number of organisms that cannibalize. Such behavior has been extensively observed in species of fish and has been related to the temperature of the body of water that they reside in (4, 6, 7, 9). However, this is the first report demonstrating the effect of temperature on the cannibalistic behavior of a microorganism.

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