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Effect of Carbon and Nitrogen on the Cannibalistic Behavior of *Bacillus subtilis*

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Abstract *Bacillus subtilis* is known to exhibit cannibalism under nutrient limitation to delay sporulation. Cells of *B. subtilis* in phosphate buffer solution (PBS) demonstrate an oscillatory behavior in cell number due to cannibalism. Since PBS did not contain any nutrients, the effect of carbon and nitrogen sources on the cannibalistic behavior is unclear. In this study, the effect of external carbon and nitrogen on the cannibalistic behavior of *B. subtilis* is presented. The studies demonstrated that when glucose as a carbon source was introduced into PBS in the absence of any other nutrients, the cannibalistic tendency was delayed. This delay increased with the increase in the amount of glucose present in the PBS. Thus, the cannibalism was observed to be very sensitive to the amount of carbon present in the medium. However, when the medium contained only ammonium sulfate as a nitrogen source and was devoid of any carbon, the effect on cannibalism was minimal. The study, therefore, demonstrated that cannibalism was more sensitive to carbon than nitrogen indicating that the phenomenon of cannibalism may be more dependent on the status of energy in the medium than on nitrogen assimilation.

Keywords *Bacillus subtilis* · Cannibalism · Glucose · Ammonium sulfate · MBRT · Endospore

Introduction

Organisms are known to demonstrate varied behavior under nutritional stress. Most of them activate their adaptive strategies in response to adverse environment. For example, microorganisms commonly form spores under stress conditions and revert back to their original vegetative form under conditions conductive for growth. *Bacillus subtilis*, a soil microorganism, is known to form spores under nutritional limitation. However, spore formation is an energy-intensive operation and the cells that have decided to form spores

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cannot reverse the process. Under transient nutritional stress, this is a disadvantage to the cells as cells dedicated to spore formation cannot respond to the presence of nutrition at a later stage. To avoid this, *B. subtilis* has evolved a strategy wherein some cells kill their own sister cells to delay sporulation by surviving on the nutrient released from the lysed cells [1–5]. This cannibalistic behavior offers the cells an advantage by responding to nutritional stress through spore formation only under continued stress. Recently, it has been demonstrated that *B. subtilis* not only uses cannibalistic tendencies but also can resort to predation under extreme nutritional stress [6]. However, it is not clear as to what constitutes as a sufficient nutritional stress level to initiate cannibalism.

Under nutritional stress, cannibalism by *B. subtilis* is carried out by producing an extracellular killing factor and an intracellular signaling protein, which causes death of the sister cells and blocks sporulation. For example, pure culture of *B. subtilis* exhibits cannibalism to activate *skfA* under a no-nutrient condition [1, 6]. This *skfA* operon is liable to produce the killing factor that lyse their own siblings and provide the released nutrients to be used by the rest of the cells [7, 8]. Experiments carried out by introducing *B. subtilis* cells into phosphate buffer saline (PBS) demonstrated that no spores were formed as neither carbon nor nitrogen was available in the medium. The cells, however, demonstrated cannibalism, and an oscillatory behavior in the cell numbers were observed. The effect of a carbon (or an energy) source or a nitrogen source on the cannibalistic behavior has not been previously reported. Numerous studies exist where the effect of carbon and nitrogen sources on growth of different organisms have been widely reported. Substrate like carbon and nitrogen has been shown to be a limiting factor for bacterial growth [9–14]. The current work attempts for the first time to demonstrate the effect of carbon and nitrogen on the cannibalistic behavior of *B. subtilis* in PBS.

In this work, the results of experiments designed to examine the effect of the cannibalistic behavior of *B. subtilis* under different levels of carbon and nitrogen sources are presented. The experiments indicate that *B. subtilis* delays cannibalism in the presence of glucose as a carbon source. However, nitrogen did not affect the dynamics of cannibalism in PBS. Experiments with different ratios of carbon and nitrogen demonstrated a range under which the delay in cannibalism occurs.

Material and Methods

B. subtilis strain and Chemicals

The wild-type *B. subtilis* 168 trpC2 was used in all experiments [6]. The strain was maintained on Luria agar slants at 4°C and was subcultured every 1 month at 37 °C. The seed culture was prepared using Luria broth for the experiments.

Cell count

A loopful of the culture from the slant was subcultured before each experiment into 100 ml of sterile Luria broth and grown for 10 h at 37 °C at 240 rpm. The cells were centrifuged at 7,500 rpm for 15 min, and the supernatant was discarded. The cells were inoculated into 100 ml sterile PBS. PBS contained NaCl, Na_2HPO_4, KCL, and KH_2PO_4 (obtained from Himedia, Mumbai, India). The cell viability was checked through plating on Luria–Bertani (LB) agar plates and using the methylene blue reduction test (MBRT) [15].

Experimental Protocol

B. subtilis was inoculated in the seed medium containing 2.5% LB medium and grown overnight at 240 rpm and 37 °C. The whole fresh cells were centrifuged at 10,000 rpm for 10 min, and the supernatant was discarded. The biomass was mixed with different concentrations of glucose (used as a carbon source) and ammonium sulfate (used as a nitrogen source) of 0.5%, 1%, 2%, and 3%. The initial colony-forming unit (CFU) in the PBS with varying carbon and nitrogen sources was always maintained to match a CFU of about 10^{14} cells. In each case, cannibalism was analyzed by MBRT, and viable cell count and the glucose and ammonia concentration were calculated by the glucose oxidase–peroxidase (GOD–POD) and Nessler's methods, respectively. Samples were obtained every 2 h for all the above experiments and analyzed for cell count and concentrations of glucose and nitrogen using the abovementioned analysis. *B. subtilis* cells were also introduced into a medium containing combination of substrates with high carbon (3%) and low nitrogen (0.5%) and low carbon (0.5%) and high nitrogen (3%).

Assay for Estimating the Potency of the Killing Factor

Fresh cells of *B. subtilis* were grown in LB media at 240 rpm and 37 °C. The supernatant was obtained every 2 h from broth of PBS containing *B. subtilis*. Depending on the carbon concentration, the first few hours represent the supernatant obtained during the maintenance of *B. subtilis*. The sample obtained after maximum consumption of carbon represents the supernatant from the region of cannibalism. The supernatant was introduced separately into fresh *B. subtilis* culture. MBRT and LB plates were used to determine the viability at the end of 20 min to obtain the reduction in cell count [6].

Assay for Spore Determination

To evaluate the number of spores formed, the culture of *B. subtilis* in PBS was boiled in a water bath at 90 °C and poured onto plates using both techniques, i.e., spread plate and streak plate. After overnight incubation at 37 °C, the numbers of *B. subtilis* colonies were evaluated.

Assay for Glucose Estimation

Glucose calculation was measured using the GOD–POD method. In this method, glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide reacts with *o*-dianisidine in the presence of peroxidase to form a colored product. Oxidized *o*-dianisidine reacts with sulfuric acid to form a more stable colored product. The optical density (OD) of the pink color measured at 540 nm is proportional to the original glucose concentration [16]. The reagents were mixed well under minimum exposure of light. The mixture was shaken for 20 min for proper mixing. Each test tube, after mixing of the reagents with a known glucose concentration, was incubated under 37 °C. The resulting solutions were cooled, and the absorbance was noted at 540 nm. A series of standard solutions with concentrations from 0.5 to 10 mM were used, and a calibration curve was obtained. The plot of standard concentrations of glucose and corresponding OD (a straight line) was regressed for the best fit. The equation for the best fit (R^2=0.99) for glucose calibration was Y=39.569x with a R^2 value of 0.999.

Assay for Ammonia Estimation

Nessler's method was used for the estimation of ammonia (supplied as ammonium sulfate). The standard solution of ammonium sulfate was prepared, and the different concentration of the mixture with distilled water was taken. A fixed quantity of Nessler's reagent was added to all the concentrations, and the OD of the solutions was taken at 500 nm. The plot of standard concentrations of ammonia and corresponding OD (a straight line) was regressed for the best fit. The equation for the best fit (R^2=0.97) for ammonia calibration was Y=0.0173x, R^2=0.97, where Y=OD at 500 nm and x=concentration of ammonia. Now, for any unknown OD, the concentration of ammonia has been calculated [17].

Results and Discussions

Firstly, *B. subtilis* was introduced into only PBS without the presence of any carbon and nitrogen sources. Figure 1 shows the CFU of *B. subtilis* versus time. It can be seen that, in about 2 h, the cannibalistic behavior was observed as an oscillatory profile. This was verified by evaluating the supernatant for the presence of the killing factor using the protocol given in "Materials and Methods" (see the section for details and Table 1 for results). Thus, *B. subtilis* demonstrated cannibalism under extreme nutritional stress. It was also observed that no spores were formed in PBS, as some nutrition as carbon and nitrogen sources was necessary for the formation of spores.

To study the effect of an energy source, various glucose concentrations were introduced into the PBS along with *B. subtilis* cells. Figure 2 shows the dynamics of CFU and glucose concentration for 0.5% (Fig. 2a), 1% (Fig. 2b), 2% (Fig. 2c), and 3% (Fig. 2d). In the presence of 0.5% of glucose, the cells maintained themselves for the first 10 h and later demonstrated the typical cannibalistic oscillatory profile. This was verified by evaluating the presence of the killing factor before 10 h. The supernatant obtained before 10 h did not have the capacity to kill *B. subtilis*. In case of 1% glucose, the cannibalistic behavior started after about 12 h (see Fig. 2b); while for 2%, it extended to beyond 18 h. Further, for 3% shown in PBS, *B. subtilis* demonstrated oscillatory behavior only after about 28 h. This indicated that with the increase in the amount of energy source, the organism delayed the

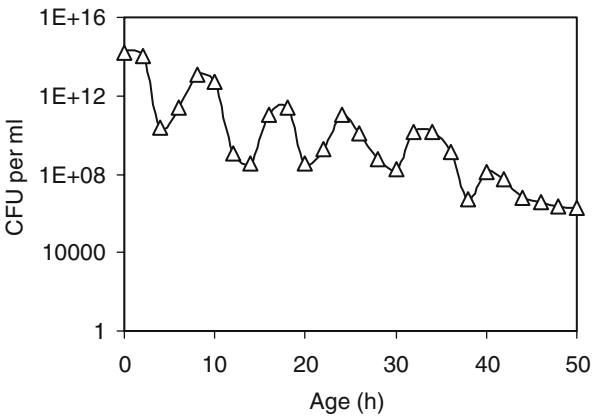

Fig. 1 Viability of *B. subtilis*, *Solid line*, CFU for the wild-type strain (*open triangle*). *B. subtilis* survived for 52 days in PBS

Table 1 Percentage drop in viability in log scale of *B. subtilis* in the presence of supernatant taken from a broth of PBS with varying glucose and ammonia concentrations along with 10^{14} cells of *B. subtilis*

	Time in hours				
	5	10	20	30	40
PBS	26	42	48	58	56
PBS+0.5% G	0	25	54	58	52
PBS+1% G	0	2	35	52	52
PBS+2% G	2	0	7	34	55
PBS+3% G	6	4	4	16	35
PBS+0.5–3% N	25	44	54	58	54

The supernatant was taken at different time points during the experiment. It should be noted that a value of 55 implies that the supernatant from an experiment could reduce the cell count of *B. subtilis* from a log scale of 14 to 6.3, where the CFU of 14 was the count in absence of the supernatant. G and N indicate glucose and nitrogen, respectively, and the data had a statistical significance of ±6 percentage points from four plates for each set.

switching on of the cannibalistic behavior. MBRT also demonstrated that the cells were viable with a slow death rate during the initial maintenance phase. The initial death rates during the maintenance of the cells were quantified as 0.37, 0.31, 0.27, and 0.2 h^{-1} for 0.5%, 1%, 2%, and 3% initial glucose concentrations, respectively. Thus, the death rate

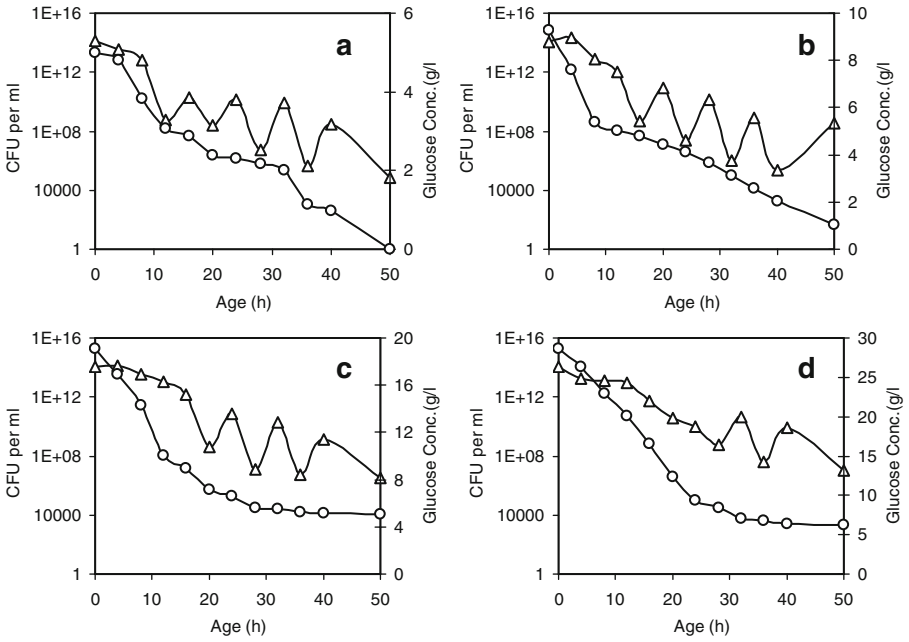

Fig. 2 Comparison of cannibalism of *B. subtilis* in the presence of different concentrations of glucose in PBS. The *left axis* shows CFU per milliliter of *B. subtilis* (*open triangle*), and the *right axis* shows glucose concentration (*open circle*) in grams per liter. **a** 0.5% of glucose in PBS, **b** 1% of glucose in PBS, **c** 2% of glucose in PBS, and **d** 3% of glucose in PBS

decreased in this maintenance phase with an increase in glucose concentration. At the onset of the oscillating phase, the death rate sharply dropped with a rate approximately equal to 2.2 h^{-1} in each case. This indicated the beginning of the cannibalistic behavior wherein the effect of the killing factor was observed (see Table 1).

The glucose profiles also demonstrated two phases. A steep drop in glucose concentration was observed in the first phase, where the cells mainly maintained themselves. A second phase was observed during which the consumption rate of glucose decreased during the period where the oscillations in the cell count was observed. The initial rate of glucose consumption was 0.15, 0.49, 0.6, and 0.8 g l^{-1} h^{-1} for 0.5%, 1%, 2%, and 3% initial glucose concentrations, respectively. Whereas in the second phase, the rates of glucose concentration were 0.085, 0.1, 0.27, and 0.19 g l^{-1} h^{-1} for 0.5%, 1%, 2%, and 3% glucose concentrations, respectively. It can be noted that with an increase in glucose concentration, the rate of consumption also increased. This essentially indicated that the cells may have used glucose as an energy source to maintain itself during the first phase. It can also be seen that the number of oscillation in the second phase decreased with an increase in glucose concentration. At the end of 50 h, it was observed that while no glucose remained unused for 0.5% of initial glucose concentration, about 1, 4.5, and 6 g/l of glucose remained unused for 1%, 2%, and 3% of the initial glucose concentration.

To study the effect of nitrogen on the cannibalistic behavior of *B. subtilis*, various concentrations of nitrogen were used along with PBS. Figure 3 shows the CFU and the concentration of ammonium sulfate as the nitrogen source at various times. Unlike the case

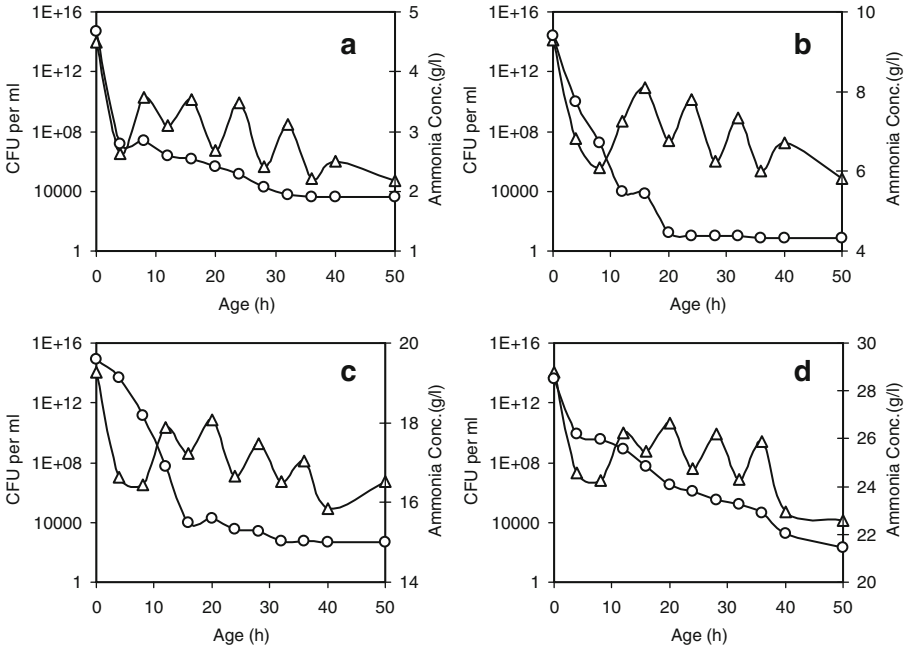

Fig. 3 Comparison of cannibalism of *B. subtilis* in the presence of different concentrations of ammonia in PBS. The *left axis* shows CFU per milliliter of *B. subtilis* (*open triangle*), and the *right axis* shows ammonia concentration (*open square*) in grams per liter. **a** 0.5% of ammonia in PBS, **b** 1% of ammonia in PBS, **c** 2% of ammonia in PBS, and **d** 3% of ammonia in PBS

of glucose addition, in the case of nitrogen, the initial phase where the cells were maintained was not observed. In this case, irrespective of the nitrogen concentration (varying from 0.5% to 3%), the cells entered the oscillatory phase similar to that observed in case of PBS alone (See Fig. 1). Further, the numbers of oscillation were also similar in all the cases, and the cell count at the end of 50 h was 10^4 cells per milliliter. The rate of the initial drop in the cell count was about 0.22 h^{-1} in all the cases, which was similar to that observed in the second phase during the addition of carbon into PBS, where the cannibalistic behavior was observed. The rate of consumption of ammonia was 0.35, 0.33, 0.26, and 0.16 g l^{-1} h^{-1} for the initial concentrations of 0.5%, 1%, 2%, and 3%, respectively. This indicated that the rate of nitrogen consumption decreased with an increase in the initial ammonia concentration. The results indicated that nitrogen, although consumed by the cells, do not seem to have any effect on the cannibalistic behavior.

Further, we also studied the effect of the presence of both carbon and nitrogen on the dynamics of cannibalism of *B. subtilis*. In this case, both glucose as a carbon source and ammonium sulfate as a nitrogen source were introduced into PBS. Firstly, we introduced a higher glucose concentration (3%) and lower nitrogen concentration (0.5%), in which case the profile of CFU were similar to that of having only glucose at 3% (see Fig. 4). The cells maintained themselves for the first 30 h and later demonstrated the oscillatory behavior. The rate of glucose consumption and drop in CFU were similar. Thus, nitrogen did not influence the behavior of *B. subtilis* in PBS. On introducing a higher nitrogen concentration (3%) and lower glucose concentration (0.5%), the initial period of the maintenance phase of 10 h was observed after which the oscillations were observed. In this case, the glucose was completely used up in 22 h, while more than half of the initial nitrogen was consumed until the end of 70 h. The death rate in the initial phase in case of high glucose and low nitrogen (0.24 h^{-1}) was four times lower than that observed in case of low glucose and high nitrogen (1 h^{-1}; Fig. 4).

The supernatant from the PBS broth for different experiments were tested for its killing potency by determining the cell count on agar plates in the presence and absence of the supernatant. Table 1 shows the percentage drop in viable cell count in the log scale due to the death of cells by the killing factor present in the supernatant. In PBS alone, the synthesis of the killing factor could be observed at $t=5$ h as the log cell count decreased from 14 to 10.4 CFU and further dropped to 6 at the end of 40 h. Thus, cannibalism was observed right after introducing the cells into PBS. On introducing glucose into PBS, the presence of the killing factor was observed after a delay depending on the glucose concentration. Table 1 show that the delay was between 10 and 30 h for a glucose range of 0.5% to 3%, respectively. These results matched with the CFU profiles shown in Fig 2. The amount of killing factor seems to saturate to yield a death of about 55% in log scale. Since it is known that the *skf* gene is responsible for the synthesis of the killing factor, which was not found to be present in the initial phase where the oscillations were not observed, the results indicated that the *skf* gene may have been switched off during this initial maintenance phase. *B. subtilis* in PBS with nitrogen demonstrated a similar profile for potency to kill the cells as that seen for PBS alone (first and last row in Table 1) indicating that nitrogen did not influence cannibalism.

In summary, experiments with *B. subtilis* in PBS containing various concentrations of carbon (glucose) and nitrogen (ammonium sulfate) indicated that glucose delayed the onset of cannibalism, while nitrogen had no influence on the dynamics of cannibalism. Thus, it may be possible that cannibalism is influenced by the energy status of the cell. It can be hypothesized that the *skf* operon responsible for the synthesis of the killing factor responsible for cannibalism may be regulated by the energy status of the cell.

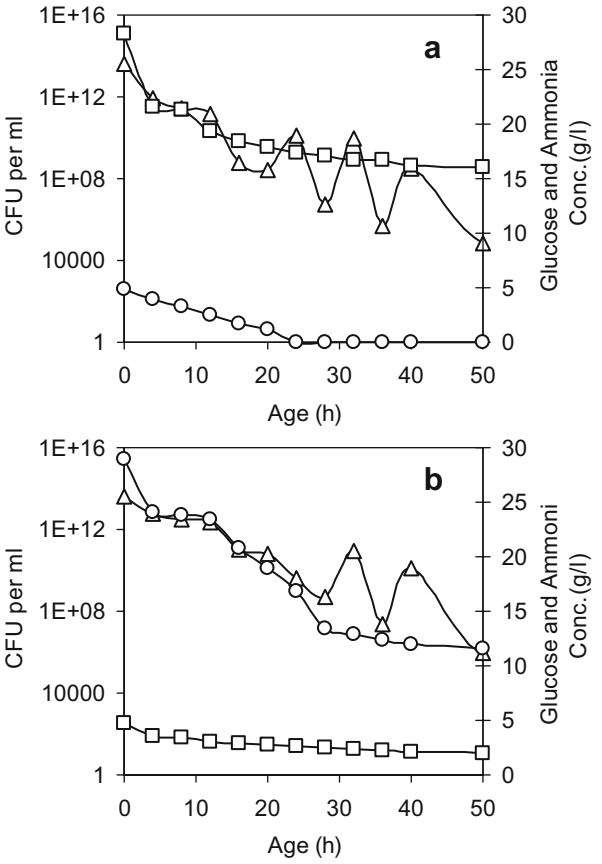

Fig. 4 Comparison of cannibalism of *B. subtilis* in the mixture of different concentrations of glucose and ammonia in PBS. The *left axis* shows CFU per milliliter of *B. subtilis* (*open triangle*), and the *right axis* shows glucose (*open circle*) and ammonia (*open square*) concentrations in grams per liter. **a** 0.5% of glucose and 3% of ammonium sulfate in PBS, **b** 0.5% of ammonium sulfate and 3% of glucose in PBS

Conclusions

It is well known that microorganisms require a sufficient amount of nutrition for their survival in an environment. However, microorganisms have evolved adaptive mechanisms to counter nutritional limitation. In such a limiting environment, *B. subtilis* is able to cannibalize its own sister cells to survive and delay sporulation. The organism has evolved this mechanism, as sporulation is an energy intensive process. The organism switches on the *skf* operon, which is under the control of Spo0A (a sporulation delaying factor), to release the killing factor and to also be immune to the killing factor. The cells that do not produce the killing factor in the population are lysed, thus releasing nutrients so that the cells producing the killing factor may survive. Studies with varying carbon and nitrogen contents demonstrated that the cannibalism of *B. subtilis* was sensitive to the extent of carbon available as an energy source in the medium. Increasing the concentration of glucose in the medium delayed the occurrence of cannibalism, indicating a delay in the production of the killing factor into the medium, whereas nitrogen does not seem to influence the cannibalism of *B. subtilis*. However, it is still unclear as to why amounts of glucose and ammonia remained unused in the PBS with *B. subtilis* cells. Further studies should address issues regarding the metabolic state of the cells during cannibalism. It is

clear that the cannibalistic behavior of *B. subtilis* is sensitive to the carbon content of the medium. It appears that *B. subtilis* has evolved to invoke cannibalism depending on the energy availability in the environment.

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