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Sporulating bacteria prefers predation to cannibalism in mixed cultures

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Abstract **Predatory behavior, a property associated with ecosystems, is not commonly observed in microorganisms. However, cannibalistic tendencies have been observed in microorganisms under stress. For example, pure culture of *Bacillus subtilis* exhibits cannibalism under nutrient limitation. It has been proposed that a fraction of cells in the population produce Spo0A, a regulatory protein that is responsible for delaying sporulation. Cells containing *spo0A* would produce a killing factor by activating *skf* operon and an associated pump to export the factor. Cells that do not contain *spo0A* in the population are lysed. However in addition to the competition among the cells of *B. subtilis*, these cells also compete with other organisms for the limited nutrients. In this work, we report the cannibalistic behavior of *B. subtilis* in presence of *Escherichia coli* under severe nutritional limitation. We demonstrate that *B. subtilis* lyses cells of *E. coli* using an antibacterial factor under the regulation of Spo0A. Our experiments also suggest that *B. subtilis* prefers predation of *E. coli* to cannibalism in mixed cultures. *B. subtilis* also demonstrated predation in mixed cultures with other soil microorganisms, such as, *Xanthomonas campestris*, *Pseudomonas aeruginosa* and *Acinetobactor lwoffi*. This may offer *B. subtilis* a niche to survive in an environment with limited nutrients and under competition from other microorganisms.**

Keywords: Predation; Cannibalism; Prokaryote; Mixed culture; *Escherichia coli*; *Bacillus subtilis*

1. Introduction

Microorganisms enter into a resting state remaining inactive for an extended period of time under nutritional limitation. For example, *Bacillus subtilis* attains a robust resting state, termed as an endospore that can remain dormant for years [1,2]. It is known that the endospore formation is an energy intensive and time consuming process. Bacteria, thus, delay spore formation, since if nutrients were to be once again available, the sporulating cells would be at a disadvantage relative to cells that are able to resume normal growth. A recent study has demonstrated that bacterial cells that have initiated the pathway to sporulation delay endospore formation by killing their siblings and feeding on the nutrients, thus, released [3,4]. Thus, *B. subtilis* demonstrates cannibalistic tendencies under nutritional stress.

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. Spo0A, a regulatory protein that governs the entry into sporulation, activates two operons *skf* and *sdp* [3–7]. The *skf* operon is responsible for the production and extracellular export of the killing factor which lyse the sister cells [8,9]. The *sdp* operon synthesizes the protein responsible for delaying sporulation. The eight-gene *skf* operon contains structural gene that is directly involved in the production of the exported killing factor (this gene is homologous to other peptide-antibiotic gene in *B. subtilis*). The operon is also able to confer resistance to the cells that produce the killing factor. Thus, it is demonstrated that some cells in a population of *B. subtilis* initiates synthesis of the killing factor and export it to the medium [3,6]. The cell that does not initiate the synthesis is vulnerable to the killing factor and is lysed [4]. Thus, cells those are able to cannibalize can delay endospore formation at the expense of the sister cells.

However in nature, *B. subtilis* cells have to compete with other organisms present in the micro flora under nutritional limitation. It is interesting to raise the question regarding the behavior of *B. subtilis* cells in mixed cultures under nutritional limitation. In this work, we report the cannibalistic behaviour of *B. subtilis* in presence of *Escherichia coli* under severe nutritional limitation. We also demonstrate that *B. subtilis* can predate on other soil microorganisms such as *Xanthomonas campestris*, *Pseudomonas aeruginosa* and *Acinetobacter lwoffi*. Our experiments indicate that the predation may be due to the release of an antibacterial factor under the regulation of Spo0A. Further, experiments also demonstrated that *B. subtilis* prefers predation to cannibalism in mixed cultures.

2. Materials and methods

2.1. E. coli *and* B. subtilis *strains*

We obtained the wild type strain of *E. coli* K12 (MTCC 1302) and *X. campestris* (MTCC 2286) from MTCC, IMTECH cell collection in Chandigarh India. *E. coli* K12 GFP was obtained from Bangalore GeNei, INDIA (Bst EII/Eco RI linker was ligated with GFP fragment and then cloned into pUC18 in Eco RI sites). The wild type *B. subtilis* 168 trpC2 was obtained from the lab of Prof. K.K. Rao, School of Biosciences and Bioengineering, IIT Bombay. The mutant strain of *B. subtilis* IRN 235/238 was obtained by the lab of Prof. Grossman, Department of Biology, MIT, Cambridge, USA [10]. The mutant strain of *B. subtilis* ybcO lacking *skfA* (168 *skfA*::pMutin1) was gifted by Prof. J.M. Van Dijl and Ms. Lidia Westers of Groningen University [9]. We obtained the strains of *P. aeruginosa* (pp4), *A. lwoffi* (isp4) from the lab of Prof. P. Phale, School of Biosciences and Bioengineering, IIT Bombay. The strains were maintained on Luria Agar (LA) slants at 4 °C and were subcultured every one-month at 37 °C.

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2.2. Cell count

A loopful of the culture from the slant was subcultured before each experiment into 100 ml of sterile Luria broth (LA) and grown for 10 h at 37°C at 240 rpm. The cells were centrifuged at 7500 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, KH_2PO_4 (obtained from Hi-media, Mumbai, India). The cell viability was checked through plating on LB agar plates and using the MBRT [11]. The data presented is an average of six experiments each conducted with three triplicates. In case of mixed culture, the cells were grown separately and later introduced into PBS after centrifugation. The MBRT was again used to differentiate the two colonies. Mcconkey agar plates were used to estimate *E. coli*, while LB resulted in the growth of both *E. coli* and *B. subtilis*. The same protocol was also implemented using mutant strains, *B. subtilis* IRN238 and *E. coli* GFP. The MBRT was also used to evaluate the viable counts of *P. aeruginosa*, *A. lwoffi* and *X. campestris*.

2.3. Image analysis of GFP strain

The imaging of *E. coli* GFP cells was conducted on a fluorescence microscope (OLYMPUS, BX 51). Every 4 h interval 10 μl of the sample from the mixed culture of *E. coli* and *B. subtilis* in PBS was used for imaging the cells and the numbers of GFP expressing cells were counted under fluorescence microscopy. We report the average of 15 images for cell count from a single time point. However, *B. subtilis* was not visible in the image of the mixed culture.

2.4. Assay for estimating the potency of the killing factor

Fresh cells of *E. coli* and *B. subtilis* were growth as detailed above. The supernatant was obtained every 2 h from broth of PBS containing both *E. coli* and *B. subtilis*. The first ten hours represents supernatant obtained during predation. The sample obtained after 10 h represents supernatant from the region of cannibalism. The supernatant was introduced separately into fresh *E. coli* and *B. subtilis* culture. MBRT and LB plates were used to determine the viability at the end of 20 min to obtain the reduction in the cell count.

2.5. Assay for spore determination

To evaluate whether *B. subtilis* formed spore in PBS, the culture of *B. subtilis* in PBS was boiled in water bath at 100 °C and pored onto plates using both techniques that is spread plate and streak plate [1]. But after overnight incubation in 37 °C no *B. subtilis* colonies were visible on the plates. This indicated that *B. subtilis* did not form any spores in PBS.

3. Results

Firstly, we investigated the individual viability of *E. coli* in phosphate buffer solution (PBS), which is devoid of any nutrients. Fig. 1a shows the viability of *E. coli* (solid line) in time. The CFU indicating viability of *E. coli* steadily decreased. There was an initial rapid drop in CFU until 4 h at 0.74 h^{-1} and a slow drop at 0.1 h^{-1} beyond 4 h. The second phase of decreased drop may be due to the cells maintaining themselves on other cells that have been naturally lysed due to cell death. No viable *E. coli* cells were found at the end of 22 days. Next, we introduced *B. subtilis* into PBS and monitored its viability (see Fig. 1c, solid line). *B. subtilis* demonstrated an oscillatory behaviour. In this case, some cells could grow on the nutrients obtained by killing the sister cells through cannibalism. The

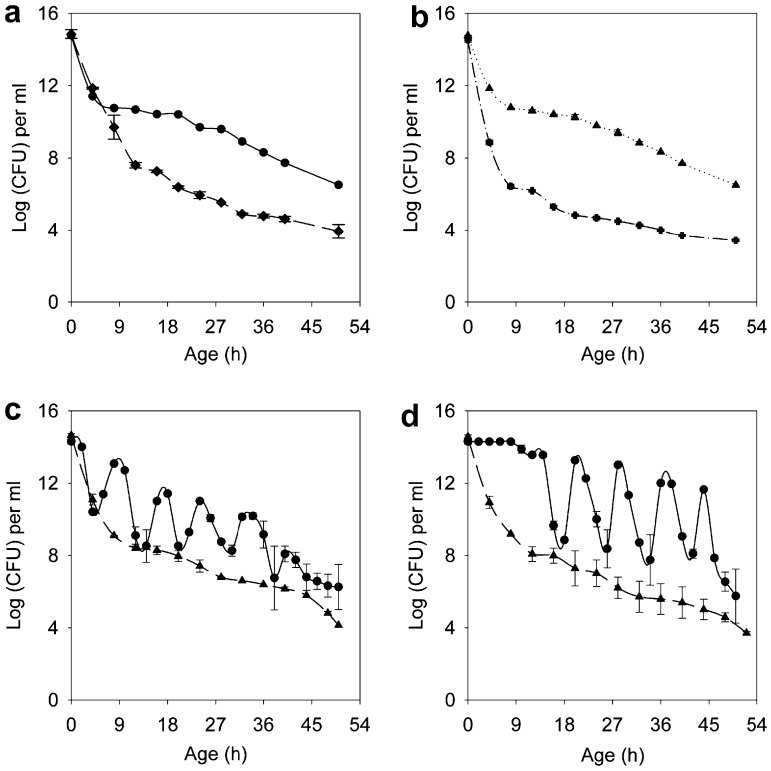

Fig. 1. Effect of *E. coli* on the cannibalistic tendencies of *B. subtilis*. (a) Viability of *E. coli* in PBS. Solid line: Colony forming units (CFU) of *E. coli* in PBS. Dashed line: CFU of *E. coli* in presence of wild type *B. subtilis*. (b) Dotted line: CFU of *E. coli* in presence of mutant *B. subtilis* IRN 238. It should be noted that the viability coincides with the profile obtained with *E. coli* alone, i.e. solid line in a. Dashed dotted line: CFU of *E. coli* in presence of mutant *B. subtilis* ybcO lacking *skfA*. (c) Viability of *B. subtilis*, Solid line: CFU for the wild type strain. *B. subtilis* survived for 52 days in PBS. Dashed line: CFU for the mutant strain of *B. subtilis* IRN235/238 lacking *spo0A*. (d) Viability of *B. subtilis* in presence of *E. coli*. Solid line: CFU for the wild type strain. Predation on *E. coli* for the first 10 h and oscillatory behaviour was observed beyond 10 h. Dashed line: CFU for the mutant strain of *B. subtilis*. The survivability of the mutant strain was not affected by the presence of *E. coli*.

oscillatory behaviour can be attributed to the cannibalistic tendencies of *B. subtilis* [12,13]. It was also observed that no viable *B. subtilis* could be found at the end of 54 days (results not shown). Further, no spores of *B. subtilis* could be identified in the PBS. This could be due to non-availability of nutrients to initiate spore formation.

Fig. 1c also shows the viability of a mutant strain of *B. subtilis* lacking the regulatory protein Spo0A (*B. subtilis* IRN238). The viability profile of the mutant did not demonstrate any oscillatory behaviour and steadily decreased (dashed line in Fig. 1c) similar to that of *E. coli* (dotted line in Fig. 1b). These have an initial sharp drop in CFU until about 9 h at 0.67 h^{-1} and a slower drop at 0.14 h^{-1} beyond 9 h. Such a mutant strain could only survive for about 16 days in PBS. This confirmed the fact that *spo0A* was necessary for the formation of the killing factor responsible for cannibalism indicated by the oscillatory behaviour. The mutant study also indicated that the phenomenon of cannibalism offers an advantage to *B. subtilis* by extending its survivability. A mutant strain of *B. subtilis* ybcO lacking *skfA* gene (responsible for producing the killing factor) was also introduced into PBS. The experiments confirmed that *skfA* gene was necessary for cannibalism as no oscillations were observed. The viability profile was similar to that of the mutant strain lacking *spo0A* (results not shown).

To study the effect of *E. coli* on the dynamics of cannibalism of *B. subtilis*, both *E. coli* and *B. subtilis* were simultaneously introduced into PBS. In such a scenario, the death rate of *E. coli* was substantially enhanced for the first ten hours (dashed line in Fig. 1a) during which time the viability of *B. subtilis* did not decrease (solid line in Fig. 1d). There was an

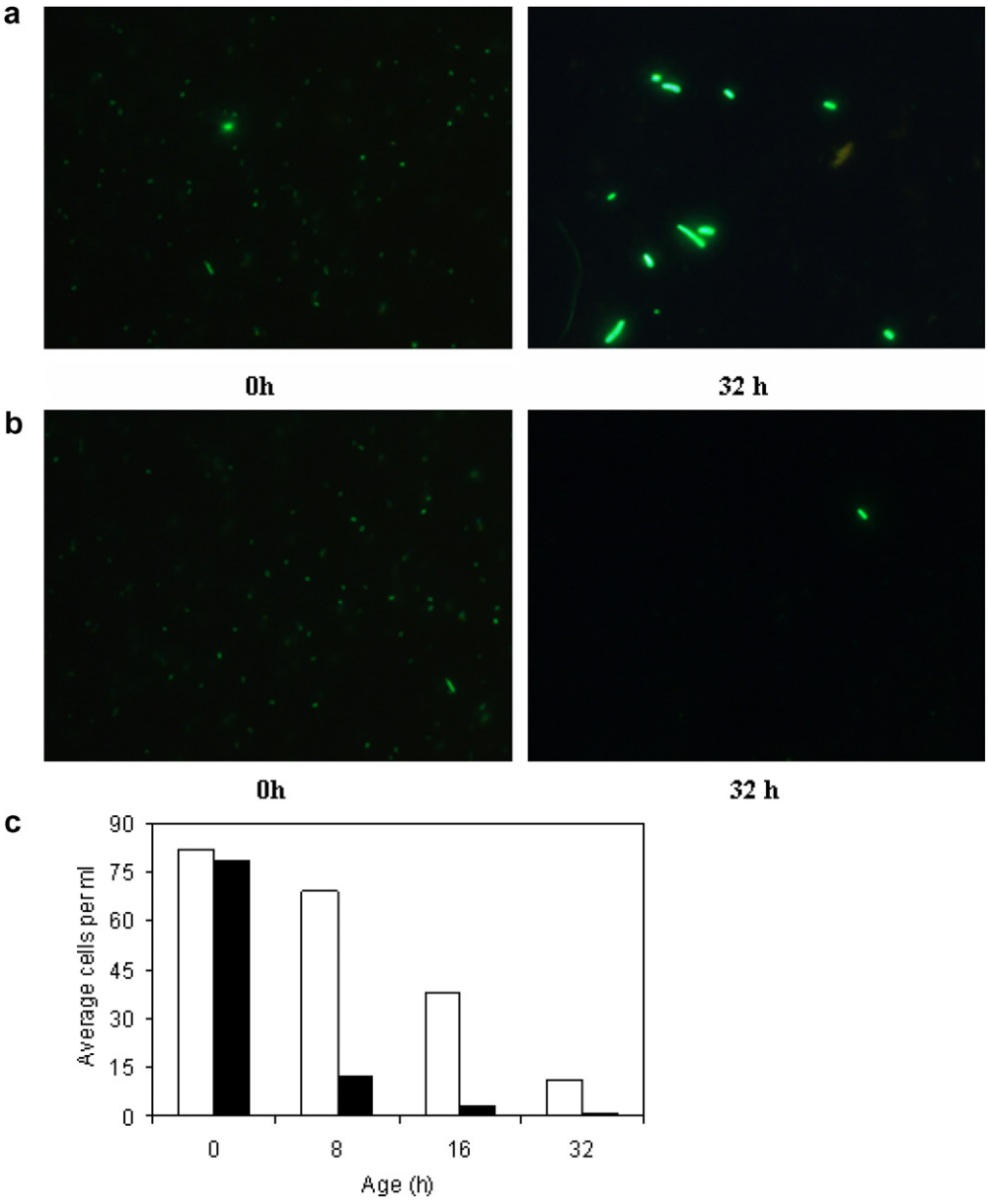

Fig. 2. Image showing *E. coli* GFP in (a) absence and (b) presence of wild-type *B. subtilis*. It can be noted that in (a) after 32 h, 12 cells can be seen in the frame from an initial count of 80, while in (b) only one cell can be seen on an average in the presence of *B. subtilis* from an initial count of 78. (c) Average cells per frame are plotted for the two cases to demonstrate the effect of predation of *E. coli* by *B. subtilis*.

eight log fold change in the CFU of *E. coli* in the first ten hours. It can be noted that during this period only four log fold change in the CFU was observed when only *E. coli* was present in PBS. This indicated that *B. subtilis* enhanced the death rate of *E. coli* and survived on its debris. During this period, no oscillation was observed implying that *B. subtilis* survived mainly on *E. coli* cells. However, after 10 h, oscillatory behaviour in the viability of *B. subtilis* recurred implying the restart of cannibalistic behaviour (Fig. 1d, solid line). Since higher cell counts existed at the end of 10 h, the amplitude of the oscillation was higher as compared to the CFU counts obtained in presence of *E. coli* (see Fig. 1c). This experiment indicated that when equal number of both *E. coli* and *B. subtilis* cells were introduced into PBS, the first ten hours demonstrated predation of *E. coli* and cannibalism began only after this period.

We have also carried out experiments with *E. coli* GFP strain, a mutant strain that is capable of expressing Green Fluorescent Protein (GFP). Both *B. subtilis* and *E. coli* GFP was introduced into PBS. Image analysis demonstrated a similar result of predation of *E. coli* followed by cannibalism. The cells per frame were evaluated through image analysis of *E. coli* GFP cells (see Fig. 2a and b). At the end of 8 h only about 10% of the *E. coli* GFP cells per frame were surviving in presence of *B. subtilis*, while 87% of the cells were surviving in absence of *B. subtilis*. Based on the image analysis, the death rate of *E. coli* in PBS was about 0.03 h^{-1}, while that in presence of *B. subtilis* was 0.08 h^{-1} (an increase of 2.6-fold due to predation). The suspended GFP observed in the supernatant in the first ten hours confirmed that the *E. coli* cells were lysed in the presence of *B. subtilis* (result not shown).

In a mixed culture of *E. coli* and the mutant strain of *B. subtilis* (*B. subtilis* IRN238) lacking *spo0A* in PBS demonstrated neither predation nor cannibalism (see dotted lines in Fig. 1b and dashed line in Fig. 1d). The viability counts of *E. coli* in presence and absence of the mutant strain in PBS were identical. The viability count of the mutant strain of *B. subtilis* also was not affected by the presence of *E. coli*. This indicates that *spo0A* was directly involved in the predation of *E. coli*. The viability of the mutant strain demonstrated a 10 log fold reduction in 50 h, both in presence and absence of *E. coli*. Further, to test whether the synthesis and export of the killing factor was essential for predation and cannibalism by *B. subtilis*, a mixed culture of *E. coli* and the mutant strain of *B. subtilis* ybcO lacking *skfA* gene were introduced into PBS. Fig. 1b shows the viability profile for such a strain (see dash-dotted

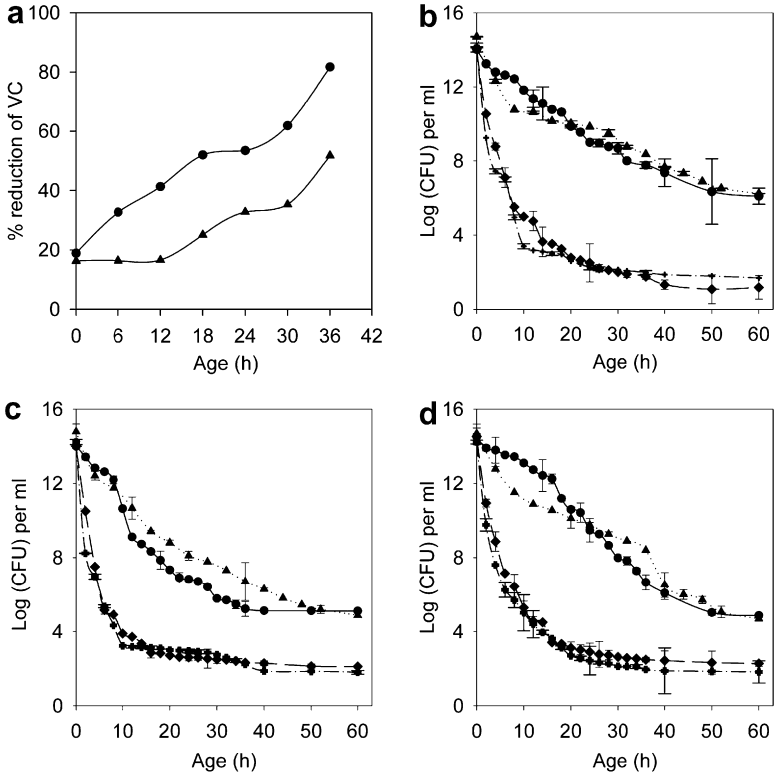

Fig. 3. (a) Comparison of percent log scale death for fresh culture of *E. coli* and *B. subtilis* due to the killing factor present in the supernatant from the flask containing *E. coli* and wild type *B. subtilis* in PBS. Circle represents percentage reduction in viable count for *E. coli* while triangles indicate for *B. subtilis*. (b) Viability of *P. aeruginosa* in PBS. Solid line: Colony forming units (CFU) of *P. aeruginosa* in PBS. Dashed line: CFU of *P. aeruginosa* in presence of wild type *B. subtilis*. Dotted line: CFU of *P. aeruginosa* in presence of mutant *B. subtilis* IRN 235/238, Dashed Dotted line: CFU of *P. aeruginosa* in presence of mutant *B. subtilis* ybcO lacking *skfA*, (c) Viability of *A. lwoffi* in PBS. Solid line: Colony forming units (CFU) of *A. lwoffi* in PBS. Dashed line: CFU of *A. lwoffi* in presence of wild type *B. subtilis*. Dotted line: CFU of *A. lwoffi* in presence of mutant *B. subtilis* IRN 235/238, Dashed Dotted line: CFU of *A. lwoffi* in presence of mutant *B. subtilis* ybcO lacking *skfA*, (d) Viability of *X. camperstris* in PBS. Solid line: Colony forming units (CFU) of *X. camperstris* in PBS. Dashed line: CFU of *X. camperstris* in presence of wild type *B. subtilis*. Dotted line: CFU of *X. camperstris* in presence of mutant *B. subtilis* IRN 235/238 and Dashed dotted line: CFU of *X. camperstris* in presence of mutant *B. subtilis* ybcO lacking *skfA*. It can be noted that in Fig. 3a–c, the viability profile of any of the three organisms in absence of *B. subtilis* matched with that obtained in presence of *B. subtilis* IRN 235/238. Further, it can also be seen that the viability profile of any of the three organisms in presence of the wild type *B. subtilis* matched with that obtained in presence of *B. subtilis* ybcO lacking *skfA*.

lines). It was observed that the death rate of *E. coli* was enhanced due to the presence of the mutant strain. A 12 log fold reduction in the viability of *E. coli* was observed in the presence of the mutant strain ybcO. However, the mutant strain did not demonstrate any oscillatory behavior indicating absence of cannibalism. This indicated that predation was possible even in the absence of *skf* operon, implying that an independent mechanism was used for predation. Thus, the killing factor synthesized through *skfA* gene was not essential to demonstrate predation but was however necessary for cannibalism. This indicates that an independent antibacterial factor was responsible for predation.

The supernatant from PBS containing both *E. coli* and *B. subtilis* was used to quantify the potency for killing *E. coli* and *B. subtilis*. The supernatant was taken at various time points from PBS containing *E. coli* and *B. subtilis*. Fig. 3a shows the percentage reduction in viable count of *E. coli* and *B. subtilis* cells by the supernatant obtained at different time points. About 20% reduction in the viability was observed in 20 min of exposure in absence of the supernatant (i.e., in control at $t = 0$). The supernatant taken during predation (before 10 h) decreased the viability of *E. coli* without affecting the viability of *B. subtilis*. However, the supernatant obtained during cannibalism (after 10 h) was able to kill both *E. coli* and *B. subtilis*. In this case, only 20% of the *E. coli* cells were viable, while about 60% of *B. subtilis* cells were able to survive. This implied that during predation the amount of killing factor from *skfA* gene was not effective to manifest cannibalism. This experiment also suggested that the killing factor from *skfA* gene may also lyse cells of *E. coli*, since the viability of *E. coli* was further reduced using supernatant during cannibalism. This was also confirmed by evaluating the potency for killing of *E. coli* by using the supernatant obtained during cannibalism when only *B. subtilis* was present in the PBS. In this case, a six-fold log reduction in *E. coli* viability was observed due to the killing factor produced by cannibalistic cells of *B. subtilis* (see Fig. 4). Further, the supernatant from PBS containing the mutant strain of *B. subtilis* ybcO was also evaluated for its potency to kill *E. coli*. A six log reduction in *E. coli* viability was observed indicating the presence of an independent antibacterial factor. This antibacterial factor was under *spo0A* regulation and was not linked to *skfA* gene.

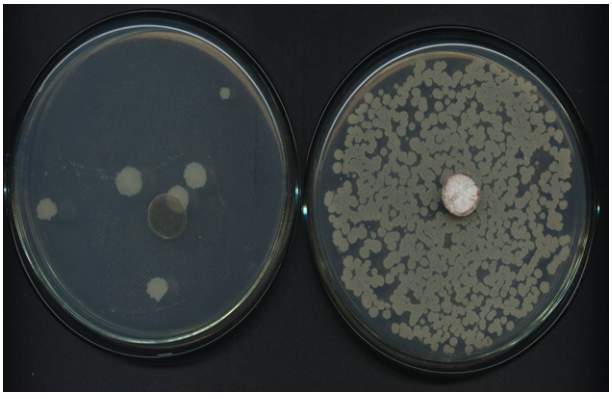

Fig. 4. CFU of *E. coli* on agar plates obtained by using supernatant of cannibalistic culture of *B. subtilis* in PBS. The agar plate on the right shows the control. The killing factor produced by *B. subtilis* in PBS was able to bring about six log fold decrease in *E. coli* viability. This demonstrates that the extracellular killing factor was able to kill *E. coli* and this mechanism was used for predation.

To evaluate the predatory behavior of *Bacillus* on other soil microorganisms, *P. aeruginosa*, *A. lwoffi* and *X. campestris* were introduced into PBS with and without *B. subtilis*. Fig. 3b–d show the viability of these organisms in PBS at various time points. The individual viability of *P. aeruginosa* in PBS is shown in Fig. 3b (solid line). As observed in the case of *E. coli*, there was a steady decline in its viability. There was an eight log fold decrease in the viability in 40 h. On introduction of wild type *B. subtilis* into PBS along with *P. aeruginosa*, the viability of *P. aeruginosa* declined sharply (see dotted line in Fig. 3b). There was an 11 log fold decline in its viability clearly demonstrating predation of *Pseudomonas* strain by *B. subtilis*. On introduction of *P. aeruginosa* into PBS containing the mutant strain of *B. subtilis* IRN238, the viability was similar to that obtained in absence of *B. subtilis*. This again implied that the predatory behavior was regulated by *spo0A*. As seen in *E. coli*, the predation was observed when the strain of *P. aeruginosa* was introduced along with the mutant strain of *B. subtilis* ybcO lacking *skfA* gene. This again implied that an independent antibiotic may be responsible for the predation of *P. aeruginosa*. Similar results were also observed for the strains of *A. lwoffi* and *X. campestris*. In case of *A. lwoffi*, a nine log fold decreases was observed when it was introduced into PBS (see solid line in Fig. 3c). The viability decreased by 12 log fold in the first ten hours in presence of *B. subtilis* indicating predation (see dotted line in Fig. 3c). Similarly, *X. campestris* demonstrated a 10 log fold decrease when it was introduced into PBS at the end of 40 h, while an 11 log fold decrease was observed at the end of 10 h. Also, in presence of *B. subtilis* lacking *spo0A*, both *A. lwoffi* and *X. campestris* were not predated upon. As observed for *E. coli* and *P. aeruginosa*, *B. subtilis* ybcO, lacking *skfA* demonstrated predation of *A. lwoffi* and *X. campestris*.

In summary, predation by *B. subtilis* was observed on all the four organisms studied. Also, *spo0A* was responsible for this predatory behavior. Further, *skfA* gene, which was responsible for producing the killing factor and was implied in cannibalism, was not solely responsible for predation. The study indicated that there may be other antibacterial factors that are under *spo0A* regulation which may be responsible for predation.

4. Discussion

Bacteria utilize ingenious signaling mechanism that enables a fraction of the bacteria in a population to cannibalize other bacterial cells in the population to use them as a food source [5,6]. This mechanism is used to delay commitment to sporulating which is expensive in terms of time and energy. Spore forming bacteria take such drastic steps to survive environment extremes when food supplies become dangerously low. The bacteria utilize a regulatory protein Spo0A to synthesize and export a killing factor and a feedback system to switch on the "immunity operon" to survive [3,4]. The sister cells lacking *spo0A* would be lysed due to lack of immunity. In this study, we demonstrated that the molecular mechanism used by *B. subtilis* to delay sporulation under nutritional stress was also used for predation in mixed cultures. Our experiments

indicate that *B. subtilis* produced antibacterial factors to kill cells of *E. coli*, *P. aeruginosa*, *A. lwoffi* and *X. campestris*, and maintain its viability on the debris thus formed. The antibacterial factors produced in mixed culture were under *spo0A* regulation. Spo0A has been shown to govern, directly or indirectly, the expression of over 500 genes [7]. Thus, secretion of an independent antibacterial factor by *B. subtilis* other than the killing factor implicated in cannibalism may be responsible for predation. The results obtained through the mixed culture studies in PBS indicate the possibility of the discovery of new antibiotics produced by *B. subtilis*. Thus, the antibacterial factors, therefore, may be advantageous to *B. subtilis* in mixed cultures. Although predatory behaviour is rarely observed in prokaryotic systems [14], our study indicates that bacterial system can use predation under extreme nutrition limitation. The signaling mechanism used by *B. subtilis* may be capable of producing various antibacterial factors other than the killing factor responsible for cannibalism. Thus, the Spo0A regulation is a generic mechanism used by *Bacillus* to predate on competitors in a mixed culture environment under conditions of nutritional stress.

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