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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

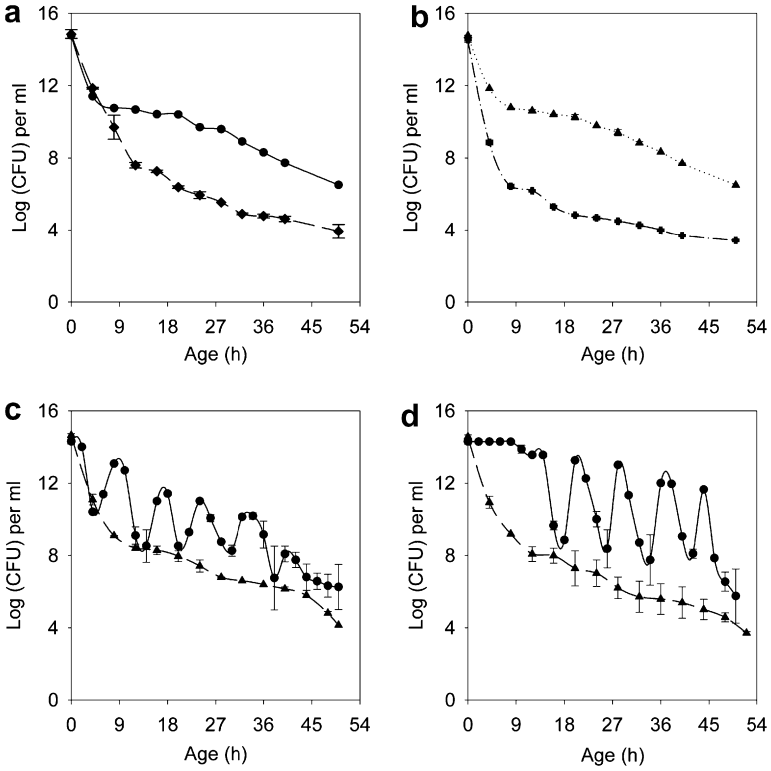

Fig. 1. Effect of *E. coli* on the cannibalistic tendencies of *B. subtilis*. (a) Viability of *E. coli* in PBS. Solid line: Colony forming units (CFU) of *E. coli* in PBS. Dashed line: CFU of *E. coli* in presence of wild type *B. subtilis*. (b) Dotted line: CFU of *E. coli* in presence of mutant *B. 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*.