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GENERAL AND APPLIED PHYSICS

Bacillus subtilis Engagement Induced via Sporulation: a Case of Bacterial Communication

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Abstract
Communication among living structures is certainly one of the most important parameters in biology leading to evolution, social, and complex behavior. It did not escape our attention, and surprise, that those important philosophers of science such as Daniel Dennet, explicitly denies bacterial communication, while other eminent molecular biophysicists present explicit theoretical modeling for it. Communication is a loose concept, and this may be the key point for disagreements. In view of the fundamental importance of the problem, we designed and performed a clean dedicated experiment, reducing at most technical jargons concerning the context of microorganisms, in order to find the correct answer under Popperian falsification paradigm test for the problem. For this purpose, we use a set of colonies of *Bacillus subtilis* accordingly elaborated: in short, two independent colonies are identically prepared, but one receives false external information and the other does not. Then, we compare their sporulation evolution, and using the Shannon concepts of the information theory, we conclude that in *Bacillus subtilis*, there exists some sort of bacterial communication.

Keywords Bacterial communication · *Bacillus subtilis* sporulation · Starvation-stress products

1 Introduction

In some way, cells know how to react to environmental conditions [1]; they can respond to stress in many different ways, by activating survival pathways, or if necessary, initiating cell death in order to eliminate damaged cells. For example, in maize, cells can fix they own ruined DNA by patching damaged segments by a new kind of genetic elements by a process called transposition [2]. Also, under environmental stress as heat shock, pollution, and absence of proper food, ciliates are able to restructure the genome by cutting the DNA into a thousand pieces and then joining them, rearranging the code [3].

Bacillus subtilis [4] under stress can produce a metabolically endospore, which is a dormant protective form resistant to time, heat, drying, ionizing radiation, and chemical insult, allowing the organism to persist until environmental conditions become favorable. Starvation-stress is the major signal regulating entry into stationary phase, but autonomous sporulation is the last resort to nutritional starvation, only employed after all alternative responses prove inadequate [5]. However, for efficient sporulation, *Bacillus subtilis* produces specific extracellular factors which work as chemical communication molecules [6, 7]. It was firstly found that these extracellular factors are increasingly secreted with cell density and that cells at high concentration sporulate efficiently under the same conditions that cells at low density sporulate poorly. Following, they shown that those extracellular factors, prepared from cells grown at high density, stimulate spore formation of cells at low density [7].

Therefore, it seems that bacterial cells communicate with each other through chemical molecules—called autoinducers, and once a specific concentration of these autoinducers

This article is part of a tribute to Professor Sergio Mascaren for his prolific career in favor of physics and scientific development in Brazil. He was a tireless encourager of people and a great source of ideas; this article is an example of his effervescent mind. Unfortunately, there was no time for this work to be expanded—and published with him alive—which will probably still be done by one of his collaborators.

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is reached, a signal is transmitted inside the cells that inform each one that other similar bacteria are nearby. This process, called quorum sensing, controls bacterial collective behavior in general [8].

However, philosophers of science, such as Daniel Dennet, negate the existence of bacterial communications [9], while other eminent molecular biophysicists present theoretical [10, 11] and mathematical modeling for it [12]. It seems that the term communication is the deadlock origin: Indeed, communication is a concept hard to define in a consistent manner because it is used to refer to many different behaviors [13]. As philosophers, Daniel Dennet argues that to be able to communicate it is necessary the ability to think, remember, and plan; that is, a complex brain is required. For him "an amoeba retreating from a harmful stimulus and a room thermostat are equally competent, but without comprehending the reason for their actions" [9]. As seen, this is a matter for philosophers and biologists to chew over [14]; here, we will follow a more practical way.

Therefore, we design and conducted a specific and very simple experiment, in whose description we suppress any elaborated technical jargons concerning the context of microorganism; the aim is to make easy to focus on the essential meaning of the term communication in this problem. We then try to demonstrate the existence of some "token-molecule" generated by the bacilli, which operates as independent message, that is, the meaning (information) contained in this message—about that particular environment conditions, or in the present case, that they have to sporulate—will be the same in any other independent cell community. This experiment is described in the following section.

2 Experimental Design, Materials, and Methods

The experimental setup consists of two main steps. In the first step, *Bacillus subtilis* were cultured in rich culture medium to provide bacteria to accomplish the experiment, as well as another colony was maintained under starvation condition in order to obtain acellular supernatant containing starvation-stress products (Sect. 2.1). In the second step, three types of medium were prepared: rich culture medium; distilled and deionized water, offering nutrient-deprived conditions to induce sporulation; and acellular fraction of *Bacillus subtilis* grown under nutrient-deprived conditions from the first step (Sect. 2.2). In Sect. 3.3, complementary details about bacillus treatments are provided.

Our strategy is to insert acellular supernatant containing starvation-stress products, which carries an environmental message (information), in another (new) bacteria colony, as false information. Then, we compare its response against the normal sporulation evolution (without external information). Both colonies are identical, except that one receives external information and the other does not.

2.1 Materials Produced by Sporulation Induced by Starvation-Stress

Samples of *Bacillus subtilis* (ATCC 9372) were kindly provided by Dr Sérgio Luiz de Souza Salvador, of the Department of Clinical, Toxicological and Bromatological Analysis, of the School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo.

Bacteria were cultured overnight at 37 °C in rich culture medium brain heart infusion (BHI) and the optical density at 623 nm adjusted with 0.89% saline solution (Gehaka, mod. 340G) to a final density of 10^8 CFU/mL (colony-forming units per milliliter). The cell suspension was transferred to a tissue culture flask and incubated at 37 °C in a shaker (Marconi MA420) at 90 rpm.

By its turn, the colony under starvation condition was maintained for 30 h (preliminary studies, data not shown). Spore release was confirmed by Wirtz-Conklin staining technique (Fig. 1). After centrifugation at 800 *g*, for 10 min at 4 °C, the acellular supernatant of the cell suspension containing starvation-stress products (SSP) was collected and transferred to another tube labeled SSP. The pellet was then washed with saline solution, centrifuged, and the remaining supernatant added to the tube SSP. Then, the suspension containing SSP was stored until use for bacillus treatment.

2.2 Experimental Design

Vegetative *Bacillus subtilis* cells were suspended in each one of the following conditions: (*i*) brain heart infusion, BHI (a rich culture medium, positive control); (*ii*) distilled and deionized water, DDW, (offering nutrient-deprived conditions to induce sporulation, negative control); and (*iii*) acellular fraction of *Bacillus subtilis* grown under nutrient-deprived conditions containing starvation-stress products (SSP) in water.

The suspension containing SSP is supposed to carry the information about an unfavorable environment or indication for sporulation. This sort of fake or hacker technique is the proceeding; we propose to use to investigate the existence of a sort of communication between two independent bacterial colonies, our hypothesis. Aliquots of these samples were collected at 2, 4, 6, 8, and 10 h after exposure to the treatments and stained using the Wirtz-Conklin staining technique [15]. An aliquot of each group was collected immediately prior to treatments exposure (time zero, internal control group). This provides the answer as quantitative results for the experiment.

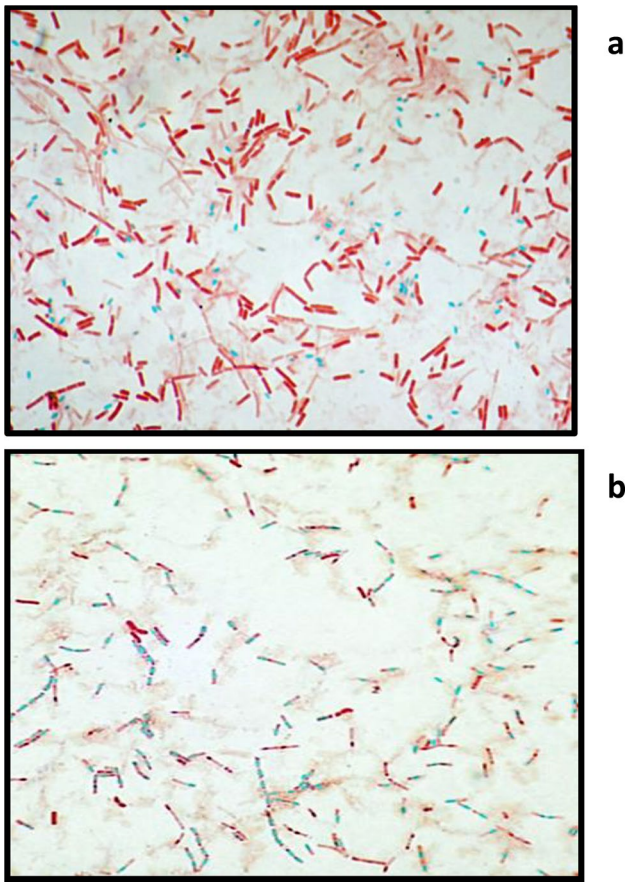

Fig. 1 Micrographs of stained *Bacillus subtilis*. Vegetative bacilli were suspended in culture medium overnight on an incubator, and then stained with Wirtz-Conklin stain. (**a**) In culture medium, bacilli are observed due to complete outgrowth to vegetative bacilli (pink rods); (**b**) after starvation period (30 h), spores stained greenish-blue, with no initiation of vegetative outgrowth

2.3 Bacillus Treatments

After growth in BHI broth, fresh colonies of *B. subtilis* were suspended in 20 mL of sterile 0.89% NaCl and then diluted to yield a final density of approximately 5×10^5 CFU/mL, which was used to prepare the three suspension of Sect. 2.2. Briefly, slides were flooded with five percent aqueous malachite green (Fisher Scientific Co. Fair Lawn, NJ, USA). Slides were intermittently heated with a Bunsen flame for approximately 5 min, to ensure that the dye remained hot but not boiling. Slides were rinsed with tap water, and then counterstained with 0.5% safranin (Sigma Chemical Co., St. Louis, MO, USA), for 1 min. After drying, the slides were examined using the oil immersion power of a light microscope (Olympus BX 51, Tokyo, Japan) and the cells were counted in 64 cm^2 (magnification of 43×). Six samples for each treatment were taken to provide the average number of counting areas. Spores were distinguished as green spherules, in contrast to vegetative cells which retain the red safranin counterstained. The results were expressed as proportions of spores in relation to total cells.

3 Results and Conclusions

Table 1 presents the average of counting results for the three treatments: vegetative *Bacillus subtilis* cells suspended in culture medium brain heart infusion, labeled **BHI**; vegetative *Bacillus subtilis* cells suspended in distilled and deionized water (nutrient-deprived conditions), labeled **DDW**; and vegetative *Bacillus subtilis* cells suspended in distilled and deionized water containing starvation-stress products, labeled **SSP**.

Figure 2, produced using the counting results of Table 1, exhibits a clear demonstration of the supposed communication among bacilli of two independent colonies, provided by this simple experimental scheme: The green line (**BHI**) shows that essentially no sporulation occurs when bacteria are in a rich culture medium, as expected for a colony free of environmental stressors. The blue line (**DDW**) describes the course of the spontaneous sporulation of bacteria under starvation condition (sporulation induced just by nutritional stress): The number of spores starts to grow slowly only after about 4 h, confirming that autonomous sporulation is the last resort to nutritional starvation. After 10 h, the number of spore reaches only about 8% of the initial number of bacteria. On the other hand, a remarkable increase on the sporulation rate is observed when the bacilli are in contact

Table 1 Average number of bacilli and spores counting (%) as a function of time; the figures correspond to the average over $n = 6$ independent counting for each treatment: DDW, patent fully healthy bacilli in distilled and deionized water; SSP, bacilli in the presence of starvation-stress products; and BHI, bacilli in brain heart infusion

	DDW		**SSP**		**BHI**	
Incubation (hours)	Bacillus	Spores	Bacillus	Spores	Bacillus	Spores
0	99.7 ± 0.8	0.3 ± 0.8	98.9 ± 1.4	1.2 ± 1.4	98.9 ± 1.7	1.1 ± 1.7
2	100 ± 0,0	0.0 ± 0.0	99.4 ± 1.6	0.7 ± 1.6	100.0 ± 0.0	0.0 ± 0.0
4	99.2 ± 1.9	0.8 ± 1.9	98.2 ± 3.1	1.8 ± 3.1	100.0 ± 0.0	0.0 ± 0.0
6	98.8 ± 2.0	1.2 ± 2.0	84.7 ± 5.8	15.3 ± 5.8	100.0 ± 0.0	0.0 ± 0.0
8	96.6 ± 4.6	3.4 ± 4.6	70.2 ± 7.6	29.8 ± 7.6	100.0 ± 0.0	0.0 ± 0.0
10	91.8 ± 4.5	8.2 ± 4.5	60.0 ± 8.4	40.0 ± 8.4	100.0 ± 0.0	0.0 ± 0.0

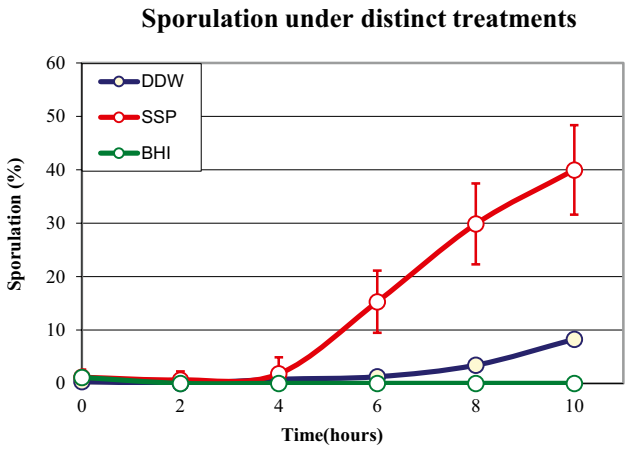

Fig. 2 Evolution of the *Bacillus subtilis* sporulation under three distinct treatments, from Table 1: green line, bacteria in rich culture medium: brain heart infusion (BHI); blue line, in just distilled and deionized water (DDW); and red line, bacteria in water plus starvation-stress products (SSP). While practically no sporulation occurs under BHI conditions, in pure DDW the number of spores grows slowly in the interval of 10 h. However, in contact with debris of sporulation of a previous colony, SSP, although latent initially, the sporulation process shows an explosive grow after about the fourth hour

with debris of sporulation of a previous different colony, the red line (**SSP**). Note that the time delay for starting the stimulated sporulation (SSP) is about the same (4h) of the autonomous sporulation (DDW).

The observed explosive growing of spores, materialized only after a time lapsed of about 4 h, confirms the existence of internal decision process that choose sporulation as the last appeal, even in the presence of debris of the previous sporulation. This internal decision could be related to intracellular storage, which can supply bacterial metabolism during periods of nutrient limitation. Bacterial storage can be especially advantageous in variable environments, by buffering an organism against variations in external resource supply or environmental conditions. However, once the message is processed, it works such like a trigger activated by the cumulated counting of contacts with such debris.

We think that unequivocal difference between the sporulation evolution with (**SSP**) and without (**DDW**) external information gives us the definitive correct answer under the Popperian falsification paradigm test [16], allowing the conclusion that we found at least one clear quantitative experimental example of bacterial communication in *B. subtilis*, under the Shannon concepts of the information theory [17]:

Message (environment condition of colony-1) → *Encoder* (generation of autoinducers) → *Chanel* (autoinducers in colony-2) → *Decoder* (cells contact autoinducers) →

Now, a number of extra careful experiments must follow to complete such exploratory results obtained in the present study, as to identify precisely the chemical molecules (token-molecules) responsible for the bacterial intercommunication, as well the function that correlates the concentration of such molecules and the sporulation rates.

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Declarations

Conflict of Interest The authors declare no competing interests.

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