Chapter 13

Synchronization of *Bacillus subtilis* Cells by Spore Germination and Outgrowth

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Abstract

This protocol defines conditions under which the germination of spores can be used to synchronize *Bacillus subtilis* cells, utilizing the time-ordered sequence of events taking place during the transition from spore to vegetative cells. The transition stages involve: phase change, swelling, emergence, initial division, and elongation. By using this method we have obtained two distinctive synchronized cell cycles, while the synchrony faded away in the third cycle. The advantage of using spore outgrowth and germination is that a highly synchronized population of bacterial cells can be obtained. Non-dividing spores stay synchronized, while synchrony rapidly decays during a few divisions. The limitations of this method are that it can be applied only for sporulating bacteria and synchrony lasts for only a limited period of time exceeding not more than two cycles.

Key words Endospore formation, Spore outgrowth, Transition stage, Vegetative cells, Permeabilization, DNA synthesis, Spectrophotometry

1 Introduction

The process of vegetative bacterial cells converted into a dormant structure is called sporogenesis and the return to the vegetative stage is known as spore germination. Contrary to endospores, vegetative cells are capable to active growth. Endospore formation is typical to three genera of Gram-positive bacteria: Bacillus (*B. anthracis*, *B. subtilis*), *Clostridium* (*C. botulinum*, *C. perfingens*, *C. tetani*), and Sporosarcina. *Bacillus subtilis* is a sporulating model organism for differentiation, gene/protein regulation, and cell cycle events [1]. That sporulation and spore germination can be used as a model system to study the transition between different cell forms has been described four decades ago [2–4]. In spore-forming bacteria, "de novo" nucleotide synthesis is not operative in the early stage of transition from spore to vegetative stage known as germination [5]. After the germination stage, the nucleoside triphosphate levels increase rapidly by the utilization of the nucleoside and

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nucleoside monophosphate pools of the dormant spores [6]. Consequently, DNA synthesis takes place only after the formation of these substrates. The processes of germination and spore outgrowth take place in a time-ordered sequence allowing to follow closely the transition between spore and vegetative stages [7–13].

The process of germination of the bacterial spore is known as a change from the heat-resistant to a heat-labile stage which does not represent the true vegetative cell. The transition has been divided into two distinct stages. The term germination is known as the first stage and the transformation of germinated spores into vegetative cells is called outgrowth [14], or post-germinative development [15]. One of the most important reactions in this chain is the initiation of germination also referred to as trigger reaction. The germination process can be triggered by heavy metals, heat, hydrostatic pressure, a variety of chemicals of nutrient, and non-nutrient origin [16]. Although little is known about the mechanism of activation, it is believed that molecular rearrangements taking place inside the spores develop into the germination process. The triggering is operationally similar to the opposite process of sporulation representing a stage of no return. One of the best triggering agent is L-alanine when present in high concentration in the germination medium. It was demonstrated that a short exposure to L-alanine caused a subsequent germination in spores of *Bacillus cereus* [17]. The quintessential germination receptor in *B. subtilis* is GerA that is activated by a single germinant, L-alanine and inhibited by its stereoisomer, D-alanine [18]. Most of what is known about Ger receptor function has been derived from studies in the gerA operon [19]. A homologous gerA operon of *B. subtilis* was isolated from *Bacillus thuringiensis* [20].

The germination process involves loss of resistance to injuring agents such as heat or heavy metals, loss of refractibility, decrease in the optical density of the spore suspension, increase in stainability [21, 22], decrease in dry weight due to the loss of picolinic acid, calcium and mucopeptides [23], the initiation of respiration [24], as well as imbibition of water [25]. These changes point to initial biochemical steps preceding visual signs of germination. Outgrowth includes four stages occurring in the following order: swelling, emergence from the spore coat, elongation of the emergent organism, and finally division of the elongated organism.

As the bacterial endospore metabolism, structure and composition differ from that of the vegetative cell, sporulation and spore germination can be applied as a simple model system to study biochemical mechanism regulating these transitions. The differentiation is cyclic and can serve the purpose of synchronizing those bacteria that undergo sporulation. This chapter describes how germination and outgrowth of *Bacillus subtilis* ATCC23857(strain 68, indole$^-$) can be adapted for cell cycle synchronization. Studies have shown that in cells of *B. subtilis* 168 rendered permeable to

small molecules by treatment with toluene, the incorporation of [^{3}H]dTTP incorporation depended on the presence of ATP and was sensitive to the inhibitor of DNA replication (6-((*p*-hydroxyphenyl)azo))uracil (HP-Ura) and to the gyrase inhibitor novobiocin [26].

2 Materials

2.1 Chemicals

1. Indole.
2. Trichloroacetic acid.
3. Casamino acids (Bacto), Becton, Dickinson and Company (Sparks, MD).
4. 60 Ci/mmol ^{3}H-dTTP, 100 Ci/mmol ^{3}H-thymidine (Amersham Pharmacia Biotech AB, Uppsala, Sweden).
5. K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, trisodium citrate.
6. $MgSO_4$ 7 H_2O, $FeCl_3$.6 H_2O.
7. Amino acids: L-alanine, L-arginine, L-asparagine, sodium glutamate, L-histidine, L-methionine, L-phenylalanine, L-serine, L-threonine, L-tryptophane.
8. Nucleotides: ATP, dATP, dGTP, dCTP.
9. Inhibitors of DNA synthesis: 1-β-D-arabinofuranosyl-cytosine--5′-triphosphate (ara-dCTP) (6-((*p*-hydroxyphenyl)azo))uracil (HP-Ura).

2.2 Disposables, Instruments

1. 1.5 ml microcentrifuge tubes (Eppendorf, Hamburg, Germany).
2. 5, 15, and 50 ml conical centrifuge tubes (BD Falcon, San José, USA).
3. Glass culture tubes and flasks.
4. Whatman GF/C glass microfilters diameter 2.4 cm, Sigma-Aldrich (St. Louis, MO).
5. Rotating incubator.
6. Microcentrifuge tubes.
7. Spectrophotometer and cuvettes.

2.3 Buffers, Solutions

1. KPO_4 buffer (K_2HPO_4/KH_2PO_4), 0.5 M, pH 7.5, 2 M $MgSO_4$, 15 mM ATP, 0.4 mM dNTP (dATP, dGTP, dCTP, each), 2 mM HP-Ura, 2 mM 1-β-D-arabinofuranosylcytosine-5′-triphosphate (ara-dCTP) (*see* **Notes 1–3**).
2. Betafluor, pre-mixed scintillation solution, National Diagnostics, Atlanta, GA.

2.4 Lyophylized Spores

1. Spores of *B. subtilis* 168 in 1.5 ml microcentrifuge tubes were kept in 50% glycerol (*see* **Note 4**).
2. Lyophylized spores: 10 mg spores plus 1 ml 50% glycerol (10 mg/ml).

2.5 Germination of Spores

1. Sterile germination medium, pH 7.4 contained 14 g K_2HPO_4, 6 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 1 g trisodium citrate, 0.5 g $MgSO_4$ 7 H_2O, 8 mg $FeCl_3$.6 H_2O, 0.5 g L-alanine, 50 mg L-arginine, 50 mg L-asparagine, 100 mg sodium glutamate, 50 mg L-histidine, 5 mg L-methionine, 50 mg L-phenylalanine, 5 mg L-serine, 100 mg L-threonine, 50 mg L-tryptophane in 1 l.
2. For germination: To 30 ml germination medium 0.3 ml 50% glucose and 1.5 ml 10 mg/ml spores were added (*see* **Note 5**).

2.6 Monitoring of Germination

1. Germination was monitored by the decrease in optical density at 525 nm on a Beckman spectrophotometer (*see* **Note 6**).

2.7 Cell Culture Medium

1. Antibiotic medium 3 (Penassay broth) (DIFCO—Becton Dickinson and Company, Franklin Lakes, NJ): 5 g/l Tryptone, 1.5 g/l yeast extract, 1.5 g/l beef extract, 3.5 g/l sodium chloride, 1 g/l dextrose, 3.68 g/l potassium phosphate dibasic, 1.32 g/l potassium phosphate monobasic. pH adjusted to 6.9.
2. Medium C, pH 7.4 contained in 1 l: 14.0 g K_2HPO_4, 6.0 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 1 g trisodium citrate, 0.2 g $MgSO_4$ 7 H_2O, 5 g glucose, 0.5 g casamino acids and 50 mg indole.

3 Methods

3.1 Cell Growth

1. To 50 ml Penassay broth, 10 µl spores in 50% glycerol were added and grown overnight in a 250 ml Erlenmeyer flask at 37 °C in a Becton Dickinson orbital incubator shaker (General Scientific Instrument Services Inc., London, UK) at 100 rpm. From the overnight culture 4 ml was added to 200 ml medium C in a 1 l flask and grown at 37 °C in the orbital shaker at 100 rpm.

3.2 Preparation of Toluene-Treated Cells

1. At 3×10^7 cells/ml density take 25 ml cells each in six 50 ml conical tubes and harvest them in a Beckmann J21 centrifuge at room temperature ($5000 \times g$, 5 min). Decant the supernatant and resuspend the pellet of each tube in 2 ml of 0.1 M KPO_4 buffer, pH 7.4. Pool the suspension in one 15 ml tube and spin down cells. Resuspend cells in 3 ml 0.1 M KPO_4 buffer, pH 7.4, add 30 µl toluene. Shake the mixture for 10 min at room temperature then chill it in ice. Add 10 ml cold 0.1 M KPO_4 buffer, pH 7.4, spin in centrifuge and resuspend cells in 1.5 ml of the phosphate buffer. The density of cells

(1.5 ml/4.5×10^9) corresponds to 3×10^9 cells/ml. Distribute toluene-treated cells in 100 µl aliquotes, freeze them in liquid nitrogen (*see* **Note 7**).

3.3 Spore Germination and Outgrowth

1. Homogenized lyophylized spores (20 mg + 2 ml H_2O) in a 5 ml test tube were subjected to a Bronson sonicator, model B-12, with a microtip and setting 4 for 5 min at 1 min intervals while immersed in ice-water bath.
2. Sonicated spores were prewarm to 37 °C for 5 min and heat-activated at 80 °C for 20 min.
3. Heat-activated spores were centrifuged at $5000 \times g$ for 10 min at +4 °C in a Sorwall RC2-B centrifuge (Ivan Sorwall, Inc., Norwalk, Co., USA).
4. The pellet of spores was resuspended in sterile 2 ml H_2O (10 mg/ml).
5. Germination was initiated in a 250 ml Erlenmeyer flask by adding to 30 ml germination medium 0.3 ml 50% glucose and 1.5 ml 10 mg/ml heat activated spores. Germination mixture was placed in a rotary shaker with the temperature set at 37 °C and a rotary speed of 100 rpm.
6. Germination was monitored by measuring the optical density at 525 nm in a spectrophotometer.

3.4 DNA Polymerase Assay in Permeable Cells of B. Subtilis

To assure that in outgrowing spores of *B. subtilis* 168 replicative DNA synthesis is dealt with, the inhibition of DNA replication can be measured in the presence and absence of both ATP and HP-Ura. Table 1 demonstrates that in toluene-treated permeable *B. subtilis* cells DNA synthesis is an ATP-dependent process and this process can be blocked by inhibitors of semiconservative replication.

3.5 DNA Synthesis in Outgrowing Spores

1. ^{3}H-thymidine incorporation is advised to be measured from zero level absorption every 10 min by adding 5 µl ^{3}H-thymidine to 0.3 ml germination medium and 0.2 ml germination mixture incubated at 37 °C for 10 min.
2. Terminate DNA synthesis by adding 5 ml 0.3 M ice-cold trichloroacetic acid (TCA).
3. Collect precipitate on Whatman GF/C glass fiber filter, wash three times with 5 ml portions of 0.3 M TCA and then with absolute alcohol.
4. Dry filters under an infrared lamp and the radioactivity determined with a toluene-based scintillation fluid.

A typical spore germination and outgrowth curve as well as the DNA synthesis profile are shown in Fig. 1. A lag period can be seen between the addition of the germination-inducing medium and the first sign of germination manifested as a decrease of absorption corresponding to the observation of Woese and Morowitz [27].

Table 1
DNA polymerase assay to prove semiconservative replication in *B. subtilis* 168 toluene-treated cells

Number of sample	^{3}H-dTTP (μl)	H_2O (μl)	KPO_4 (μl)	$MgSO_4$ (μl)	ATP (μl)	dNTP (μl)	HP-Ura (μl)	+ara-dCTP-dCTP (μl)	*B. subtilis* (μl)	Counts (cpm)
1	5	55	14	6	10	–	–	–	10	54 ± 21
2	5	55	14	6	–	10	–	–	10	36 ± 16
3	5	45	14	6	10	10	–	–	10	1180 ± 38
4	5	35	14	6	10	10	10	–	10	337 ± 29
5	5	35	14	6	10	10	–	10	10	78 ± 26

The final volume of each reaction mixture was 100 μl. Incubation lasted for 30 min at 37 °C. Termination took place by the addition of ice-cold 0.3 M trichloroacetic acid (TCA). Precipitate was collected on Whatman GF/C glass fiber filters washed with three 5 ml portions of 0.3 M TCA and then with 3 × 5 ml ethanol. Filters were dried under an infrared lamp and the radioactivity was determined in a toluene-based scintillation fluid

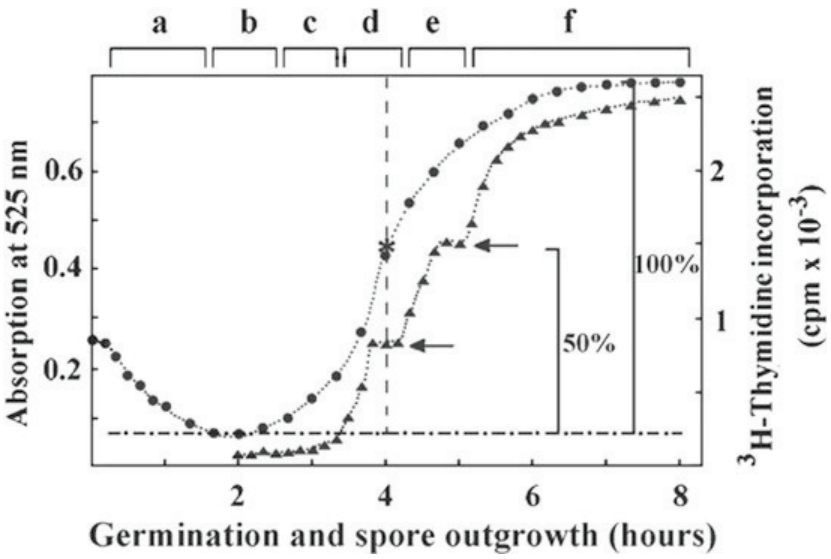

Fig. 1 Synchronization of *B. subtilis* 168 cells by spore outgrowth and germination. Lyophylized spores were homogenized, heat-activated and germinated as described in the Subheading 2. Germination is given as decrease in optical density vs. time. The germination and outgrowth curve consists of (*a*) phase change (germination), (*b*) swelling stage (zero level), (*c*) emergence, (*d*) 1st cell cycle, (*e*) 2nd cell cycle, (*f*) stationary phase (●…●). DNA synthesis showing (*b*) the lack of ^{3}H-thymidine incorporation in the swelling stage, (*c*) emergence, (*d*) initiation of DNA synthesis and the 1st cell cycle, (*e*) elongation and 2nd cell cycle, (*f*) stationary phase (▲…▲). The swelling stage is regarded as the zero level of absorption (-●-). For the best synchrony stop outgrowth of spores after the swelling stage when the optical density at 525 starts to increase and does not exceed twice the value of its original absorption before germination. The upper limit of synchrony corresponding to the inflexion point of DNA synthesis (~50 % of maximal outgrowth) is indicated by the *asterisk* (*). The two *arrows* (←) indicate the end of the first and second round of the cell cycle

The germination phase (Fig. 1a) continues till the absorption reaches its minimum referred to as zero level (Fig. 1b). The zero level of absorption is followed by spore outgrowth involving RNA and protein synthesis and finally DNA synthesis. The outgrowth consists of several subphases (Fig. 1c–f) including swelling and emergence from spore coat (Fig. 1c), elongation of the emerging vegetative cells from the spore coats proceeding to the early phase of logarithmic growth and cell division (Fig. 1d), to mid logarithmic phase and cell growth (Fig. 1e) and to late log and the stationary phase (Fig. 1f). Synchrony is indicated by the biphasic curve of ^{3}H-thymidine incorporation, involving back-to-back division cycles such that the population doubles in number every generation time (Fig. 1d and e). Synchrony is lost in late logarithmic and stationary phase (Fig. 1f) (*see* **Note 8**).

However, this does not mean that the stationary phase could not be selected for the synchronization of bacteria. Cultures of *Escherichia coli* and *Proteus vulgaris* have been synchronized by the stationary-phase method. This method consists of growing the bacteria to an early stationary phase, harvesting them quickly under minimal conditions of stress, and inoculating them into fresh medium at a dilution of about sevenfold. By repeating this procedure on partially synchronized cultures up to four-generation cycles of high percentage of phasing were obtained [28]. Other early methods used filtration to synchronize bacteria [29–31], the principle of binding bacteria to various surfaces and elute unbound sister cells from the surface [32, 33], by means of temperature shift [34], or by amino acid starvation [35].

4 Notes

1. Unless otherwise stated, all solutions should be prepared with distilled water that has an electric conductance of <0.055 mS and an organic content of less than five parts per billion. The solvent is mentioned as “distilled water” in the text.
2. KPO_4 buffer is best stored at 4 °C and can be used for up to 2 months.
3. dNTP solutions (dATP, dGTP, dCTP) have been prepared with distilled water and neutralized with 0.1 M NaOH to pH 7.5.
4. Freshly harvested spores germinate more reproducibly, but spores kept in glycerol also gave reproducible results. For long-term storage, spores were stored in 50% glycerol at –20 °C.
5. To start germination, initial absorbance values at 525 nm should be between 0.25 and 0.3 optical density.
6. To obtain best synchrony stop outgrowing spores after the swelling stage. The best choice regarding synchrony is to take

outgrowing spores after zero level absorption when the OD 525 nm value starts to increase and reaches the initial optical density of spores before germination.

7. After toluene treatment work as quickly as possible, toluene-treated cells are vulnerable. For short-term storage (1 day) keep toluene-treated cells in dry ice–ethanol mixture; for long-term store permeable *B. subtilis* cells at –80 °C or in liquid nitrogen.
8. Do not use outgrowing spores after the inflexion point of the growth curve as the vegetative culture will be already in stationary phase.

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