

Chapter 13

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

Gaspar Banfalvi

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. perfringens*, *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

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 [³H]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 ³H-dTTP, 100 Ci/mmol ³H-thymidine (Amersham Pharmacia Biotech AB, Uppsala, Sweden).
5. K₂HPO₄, KH₂PO₄, (NH₄)₂SO₄, trisodium citrate.
6. MgSO₄ 7 H₂O, FeCl₃·6 H₂O.
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. KPO₄ buffer (K₂HPO₄/KH₂PO₄), 0.5 M, pH 7.5, 2 M MgSO₄, 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 Lyophilized Spores

1. Spores of *B. subtilis* 168 in 1.5 ml microcentrifuge tubes were kept in 50% glycerol (*see Note 4*).
2. Lyophilized 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 K₂HPO₄, 6 g KH₂PO₄, 2 g (NH₄)₂SO₄, 1 g trisodium citrate, 0.5 g MgSO₄ 7 H₂O, 8 mg FeCl₃·6 H₂O, 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 K₂HPO₄, 6.0 g KH₂PO₄, 2 g (NH₄)₂SO₄, 1 g trisodium citrate, 0.2 g MgSO₄ 7 H₂O, 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 KPO₄ buffer, pH 7.4. Pool the suspension in one 15 ml tube and spin down cells. Resuspend cells in 3 ml 0.1 M KPO₄ 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 KPO₄ 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 × 10⁹) corresponds to 3 × 10⁹ 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 lyophilized spores (20 mg + 2 ml H₂O) 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 × *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 H₂O (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. ³H-thymidine incorporation is advised to be measured from zero level absorption every 10 min by adding 5 µl ³H-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 ₂ O (μl)	KPO ₄ (μl)	MgSO ₄ (μl)	ATP (μl)	dNTP (μl)	HP-Ura (μl)	+ara-dCTP- dCTP (μl)	<i>B. subtilis</i> (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 infra-red lamp and the radioactivity was determined in a toluene-based scintillation fluid

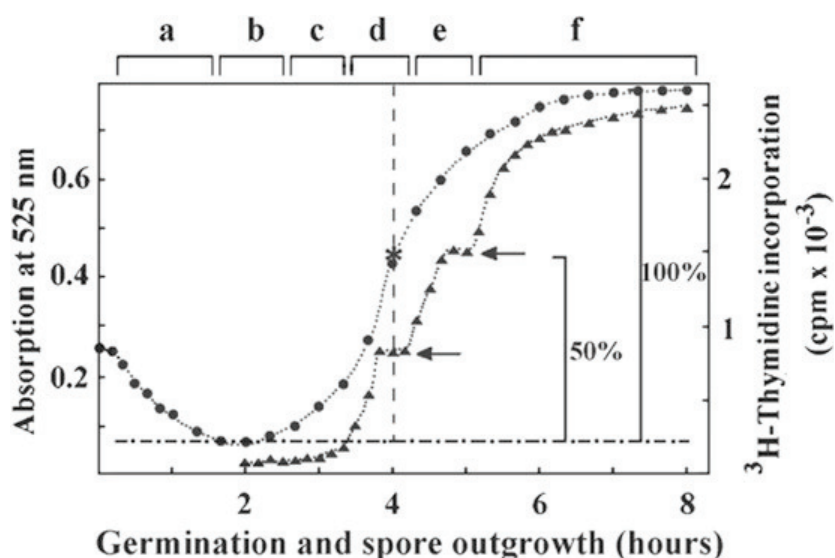


Fig. 1 Synchronization of *B. subtilis* 168 cells by spore outgrowth and germination. Lyophilized spores were homogenized, heat-activated and germinated as described in the Subheading 2. Germination is given as decrease in optical density vs. time. The germination and outgrowth curve consists of (a) 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 ³H-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 ^3H -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 $-80\text{ }^{\circ}\text{C}$ 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.

References

1. Graumann P (ed) (2007) *Bacillus: cellular and molecular biology*, 1st edn. Caister Academic Press, Haverhill, UK, <http://www.horizonpress.com/bac>
2. Hansen JN, Spiegelman G, Halvorson HO (1970) Bacterial spore outgrowth: its regulation. *Science* 168:1291–1298
3. Keynan A (1973) The transformation of bacterial endospores into vegetative cells. *Symp Soc Gen Microbiol* 23:85–123
4. Mandelstam J (1976) Bacterial sporulation: a problem in the biochemistry and genetics of a primitive developmental system. *Proc R Soc B* 193:89–106
5. Nelson DL, Kornberg A (1970) Biochemical studies of bacterial sporulation and germination. XVIII. Free amino acids in spores. *J Biol Chem* 245:1128–1136
6. Setlow P, Kornberg A (1970) Biochemical studies of bacterial sporulation and germination. XXIII. Nucleotide metabolism during spore germination. *J Biol Chem* 245:3645–3652
7. Balassa G (1965) Synthèse macromoléculaire au cours de la germination des spores de *B. Subtilis*. I. Cinq. *Ann De l'Institut Pasteur* 109:13–35
8. Balassa G (1969) Biochemical genetics of bacterial sporulation. I. Unidirectional pleiotropic interactions among genes controlling sporulation in *Bacillus subtilis*. *Mol Gen Genet* 104:73–103
9. Kobayashi I, Steinberg W, Higa A, Halvorson HO, Levinthal C (1965) Sequential synthesis of macromolecules during outgrowth of bacterial spores. In: Campbell LL, Halvorson HO (eds) *Spores IZZ*. American Society for Microbiology, Washington, DC, pp 200–212
10. Torriani A, Levinthal C (1967) Ordered synthesis of proteins during outgrowth of spores of *B. cereus*. *J Bacteriol* 94:176–183
11. Armstrong RL, Sueoka N (1968) Phase transition in ribonucleic acid synthesis during germination of *B. Subtilis* spores. *Proc Natl Acad Sci U S A* 59:153–160
12. Steinberg W, Halvorson HO (1968) Timing of enzyme synthesis during outgrowth of *Bacillus cereus*. I. Ordered enzyme synthesis. *J Bacteriol* 95:469–478
13. Kennett RN, Sueoka N (1971) Gene expression during outgrowth of *B. subtilis* spores. The relationship between order on the chromosome and temporal sequence of enzyme synthesis. *J Mol Biol* 60:31–44
14. Campbell LL Jr (1957) Bacterial spore germination. Definitions and methods of study. In *Spores*. HO Halvorson (ed.). American Institute of Biological Sciences, Washington, DC, pp. 33–38
15. Levinson HS, Hyatt MT (1956) Correlation of respiratory activity with phases of spore germination and growth in *Bacillus megaterium* as influenced by manganese and L-alanine. *J Bacteriol* 72:176–183
16. Halvorson HO (1959) Symposium on initiation of bacterial growth. *Bacteriol Rev* 23:267–272
17. Harrell WK, Halvorson H (1955) Studies on the role of L-alanine in the germination of spores of *Bacillus terminalis*. *J Bacteriol* 69:275–279
18. Yasuda Y, Tochikubo K (1984) Relation between D-glucose and L- and D-alanine in the initiation of germination of *Bacillus subtilis* spores. *Microbiol Immunol* 28:197–207
19. McCann KP, Robinson C, Sammons RL, Smith DA, Corfe BM (1996) Alanine germination receptors of *Bacillus subtilis*. *Letts Appl Microbiol* 23:290–294
20. Liang L, Gai Y, Hu K, Liu G (2008) The *gerA* operon is required for spore germination in

- Bacillus thuringiensis*. Wei Sheng Wu Xue Bao 48:281–286 (article in Chinese)
21. Powell JF (1950) Factors affecting the germination of thick suspensions of *Bacillus subtilis* spores in L-alanine solution. *J Gen Microbiol* 4:330–338
 22. Pulvertaft RJV, Haynes JA (1951) Adenosine and spore germination; phasecontrast studies. *J Gen Microbiol* 5:657–663
 23. Powell JF, Strange RE (1953) Biochemical changes occurring during the germination of bacterial spores. *Biochem J* 54:205–209
 24. Murrell WG, Scott WJ (1958) The permeability of bacterial spores to water. In: Proceedings of the 7th international congress of microbiology, Stockholm, p. 26
 25. Powell JF (1957) Biochemical changes occurring during spore germination in bacillus species. *J Appl Bacteriol* 20:349–358
 26. Bhattacharya S, Sarkar N (1981) Study of deoxyribonucleic acid replication in permeable cells of *Bacillus subtilis* using mercurated nucleotide substrates. *Biochemistry* 20:3029–3034
 27. Woese C, Morowitz HJ (1958) Kinetics of the release of dipicolinic acid from spores of *Bacillus subtilis*. *J Bacteriol* 76:81–83
 28. Cutler RG, Evans JE (1966) Synchronization of bacteria by a stationary-phase method. *J Bacteriol* 91:469–476
 29. Maruyama Y, Yanagita T (1956) Physical methods for obtaining synchronous culture of *Escherichia coli*. *J Bacteriol* 71:542–546
 30. Abbo FE, Pardee AB (1960) Synthesis of macromolecules in synchronously dividing bacteria. *Biochim Biophys Acta* 39:473–485
 31. Lark KG, Lark C (1960) Changes during the division cycle in bacterial cell wall synthesis, volume, and ability to concentrate free amino acids. *Biochim Biophys Acta* 43:520–530
 32. Helmstetter CE, Uretz RB (1963) X-ray and ultraviolet sensitivity of synchronously dividing *Escherichia coli*. *Biophys J* 3:35–47
 33. Helmstetter CE, Cummings DJ (1963) Bacterial synchronization by selection of cells at division. *Proc Natl Acad Sci U S A* 50:767–774
 34. Lark KG, Maaloe O (1954) The induction of cellular and nuclear division in *Salmonella typhimurium* by means of temperature shift. *Biochem Biophys Acta* 15:345–356
 35. Ron EZ, Rozenhak S, Grossman N (1975) Synchronization of cell division in *Escherichia coli* by amino acid starvation: strain specificity. *J Bacteriol* 123:374–376