Tips and Tools

JMBE Journal of Microbiology & Biology Education

An Adaptable and Modular Set of Laboratory Exercises Connecting Genotype to Phenotype in Sporulating *Bacillus subtilis*

Anna L. McLoon,[a] Amy H. Camp,[b] and Lilah Rahn-Lee[c]
[a]*Biology Department, Siena College, Loudonville, New York, USA*
[b]*Department of Biological Sciences, Mount Holyoke College, South Hadley, Massachusetts, USA*
[c]*Biology Department, William Jewell College, Liberty, Missouri, USA*

KEYWORDS laboratory exercise, sporulation, gene regulation, gene expression, genetic pathway, bacterial morphology, *Bacillus subtilis*

INTRODUCTION

Endospore formation (henceforth "spore formation" or "sporulation") is an important topic in college-level microbiology. Sporulating bacteria are abundant in soil and critical for plant health (1, 2). The durability of spores from pathogens like *Clostridioides difficile* pose challenges for human health (3). The morphological processes and genetic regulation of spore formation are fascinating aspects of prokaryotic cellular biology and molecular genetics. Therefore, lab activities investigating spore formation are excellent additions to both medical and basic science-focused microbiology courses.

When sporulating bacteria are starved, they begin a well-defined set of morphological and genetic changes best characterized in the model bacterium *Bacillus subtilis* (Fig. 1) (4, 5). Directed by the transcription factor Spo0A, cells divide asymmetrically, generating a larger mother cell and a smaller forespore. This leads to the activation of two alternative sigma factors, first σ^F in the forespore, then σ^E in the mother cell. The mother cell then engulfs the forespore, at which point σ^G becomes active in the forespore, followed by σ^K in the mother cell. These sigma factors direct the formation of a spore coat around the forespore, drive forespore dormancy, and ultimately induce mother cell lysis, freeing the spore. The mechanisms regulating this sigma factor cascade have been extensively studied. We recommend introducing students to a review (5, 6). Advanced students can also read primary literature.

While this complex process will fascinate advanced students, sporulation is a linear genetic pathway that intermediate students can use to make and test predictions linking genotype to phenotype. Here, we present five protocols implementable in different combinations to suit different courses, student levels, and modes of instructions, such as in-person or virtual labs.

PROCEDURE

Using a set of five nonsporulating *B. subtilis* mutants in which either *spo0A* or one of the alternative sigma factor genes has been deleted, we have developed five protocols that can be used in any combination by students to identify which mutant they received (Table 1).

Malachite green staining

Malachite green is a traditional microbiology stain. It does not bind to cells, but intense heat (produced by placing the slide over boiling water) drives it irreversibly into the coat of mature spores. All other cells, including mother cells and partially formed spores, are stained only by the safranin counterstain. This protocol reinforces mounting, staining, and microscopy skills. Students then hypothesize whether their unknown strain has a sporulation defect.

Sporulation efficiency tests

Sporulation efficiency (the percentage of cells that have completed sporulation) can be calculated by comparing the number of colony forming units (CFU) produced before and after boiling, given that only mature spores survive boiling. Mutant and wild-type (WT) strains are induced to sporulate on a nutrient-poor medium (DSM) for 24 h. Students then make serial dilutions of these cells and plate them onto nutrient-rich medium (LB). The dilutions are then boiled and plated again. Colonies from the boiled dilutions represent only germinated spores, whereas colonies from the preboiled dilutions represent all cells (sporulated or not). Sporulation efficiency (SE) is defined as SE = (CFU/mL boiled)/(CFU/mL preboiled) × 100. Under these conditions, WT strains can achieve high sporulation efficiency, while the mutants should not produce spores. This protocol confirms whether students' unknowns have a sporulation defect and introduces them to serial dilution and the concept of CFU.

Address correspondence to Biology Department, Siena College, Loudonville, New York, USA. E-mail: amcloon@siena.edu.
The authors declare no conflict of interest.
Received: 28 April 2021, Accepted: 22 November 2021, Published: 15 December 2021

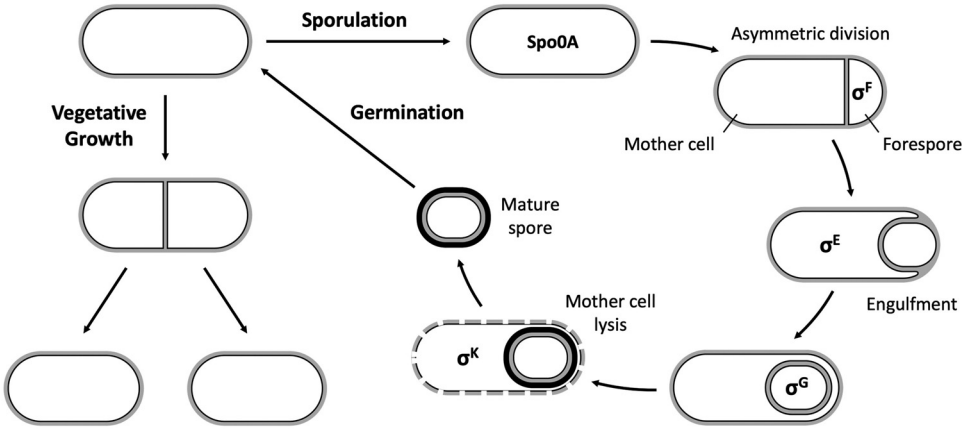

FIG 1. Diagram of vegetative growth, sporulation, and germination by the Gram-positive model bacterium *Bacillus subtilis*. During vegetative growth, *B. subtilis* divides symmetrically by binary fission, creating two identical daughter cells. When nutrients become scarce, *B. subtilis* can initiate a developmental pathway, sporulation, that culminates in the production of a metabolically dormant, environmentally resistant cell type called a *spore*. The major morphological events that occur during sporulation are shown, including asymmetric division, forespore engulfment, and mother cell lysis. The transcriptional regulators responsible for sporulation-specific gene expression—Spo0A, σ^F, σ^E, σ^G, and σ^K—are shown in the cell in which they are active, in order. When nutrients again become available, a spore can resume vegetative growth in a process called *germination*.

Fluorescence microscopy using MitoTracker green

MitoTracker green is a membrane permeable dye. Though blocked by the spore coat, it stains membranes at all other stages of sporulation, including the forespore membrane after engulfment. *spo0A* mutants, which do not divide asymmetrically, σ^F and σ^E mutants, which do not begin engulfment, and σ^K and σ^G mutants, which complete engulfment but do not form phase bright spores, are distinguishable here, differentiating mutants that appear similar with malachite green.

This technique uses fluorescence microscopy, but prepared images are available for students without microscope access or who are learning remotely. We recommend the free analysis program ImageJ for counting cells at each stage of sporulation. Students become familiar with image processing and quantitative data analysis and can refine their hypothesis about their mutant's sporulation defect.

lacZ reporters

The *lacZ* gene is a common transcriptional reporter visualizable on plates using the colorimetric substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). We fused *lacZ* to five promoters whose expression is activated by Spo0A or one of the alternative sigma factors. These fusions can be easily introduced into any *B. subtilis* strain. Students receive a set of reporters in the WT or mutant background and plate them onto nutrient-poor medium with X-gal. All reporters turn blue in the WT background, while the mutant strains turn blue for reporters genetically upstream, but remain white for reporters genetically downstream, of their sporulation block. This assay distinguishes mutant pairs, such as σ^F and σ^E, that appear identical using MitoTracker green and enables students to predict which gene has been disrupted in their mutant.

Kinetic luciferase assays

The gene *lacZ* gives a binary answer about reporter gene activation. In contrast, a luminometer plate reader monitors gene expression from luciferase reporters in real time. With these data, students can observe when (not only if) reporters activate. In our experience, it is impractical for students to run this assay. Instead, we provide data, introducing the concept of multiple replicates and teaching students to manipulate and

TABLE 1
Summary of the 5 modular experiments

Technique	Confirm defect in spore formation	Determine stage of sporulation affected/ identity of mutant	Data available for remote analysis upon request?
Malachite green staining	✓		✓
Sporulation efficiency assay	✓		✓
Fluorescence microscopy		✓	✓
Plate-based *lacZ* (β-galactosidase) reporter gene assay		✓	✓
Kinetic *lux* (luciferase) reporter gene assay		✓	✓

graph large amounts of data. Their graphs support or refute their hypothesis about their mutant.

Safety issues

1. Protocols using boiling water require proper personal protective equipment (PPE).
2. Malachite green requires environmentally safe waste disposal.
3. *B. subtilis* requires biosafety level 1 (BSL1) handling, including autoclaving before disposal. Endospores are resistant to chemical disinfectants.

Adaptability

Depending on your goals, each protocol can stand alone, or the protocols can be combined into a module. All experiments have prepared data available to accommodate remote work or equipment limitations (see "Data Availability," below). These 5 protocols fit into two 3- to 4-h labs. With more time, students could move the reporter genes into their own mutant strain, introducing transformation, selection, and strain validation techniques. Though these protocols do not use true unknowns, they could be made more open-ended. For instance, students could isolate then characterize novel sporulation mutants using mutagenesis and genetic screening.

Data availability

Data sets for all 5 modules are available and will be shared upon request. Strains are available from the Bacillus Genome Stock Center: http://www.bgsc.org.

CONCLUSION

In addition to practical microbiology skills, these exercises introduce critical thinking. For example, students formulate then reformulate their hypothesis about their mutant's identity as they move through the protocols. Students must understand how each assay relates to their biological question, as well as each assay's limitations. Because of the roles spore formers play in human and environmental health, these labs complement classroom discussions about pathogenesis, soil microbiomes, or biogeochemistry. In summary, these experiments can be implemented in a variety of ways to reinforce practical techniques and key concepts in molecular genetics and microbiology.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

ACKNOWLEDGMENTS

We thank A. Sullivan for help with cloning and optimizing the fluorescence microscopy and R. Harper for help with medium preparation. We also thank R. Losick, who introduced us to *B. subtilis* sporulation and one another.

Preliminary work was presented as a poster at the *20th International Conference on Bacilli and Gram-Positive Bacteria*.

REFERENCES

1. Saxena AK, Kumar M, Chakdar H, Anuroopa N, Bagyaraj DJ. 2020. *Bacillus* species in soil as a natural resource for plant health and nutrition. J Appl Microbiol 128:1583–1594. https://doi.org/10.1111/jam.14506.
2. Fira D, Dimkić I, Berić T, Lozo J, Stanković S. 2018. Biological control of plant pathogens by *Bacillus* species. J Biotechnol 285:44–55. https://doi.org/10.1016/j.jbiotec.2018.07.044.
3. Swick MC, Koehler TM, Driks A. 2016. Surviving between hosts: sporulation and transmission. Microbiol Spectr 4:4.4.26. https://doi.org/10.1128/microbiolspec.VMBF-0029-2015.
4. Rudner DZ, Losick R. 2001. Morphological coupling in development: lessons from prokaryotes. Dev Cell 1:733–742. https://doi.org/10.1016/s1534-5807(01)00094-6.
5. Tan IS, Ramamurthi KS. 2014. Spore formation in *Bacillus subtilis*. Environ Microbiol Rep 6:212–225. https://doi.org/10.1111/1758-2229.12130.
6. Riley EP, Schwarz C, Derman AI, Lopez-Garrido J. 2020. Milestones in *Bacillus subtilis* sporulation research. Microb Cell 8:1–16. https://doi.org/10.15698/mic2021.01.739.