Appendix 1: suggested reading list

Suggested Reading List

Review Articles
Riley, E.P., Schwarz, C., Derman, A.I., and Lopez-Garrido, J. (2020). Milestones in *Bacillus subtilis* sporulation research. Microb Cell 8, 1–16. DOI: 10.15698/mic2021.01.739

Shen, A., Edwards, A.N., Sarker, M.R., and Paredes-Sabja, D. (2019). Sporulation and Germination in Clostridial Pathogens. Microbiol Spectr 7. DOI: 10.1128/microbiolspec.GPP3-0017-2018

Tan, I.S., and Ramamurthi, K.S. (2014). Spore formation in *Bacillus subtilis*. Environ Microbiol Rep 6, 212–225. DOI: 10.1111/1758-2229.12130

Primary Research Articles
Bradshaw, N., and Losick, R. (2015). Asymmetric division triggers cell-specific gene expression through coupled capture and stabilization of a phosphatase. Elife 4, e08145. DOI: 10.7554/eLife.08145

Camp, A.H., and Losick, R. (2009). A feeding tube model for activation of a cell-specific transcription factor during sporulation in *Bacillus subtilis*. Genes Dev 23, 1014–1024. DOI: 10.1101/gad.1781709

Doan, T., Coleman, J., Marquis, K.A., Meeske, A.J., Burton, B.M., Karatekin, E., and Rudner, D.Z. (2013). FisB mediates membrane fission during sporulation in *Bacillus subtilis*. Genes Dev 27, 322–334. DOI: 10.1101/gad.209049.112

Ellermeier, C.D., Hobbs, E.C., Gonzalez-Pastor, J.E., and Losick, R. (2006). A three-protein signaling pathway governing immunity to a bacterial cannibalism toxin. Cell 124, 549–559. DOI: 10.1016/j.cell.2005.11.041

Fujita, M., and Losick, R. (2003). The master regulator for entry into sporulation in *Bacillus subtilis* becomes a cell-specific transcription factor after asymmetric division. Genes Dev 17, 1166–1174. DOI: 10.1101/gad.1078303

Fujita, M., and Losick, R. (2005). Evidence that entry into sporulation in *Bacillus subtilis* is governed by a gradual increase in the level and activity of the master regulator Spo0A. Genes Dev 19, 2236–2244. DOI: 10.1101/gad.1335705
Halder, S., Parrell, D., Whitten, D., Feig, M., and Kroos, L. (2017). Interaction of intramembrane metalloprotease SpoIVFB with substrate Pro-σK. Proc Natl Acad Sci U S A 114, E10677–E10686. DOI: 10.1073/pnas.1711467114

Karow, M.L., Glaser, P., and Piggot, P.J. (1995). Identification of a gene, spoIIR, that links the activation of σE to the transcriptional activity of σF during sporulation in Bacillus subtilis. Proc Natl Acad Sci USA 92, 2012–2016. DOI: 10.1073/pnas.92.6.2012

Laue, M., Han, H.-M., Dittmann, C., and Setlow, P. (2018). Intracellular membranes of bacterial endospores are reservoirs for spore core membrane expansion during spore germination. Sci Rep 8, 11388. DOI: 10.1038/s41598-018-29879-5

Luhur, J., Chan, H., Kachappilly, B., Mohamed, A., Morlot, C., Awad, M., Lyras, D., Taib, N., Gribaldo, S., Rudner, D.Z., et al. (2020). A dynamic, ring-forming MucB / RseB-like protein influences spore shape in Bacillus subtilis. PLoS Genet 16, e1009246. DOI: 10.1371/journal.pgen.1009246

Martínez-Lumbreras, S., Alfano, C., Evans, N.J., Collins, K.M., Flanagan, K.A., Atkinson, R.A., Krysztofinska, E.M., Vydyanath, A., Jackter, J., Fixon-Owoo, S., et al. (2018). Structural and functional insights into Bacillus subtilis sigma factor inhibitor, CsfB. Structure 26, 640-648.e5. DOI: 10.1016/j.str.2018.02.007

Mearls, E.B., Jackter, J., Colquhoun, J.M., Farmer, V., Matthews, A.J., Murphy, L.S., Fenton, C., and Camp, A.H. (2018). Transcription and translation of the sigG gene is tuned for proper execution of the switch from early to late gene expression in the developing Bacillus subtilis spore. PLoS Genet 14, e1007350. DOI: 10.1371/journal.pgen.1007350

Ramos-Silva, P., Serrano, M., and Henriques, A.O. (2019). From Root to Tips: Sporulation evolution and specialization in Bacillus subtilis and the intestinal pathogen Clostridioides difficile. Molecular Biology and Evolution 36, 2714–2736. DOI: 10.1093/molbev/msz175

Riley, E.P., Lopez-Garrido, J., Sugie, J., Liu, R.B., and Pogliano, K. (2021). Metabolic differentiation and intercellular nurturing underpin bacterial endospore formation. Science Advances 7, eabd6385. DOI: 10.1126/sciadv.abd6385

Rudner, D.Z., Pan, Q., and Losick, R.M. (2002). Evidence that subcellular localization of a bacterial membrane protein is achieved by diffusion and capture. Proc Natl Acad Sci USA 99, 8701–8706. DOI: 10.1073/pnas.132235899

Tan, I.S., Weiss, C.A., Popham, D.L., and Ramamurthi, K.S. (2015). A quality-control mechanism removes unfit cells from a population of sporulating bacteria. Dev Cell 34, 682–693. DOI: 10.1016/j.devcel.2015.08.009
Appendix 2: general background for lab manual

Endospore formation

Objectives:

Some species of bacteria are able to form dormant spores. These spores are morphologically and physiologically distinct from vegetative cells, and the differences can have profound consequences for the species in their environment, and profound consequences for human health if the bacterium is part of the human microbiome or is a human pathogen. There are several different types of spores formed by different bacteria: actinomyces form spores at the end of long filamentous hyphae, and for many other spore-forming bacteria, cells simply round up and become slightly more resistant. In contrast, endospores are produced inside of another cell (the so-called “mother cell”), and are ultimately released by lysis (destruction) of that cell. Endospore forming pathogens include Bacillus anthracis (the causative agent of anthrax), Clostridium botulinum (the causative agent of botulism), and Clostridioides difficile (the causative agent of severe gastrointestinal diseases including diarrhea, pseudomembranous colitis, and toxic megacolon).

In this lab, we will use the harmless, model endospore-forming bacterium Bacillus subtilis to learn more about endospores, the process by which they arise, and the techniques used to study them. More specifically, the learning objectives of this laboratory are:

- become familiar with endospores
- follow the specific stages of endospore formation
- identify the specific gene disrupted in a mutant strain of B. subtilis that cannot initiate or complete the process of endospore formation

Background:

Many groups of bacteria form some sort of dormant cell-type, typically called a “spore”, that is resistant to adverse conditions. The spores formed by various taxonomic groups are morphologically unique and form in different ways: actinomyces form spores at the end of long filaments, while other spore-forming bacteria like the myxobacteria simply round up and become slightly more resistant. The toughest spores, however, are the endospores that are formed exclusively by certain groups of low-GC Gram positive bacteria (certain species of firmicutes including Bacillus and Clostridium species). In fact, the species Bacillus subtilis, the most extensively studied model endospore former, was first isolated from an infusion of hay that was boiled—vegetative cells and spores from most other species were killed by the high temperatures, while B. subtilis endospores survived!
Figure 1. Cartoon depiction of vegetative growth, endospore formation ("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. 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 and order in which they are active. When triggered by nutrients, a spore can resume vegetative growth in a process called germination.

During endospore formation, spores form within another cell (the prefix “endo” means “in” or “within”) (Figure 1). In the presence of plentiful nutrients, endospore-forming bacteria will grow “vegetatively” by binary fission, dividing at the cell midpoint forming two identical daughter cells. In contrast, when nutrients become scarce, endospore formation can occur. This process, referred to hereafter simply as “sporulation”, begins with an asymmetric cell division, where the division septum forms closer to one cell pole. The smaller cell, called the forespore, is fated to become the spore. The larger cell, which helps the forespore develop but ultimately dies, is called the mother cell. Next, the mother cell membrane migrates around the forespore in a process called engulfment. Once the mother cell membranes fuse on the other side of the forespore, engulfment is complete, and the forespore is suspended within the mother cell’s cytoplasm. The forespore then matures as cell wall material called the cortex is deposited in the space between the two forespore membranes, and a spore coat composed of proteins is layered outside the membranes. Finally, the mother cell lyases, releasing the fully-developed, metabolically-dormant, environmentally-resistant spore into the
environment. Spores of *B. subtilis* and related species are thought to be able to survive for hundreds, if not thousands of years or more. When nutrients again become available, spores can return to vegetative growth in a process called germination.

The morphological events of sporulation described above are driven by the expression of hundreds of genes in the appropriate cell at the appropriate time. This feat of gene expression is orchestrated by a set of transcriptional regulators that have been studied most extensively in the model organism *B. subtilis*. First, a transcription factor called Spo0A ("spo-zero-A") triggers entry into this developmental pathway through the activation of sporulation-specific genes. Subsequently, a series of four alternative sigma (σ) factors recruit RNA polymerase to transcribe large sets of sporulation genes in the forespore or mother cell, at early or late times, depending on the sigma factor. Soon after asymmetric division, σ^F ("sigma-F") becomes active in the forespore. This is followed by activation of σ^E ("sigma-E") in the mother cell. After the completion of forespore engulfment, σ^G ("sigma-G") becomes active in the forespore, taking over for σ^F. Last but not least, σ^K ("sigma-K") becomes active in the mother cell, replacing σ^F.

How are these transcriptional regulators themselves regulated, such that they become active in the correct cell at the correct time? The picture that has emerged from decades of research is that the activity of each transcriptional regulator relies on the one(s) that came before, in the following order: Spo0A (pre-divisional cell) → σ^F (forespore) → σ^E (mother cell) → σ^G (forespore) → σ^K (mother cell). This “cascade” of activation, which involves three intercellular communication events, ensures that gene expression in the two cells stays in register, and that late gene expression does not begin until early gene expression is complete. If you are interested to learn more about the molecular details of this sporulation gene expression cascade, a good place to start would be a review article such as Tan and Ramamurthi (2014) (DOI: https://doi.org/10.1111/1758-2229.12130) or Riley et al. (2020) (DOI: https://doi.org/10.15698/mic2021.01.739).

Given their central role in driving sporulation-specific gene expression, it makes good sense that *B. subtilis* mutants lacking the genes encoding Spo0A, σ^F, σ^E, or σ^K are unable to form spores. Moreover, each of these mutants arrest at a predictable stage of sporulation, depending on where they act in the cascade (Table 1). In this lab, you will receive one such mutant, as well as a non-mutant “wild type” control strain. First, you will experimentally confirm that your mutant has a defect in sporulation. Second, you will determine the specific stage of sporulation at which your mutant is blocked, thereby allowing you to identify which gene has been deleted!
Table 1. Processes controlled by sporulation-specific transcriptional regulators and the stage of sporulation at which their mutants arrest.

| Transcriptional Regulator | Processes Controlled | Appearance of Mutant |
|---------------------------|----------------------|----------------------|
| Spo0A                     | Expression of σ\text{F} and σ\text{E}, asymmetric division |
| σ\text{F}                | Early forespore development, activation of σ\text{E}, expression of σ\text{G} |
| σ\text{E}                | Engulfment, activation of σ\text{G}, expression of σ\text{K} |
| σ\text{G}                | Late forespore development, activation of σ\text{K} |
| σ\text{K}                | Forespore coat assembly, mother cell lysis |

*Mutants lacking σ\text{F} or σ\text{E} cannot progress past the stage of asymmetric division. These mutants often initiate a futile second asymmetric division event, leading to a “disporic” phenotype and even after prolonged periods of time, the formation of short mini-cells corresponding to the forespore.*
Appendix 3: Malachite green stain, lab manual section

Protocol 1: Confirm a sporulation block using malachite green stain

Background:
Malachite green is one of the classic microbiology staining protocols traditionally used to identify endospore-forming bacteria. The dye is an organic molecule composed of three benzene rings. This strong aromatic character causes the molecule to absorb light at 621 nm, producing a brilliant green color. To force the stain into endospores, which have many protective layers and are difficult for molecules to penetrate, cell smears are covered in stain and then steamed over boiling water. Unlike many stains used in microbiology, malachite green does not interact strongly with cellular material. This is the principle behind this differential staining protocol. A quick water rinse after the steaming easily removes malachite green from vegetative cells, while leaving the stain trapped within the many layers of the endospore. To visualize the now colorless vegetative cells, a safranin counterstain is used.

If your strain of bacteria is able to produce mature endospores with malachite green-trapping protective layers, you should expect to see green-stained endospores in a sea of red vegetative cells after staining cells grown under conditions that trigger endospore formation. However, even if grown under such starvation conditions, strains that have mutations preventing sporulation from occurring will be unable to produce an endospore that can retain the malachite green dye.

Materials:

- WT and mutant strains of *Bacillus subtilis*
- DSM agar plates
- Glass slides
- Sterile water
- Inoculating loop or sterile pipette tips
- Malachite green (1% in water)
- Safranin (0.25% in water from a 2.5% stock in ethanol)
- Hot plate
Beaker

Slide holder to hold slide in steam over beaker

Paper towel

Water

Brightfield microscope with 100X oil immersion lens

Immersion oil

Recipes:

DSM agar plates

Per liter: 8 g Bacto Nutrient Broth, 10 mL 10% (w/v) KCl, 10 mL 1.2% (w/v) MgSO₄·7H₂O. pH to 7.6 with NaOH. Add 1% (w/v) agar. Autoclave, then cool to 50 °C. Add sterilized: 1 mL each of 1M Ca(NO₃)₂, 0.01 M MnCl₂, 1 mM FeSO₄. Pour into sterile petri plates and allow to cool before use.

Protocol:

1. 24-36 hours before class, streak the control WT strain and your unknown mutant onto a DSM plate to induce sporulation. Multiple strains can share one plate, and streaking for single colonies is not required at this step. Incubate at 37 ℃.

2. For each strain, prepare bacterial smears on a glass slide by using a sterile loop or pipette tip to suspend cells from the DSM plate in a drop of sterile water. Allow cells to air dry or use a slide drier.

3. Cut a small piece of paper towel to the same size as the slide and place it over the slide.

4. Flood the paper towel with malachite green and allow it to stand for 30 seconds.

5. Place the slides on a slide holder over a beaker of boiling H₂O for 5 minutes, adding additional stain as necessary. As the stains and water from the waterbath can splash and burn or stain, use lab coats, safety glasses, and gloves. Be careful not to drip stain into the boiling water or the hot plate surface, and not to let the beaker boil dry.

6. Remove slides from steam using forceps, allow them to cool, and remove the paper using forceps. Rinse with water for 30 seconds.
7. Counterstain with safranin for 60 seconds.

8. Wash the slides with water for 30 seconds and blot dry.

9. Examine the slides under 100x oil immersion, and draw/describe the stained cells in your notebook. Image using the digital workstations if available.

**Analysis**

1. Describe your observations for your WT and mutant strain. Include a drawing or digital micrograph of each. Do you see green spores? What color are non-spore cells? Can you see green spores within mother cells? Make note of relative position and shape of spore and mother cell, as this is a distinguishing characteristic of some *Bacillus* species.

2. Does it appear that your mutant does indeed have a problem with sporulation? Explain your findings by referring to your observations.
Protocol 2: Determine the sporulation efficiency of WT and mutant strains

Background:
Because boiling kills vegetative cells but not spores, we can determine the sporulation efficiency of a strain, or the percent of cells that have successfully completed sporulation, by comparing the number of colony forming units (CFUs) produced by a culture before and after boiling. Often when we streak or spread bacteria on a plate of solid medium, we think of a single colony as having arisen from a single cell that was isolated on the surface of the medium away from other cells. But a better way to think about colonies is that they are descended from colony forming units. A CFU might be a single cell, or it could be a collection of several cells that have clumped together. For the purposes of this experiment it's important to note that a CFU could be a vegetative cell or a spore that has germinated on the plate.

To determine how effective our strains are at undergoing endosporulation, we want to know how many vegetative cells versus spores our cultures contain after we have incubated them in nutrient poor conditions. Because the colonies produced from vegetative cells or from germinated spores look the same, we will distinguish between them by comparing the number of colonies produced on nutrient rich medium by a particular volume of culture before and after boiling. The colonies from the culture before boiling could have originated from vegetative cells or from spores, but the colonies that grow after boiling must have come from germinated spores.

Once we have counted the numbers of colonies produced in each condition, we will calculate sporulation efficiency using the equation:

\[ SE = \frac{\text{CFU/mL boiled}}{\text{CFU/mL pre-boiled}} \times 100 \]

Before we perform our experiment, we won’t know how many CFUs there are per milliliter of our culture. If we put too many CFUs on a plate, they will form a lawn and we won’t be able to count them, but if we put too few we won’t have enough to count. To get around this problem, we will create a dilution series and plate several dilutions of our cultures to ensure we have at least some plates with a good number of colonies to count.
**Materials:**

- WT and mutant strains of *Bacillus subtilis*
- DSM agar plates or liquid DSM
- Sterile water
- Sterilized borosilicate glass test tubes (6 per strain) and tube rack
- Water bath capable of heating to 80-100 °C
- LB agar plates (12 per strain)
- Sterilized glass beads for spread plating
- Micropipettes and sterile pipette tips

**Recipes:**

**DSM agar plates**

Per liter: 8 g Bacto Nutrient Broth, 10 mL 10% (w/v) KCl, 10 mL 1.2% (w/v) MgSO₄·7H₂O. pH to 7.6 with NaOH. Add 1% (w/v) agar. Autoclave, then cool to 50 °C. Add sterilized: 1 mL each of 1M Ca(NO₃)₂, 0.01 M MnCl₂, 1 mM FeSO₄. Pour into sterile petri plates and allow to cool before use.

**DSM liquid**

As recipe for agar plates above, except omit agar.

**Protocol:**

1. 24-36 hours before class, streak the control WT strain and your unknown mutant onto a DSM plate to induce sporulation. Multiple strains can share one plate, and streaking for single colonies is not required at this step. Alternatively, for more efficient sporulation, inoculate the strains into liquid DSM. Incubate plates 24-36 hours at 37 °C. Incubate liquid cultures with shaking at 37 °C for 24-72 hours.

2. Using sterile technique, add 900 uL of sterile water to each of 5 glass test tubes for each strain under investigation. If using liquid DSM medium, add 1 mL of the 24 hour culture to the remaining tube. If using DSM agar plates, add 1 mL sterile water
to the remaining tube, and use an inoculating loop or pipette tip to scrape some cells off the agar plate into the 1 mL of sterile water.

3. We will now create a dilution series. Transfer 100 μL of liquid from the tube containing your cells (the undiluted tube) to the next tube (the 10⁻¹ tube). Pipette up and down, then replace your pipette tip. With your new tip, transfer 100 μL from the second tube (the 10⁻¹ tube) to the third tube (the 10⁻² tube). Pipette up and down, then replace your pipette tip. Repeat this process until each tube in the 6-tube series has been inoculated, creating a series of 1:10 dilutions starting with the undiluted tube, and finishing with the 10⁻⁵ tube. Create one dilution series for each strain under investigation. *NOTE:* It is important to change pipette tips between each dilution for accuracy because cells stick to the plastic tip and are carried over from tube to tube if only one tip is used.

4. Pipette 100 μL of each of your dilutions onto LB agar plates (6 plates per strain). Using sterilized glass beads, spread the liquid evenly across the surface of the agar. *NOTE: because spores survive ethanol, a reusable glass spreader is not recommended for spread plating endospore forming bacteria. Glass beads, which are sterilized by autoclaving between use, are preferred.*

5. Using lab coats, safety glasses, and gloves, carefully place the rack containing the glass tubes with your dilution series in the 100 °C water bath and incubate for 10 minutes. As cool tubes are placed inside, the temperature of the bath may drop. It’s important to start with a bath as hot as possible, so that a temperature of above 80 °C is maintained over the 10 minute incubation.

6. Remove the rack of tubes from the water bath and allow to cool. Pipette 100 μL of each of your boiled dilutions onto LB agar plates (6 plates per strain). Spread plate using glass beads.

7. Incubate your LB agar plates (12 per strain) at 37 °C for 18-24 hours. Count the colonies on each plate.

**Analysis:**

1. What sporulation efficiency do you expect for WT? What do you expect for mutants missing spo0A or one of the alternative sigma factors? Why?
2. Fill out the table below with your colony counts. If a plate is completely covered with uninterrupted growth, write “lawn”. If there are individual colonies visible, but they have grown together so much that you can’t tell how many they are, write “too numerous to count”. If there are more than several hundred colonies, you can divide the plate into even quarters or eights, and count only a portion of the plate, then multiply by 4 or 8 (only use this trick if the colonies are evenly spread across the surface of the plate).

|        | Undiluted | 10⁻¹ | 10⁻² | 10⁻³ | 10⁻⁴ | 10⁻⁵ |
|--------|-----------|------|------|------|------|------|
| WT     | Pre-Boiled|      |      |      |      |      |
|        | Boiled    |      |      |      |      |      |
| Mut    | Pre-Boiled|      |      |      |      |      |
|        | Boiled    |      |      |      |      |      |

3. Using your data above, select the 2 or 3 most usable dilutions for each condition, and calculate CFU/mL, using the formula:

\[
\text{CFU/mL} = (\# \text{ colonies})(10)(\text{dilution factor})
\]

Where dilution factor would be 1 for undiluted, 10¹ for the 10⁻¹ plate, 10² for the 10⁻² plate, etc. Average the CFU/mL value you receive from the 2 or 3 most useful dilutions for each condition.

4. Calculate the sporulation efficiency for each strain using the formula:

\[
\text{SE} = (\text{CFU/mL boiled})/(\text{CFU/mL pre-boiled}) \times 100
\]
5. How do your sporulation efficiencies compare between the WT and your mutant? Are these numbers what you would expect? Why or why not?

6. Based on your data here, do you think your mutant has a defect in sporulation? Does this agree or disagree with your malachite green staining data (if applicable)?

7. Why did we multiply by 10 in the equation in question 3? (HINT: what volume did we add to our plates?)

8. Did your CFU/mL answer from different dilutions of the same condition agree or disagree with each other? Why would these numbers be different? Is averaging them a good practice? Why or why not?

9. The sporulation efficiency for the WT strain of *B. subtilis* will differ between cultures sporulated on DSM agar and DSM liquid media. Given your understanding of sporulation from your reading of review articles and/or the primary literature, why do you think this might be?
Appendix 5: MTG fluorescence microscopy, lab manual section

Protocol 3: Use fluorescence microscopy to identify a specific sporulation defect

Background:
Light microscopy and malachite green staining only allow visualization of mature spores. However, there can be defects in stages of sporulation that occur prior to deposition of material in the spore coat and spore cortex. Using a fluorescent membrane dye such as MitoTracker Green (MTG), it is possible to see the asymmetric cell division that leads to formation of the forespore and mother cell, and to identify cells whether engulfment has started or completed. MTG can pass through the cellular membrane, labeling a fully engulfed forespore, but does not bind effectively once the spore coat has formed. One possible complication of this staining method is that spores sometimes autofluoresce weakly, which is possible to confuse with MTG staining.

Materials:
- WT and mutant strains of *Bacillus subtilis*
- DSM agar plates
- MitoTracker Green stain (MTG) (Invitrogen cat. number M7514)
- Coverslips
- Poly-L-lysine solution (0.1% w/v in water, purchase as solution)
- Laboratory tape
- 1% agar in 1x phosphate buffered saline (PBS)
- Glass slides
- Micropipettes and sterile pipette tips

Recipes:

**DSM agar plates**
Per liter: 8 g Bacto Nutrient Broth, 10 mL 10% (w/v) KCl, 10 mL 1.2% (w/v) MgSO₄·7H₂O. pH to 7.6 with NaOH. Add 1% (w/v) agar. Autoclave, then cool to 50 °C. Add sterilized: 1 mL each of 1M Ca(NO₃)₂, 0.01 M MnCl₂, 1 mM FeSO₄. Pour into sterile petri plates and allow to cool before use.

**MTG stain**
MTG should be resuspended at a stock concentration in DMSO, then diluted to a working concentration of 10-20 μg/ml in PBS. Different concentrations may be optimal depending on your microscope optics.

**PBS**
Per liter: 8g NaCl, 0.2g KCl, 1.44g Na₂HPO₄, 0.42g KH₂PO₄

**Protocol:**

1. 24-36 hours before class, streak the control WT strain and your unknown mutant onto a DSM plate to induce sporulation. Multiple strains can share one plate, and streaking for single colonies is not required at this step. Incubate at 37 °C.

2. Prepare poly-L-lysine treated coverslips for each sample by dipping a coverslip into the poly-L-lysine solution, then allowing the solution to dry. Dab off any big drops, as they will otherwise leave a ring.

3. Prepare agar pads. Tape the left and right sides of a microscope slide to the bench with three layers of laboratory tape, then pipette on approximately 500 μL of melted 1% agar in PBS between the tape stacks. Place a second clean slide directly on top of the agar. Once the agar has solidified, slide off the top slide and peel off the tape, leaving a thin, level agar pad.

4. Mix your mutant and the control into separate tubes containing 30 μl of the MTG solution.

5. Spot 5 μl of your suspended cells onto the agar pad, then cover with a poly-L-lysine treated coverslip.

6. Take images of multiple fields of your cells using the digital workstations using the 100x oil immersion lens. Work very carefully to get as many cells in focus as possible, then take images of MTG and phase or DIC. For each field it is ESSENTIAL that you take an image using the green channel and phase without touching the stage controls. If the slide moves, it can be difficult or impossible to carry out the analysis. I would recommend taking image sets of at least 5-6 different fields of view for both your positive sporulation control and your mutant.

7. Save your images, making sure the file titles reflect the sample (WT or mutant) so that you know which MTG and phase images correspond.

**Optional: Cell counting with ImageJ**

1. Install ImageJ on your computer by going to https://imagej.nih.gov/ij/download.html and following the instructions for your computer platform. (note: current versions of ImageJ are available for Linux, Windows and Mac machines).
2. Install the CellCounter plugin by going to https://imagej.nih.gov/ij/plugins/cell-counter.html and following the directions to download the file and move it to the correct directory.

3. Open the ImageJ program. Open the image file(s) you wish to analyze using the ImageJ menu: File>open then navigate to the image file that you wish to open.

   Hint: You can use the ImageJ menu to Zoom in or out by using the magnifying glass in the imageJ window, then left clicking on the image to zoom in, and right clicking on the image to zoom out. If you zoom in, you can then use the scrolling tool (hand outline) on the ImageJ menu to move the section of the image you are viewing. To begin counting, turn back on the “rectangular selection” tool (far left).

4. Open the cell counter plugin using the ImageJ menu: Plugin>Cell Counter

5. Initialize the image for counting by clicking the Initialize button in the cell counter window.
6. The cell counter counts generically by type. In order to count cells in different stages of sporulation, you should define each type in terms of sporulation stage. For instance, you could use this key:

| Cellcounter type | Stage of sporulation |
|------------------|-----------------------|
| Type 1           | Vegetative cells      |
| Type 2           | Asymmetric division (this can include dipsporic cells in a $\sigma^F$ or $\sigma^E$ mutant) |
| Type 3           | Engulfment is occurring/has occurred but phase dark |
| Type 4                          | Engulfment complete and phase bright |
|--------------------------------|-------------------------------------|
| Type 5                          | Free spores*                        |

*Remember that free spores MUST be oval and phase bright. Otherwise you may be looking at debris or minicells that sometimes split off if a cell spends too long at the “asymmetric division” step.

7. To begin counting, click on the round button in the Cell Counter window corresponding to the cell type you wish to count.

8. Then, click on every cell you wish to count in the image that belongs to that cell type. For instance, if “type 1” is selected, click on all cells that are vegetative with central division septa. A point with the number 1 should appear on the image where you clicked.

   **Hint:** If you accidentally click in the wrong place on the image, you can use the delete button in the Cell Counter window to remove the most recent count. If there is an earlier marker that you want to remove or there are multiple markers that you want to remove, you can use the menu to switch into “delete mode” to remove markers by clicking on them. Just be sure to exit “delete mode” before beginning to count again.

9. Once you have completed counting cells corresponding to that stage of sporulation, click on the next “type” that you wish to count in the Cell Counter window, and click on the cells you wish to count in the image. Markers should appear in the new color.

10. When ensuring that a small cell is actually a spore or when differentiating between “engulfment occurring but phase bright” and “engulfment complete and phase bright”, be sure you are examining both the MTG image (showing all membranes) and the phase or DIC image.

**Analysis:**

1. Do you see phase-bright spores? Describe the spores and mother cells. How do your fluorescence microscopy results compare to Malachite green staining (if applicable)?
2. Now, look at the fluorescence images you have generated. Count all the cells systematically in a field, assigning each to a stage of sporulation. You should aim to count at least 200 cells each for the control and mutant.

|                                              | Number of WT cells that are: | Number of mutant cells that are: |
|----------------------------------------------|------------------------------|---------------------------------|
| Vegetative cell                              |                              |                                 |
| Asymmetric cell division complete            |                              |                                 |
| Engulfment                                   |                              |                                 |
| Spore coat/cortex production started/completed |                              |                                 |
| Mature spores following mother cell lysis    |                              |                                 |

3. Can you hypothesize the step of sporulation that is not occurring properly in your mutant?

4. Consider what you expect to see for each of the following genetic defects:

   a. What would you expect to see in a Spo0A mutant? Which processes fail to happen and which sigma factors fail to activate?

   b. What would you expect to see in a $\sigma^F$ mutant? Which processes fail to happen and which sigma factors fail to activate?
c. What would you expect to see in a $\sigma^c$ mutant? Which processes fail to happen and which sigma factors fail to activate?

d. What would you expect to see in a $\sigma^0$ mutant? Which processes fail to happen and which sigma factors fail to activate?

e. What would you expect to see in a $\sigma^K$ mutant? Which processes fail to happen and which sigma factors fail to activate?

5. Considering your phenotype described in question 2 and your predictions about each mutant described in question 4, formulate a hypothesis as to which protein or sigma factor is missing in your unknown mutant.
Appendix 6: LacZ plate assays, lab manual section

Protocol 4: Use semi-quantitative LacZ plate assays to identify the transcriptional activation of sporulation genes

Background:
The lacZ gene encodes the enzyme Beta-galactosidase (β-gal), which digests the glycosidic bond between a galactose sugar and other molecules, such as the bond between galactose and glucose in the disaccharide lactose. X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) is a colorimetric substrate for β-gal. It is colorless when intact, but when cleaved by the enzyme turns blue. We can take advantage of this property to track the presence and relative amount of Beta-galactosidase by observing a blue color in the presence of X-gal.

For this experiment, we have created “reporters”: genetic fusions of promoters from Bacillus subtilis to the lacZ gene. These gene fusions have then been introduced into the chromosome of the Wild Type or mutant B. subtilis strains under investigation here, creating reporter strains. When a reporter strain is grown with X-gal under conditions that activate the promoter fused to lacZ, the cells will turn blue, but they will remain colorless under conditions where the promoter is not activated. Reporter strains are a simple way to observe if a promoter of interest is activated or not.

We have selected five promoters for our reporter strains, choosing promoters that are known to be activated by the sporulation transcription factor and sigma factors:

1. P_{spolIIG}lacZ (Spo0A reporter)
2. P_{spolIIQ}lacZ (σ^F reporter)
3. P_{spolIId}lacZ (σ^E reporter)
4. P_{sspB}lacZ (σ^G reporter)
5. P_{gerE}lacZ (σ^K reporter)

In a properly functioning cell, we would expect all five reporter strains to remain white in the presence of X-gal on a nutrient-rich growth medium, but to turn blue under starvation conditions as the cells move through the sporulation process. But in the case of sporulation mutants, a block earlier in the sporulation pathway may prevent the activation of a promoter that is normally turned on by a downstream sigma or transcription factor. We will use this principle in this experiment to investigate what part of the sporulation pathway is disrupted in our mutant strains.
**Materials:**

- DSM plates with 100 ug/mL X-gal (for Spo0A, σE, σG, and σK reporters)
- DSM plates with 140 ug/mL X-gal (for σF reporter)
- LB plates struck out with WT and mutant strains of *Bacillus subtilis* carrying the 5 reporter constructs to be investigated (5 strains per WT or mutant background)
- Materials for streaking bacteria (inoculating loop and bunsen burner, or sterile sticks)

**Recipes:**

**DSM agar plates with X-gal**

Per liter: 8g Bacto Nutrient Broth, 10 mL 10% (w/v) KCl, 10 mL 1.2% (w/v) MgSO₄·7H₂O. pH to 7.6 with NaOH. Add 1% (w/v) agar. Autoclave, then cool to 50 °C. Add sterilized: 1 mL each of 1M Ca(NO₃)₂, 0.01 M MnCl₂, 1 mM FeSO₄. Add the appropriate amount of X-gal dissolved in DMSO. Pour into sterile petri plates and allow to cool before use. Store X-gal containing plates in the dark.

**Protocol:**

1. Make a plan for how you will streak out your strains on your DSM X-gal plates. You don’t need to streak for single colonies, so you could patch your strains, or streak once across a quarter or smaller section of your plate. All of the reporter constructs should be struck out on the DSM with 100 ug/mL X-gal, except for the weaker σF reporter, which should be struck out on 140 μg/mL X-gal. Label your plates.

2. Streak the reporter constructs (5 strains per WT or mutant background) onto your DSM X-gal plates.

3. Incubate the plates at 37 °C for 48 hours, then photograph and record the color of your streaks.
**Analysis:**

1. Before the lab, fill out this table. For each mutant, predict which (if any) reporters should be activated.

| Spo mutant background | Spo0A reporter | σ^F reporter | σ^E reporter | σ^G reporter | σ^K reporter |
|------------------------|----------------|--------------|--------------|--------------|--------------|
| Wildtype PY79 (no mutations) |                |              |              |              |              |
| Δspo0A                  |                |              |              |              |              |
| ΔsigF                   |                |              |              |              |              |
| ΔsigE                   |                |              |              |              |              |
| ΔsigG                   |                |              |              |              |              |
| ΔsigK                   |                |              |              |              |              |

2. Given any previous experiments you have carried out, which gene(s) may be mutated in your mutant strain? Which reporters in your prediction table above could be used to rule out or confirm your hypotheses?

3. After the lab, fill out this table with your observations. Which strains are blue, and which are not blue? (since some promoters are under more strict repression than others, a faint color may or may not indicate that the promoter has been activated. To help with your analysis, be sure to indicate intensity)

| Spo mutant background | Spo0A reporter | σ^F reporter | σ^E reporter | σ^G reporter | σ^K reporter |
|------------------------|----------------|--------------|--------------|--------------|--------------|
| Wildtype (PY79)         |                |              |              |              |              |
4. What does it mean in this experiment when a streak looks white on the plate? When a streak looks blue on the plate? When a streak has a very light or a very dark color?

5. Comparing your results with your predictions, which mutant did you receive? Explain your answer, making sure to include any necessary information from your reading of review articles and/or the primary literature. Describe any ambiguities.

6. If applicable, do your results here agree or disagree with previous experiments (such as microscopy or sporulation efficiency results)?
Appendix 7: Kinetic luciferase assays, lab manual section

Protocol 5: Analyze kinetic luciferase assays to identify the transcriptional activation of sporulation genes

Note: Here the experimental work was carried out for you. For this protocol, you will be analyzing the data.

Background:
Luciferase is a small molecule that produces light; this isn’t fluorescence, this molecule actually glows. Luciferase is produced by the enzymes encoded by a 5-gene operon called lux. In this experiment, we have created luciferase reporter constructs by fusing the promoter from the gene of interest to the lux operon. These gene fusions have then been introduced into the chromosome of the Wild Type or mutant B. subtilis strains under investigation here, creating reporter strains. In this experimental setup, the bacteria will glow if and only if that promoter is transcriptionally active. Reporter strains are a simple way to observe if and when a promoter of interest is activated.

We have selected five promoters for our reporter strains, choosing promoters that are known to be activated by the sporulation transcription factor and sigma factors:

1. $P_{spoIIG}$-luxABCDE ($spoA$ reporter)
2. $P_{spoIIQ}$-luxABCDE ($\sigma^F$ reporter)
3. $P_{spoIID}$-luxABCDE ($\sigma^E$ reporter)
4. $P_{spssB}$-luxABCDE ($\sigma^G$ reporter)
5. $P_{gerE}$-luxABCDE ($\sigma^K$ reporter)

While luciferase produces light, it is not possible to monitor these strains by eye because the amount of light produced is very small. Instead, the reporter strains are grown in clear, flat bottom plates inside a plate reader machine with a luminometer, which can quantify the amount of light produced from each reporter over time.

For the prepared data provided here, we grew three replicates of each reporter strain in a rich growth medium (in which sporulation does not occur) for 5 hours. 10 µL of each was spotted into a single well of a white 96-well plate in which 200 µL Difco Sporulation Medium (DSM) agar pads had been poured. DSM is a nutrient-poor medium that induces sporulation. The plate reader incubated the plates at 37 °C, and recorded luminescence every 10 minutes for 24 hours.
We used the same promoters in our reporter constructs for protocol 4 and protocol 5. There are two main differences between the types of information gleaned from these experiments. First, the data for the intensity of promoter activation is quantitative (the luminescence readings) in protocol 5, while it is qualitative in protocol 4 (your observations about the color intensity on your plates). Second, you can observe the timing of reporter activation in protocol 5, while in protocol 4 you only know if (not when) your reporters were active.

**Protocol:**

1. Open the data files for this experiment. You will need to pull out the data for your mutant and for the WT for each of the 5 reporters listed above.

2. Use Excel to calculate average luminescence and standard deviations for each timepoint for each reporter for your mutant, for WT, and for the WT strain without any reporter.

3. Use Excel to graph your averages with time on the x axis and luminescence on the y axis. Add error bars to your data series showing the standard deviations. You will need to make 5 graphs for each strain you are investigating, one for each of the reporter constructs. Each graph should have 3 lines: the luminescence data from your mutant, the luminescence data from the Wild Types positive control, and the luminescence data for the WT strain without any reporter.

4. Use the data in the graphs to answer the following questions about your mutant.

**Analysis:**

1. Before the lab, fill out this table. For each mutant, predict which (if any) reporters should be activated.

| Spo mutant background | Spo0A reporter | σF reporter | σE reporter | σG reporter | σK reporter |
|-----------------------|----------------|-------------|-------------|-------------|-------------|
| Wildtype PY79 (no mutations) |               |             |             |             |             |
| Δspo0A                |               |             |             |             |             |
| ΔsigF                 |               |             |             |             |             |
| ΔsigE                 |               |             |             |             |             |
2. Given any previous experiments you have carried out, which gene(s) may be mutated in your mutant strain? Which reporters in your prediction table above could be used to rule out or confirm your hypotheses?

3. After making your graphs, describe your data qualitatively. How does this data compare with your predictions in question 1? Does this agree or disagree with any previous data you have collected (if applicable)?

4. Using the data analyzed here, and any previous data from earlier experiments, which mutant did you receive? Explain your answer, including justification from review articles or other primary literature that you read.
