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Richard Losick's Lecture Part 1:
How *Bacillus subtilis* Makes a Spore

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1. Keywords and Terms

Bacillus subtilis, spore, sporangium, asymmetric septation, forespore, mother cell, FtsZ, Z-ring, nucleoid, axial filament, RacA, DivIVA, DNA translocase, engulfment, cortex, coat, Spo0A, σF, σE, σG, σK, σ factor, reporter gene, anti-σ factor, anti-anti-σ factor, intercellular communication, pro-protein, regulated intramembrane proteolysis.

2. Lecture Notes

Ferdinand Cohn discovered the bacterium *Bacillus subtilis* and its spores in 1877.

B. subtilis is related to the anthrax-causing bacterium *Bacillus anthracis*.

Spore formation by *B. subtilis* is a powerful model system in which to ask fundamental questions about cellular differentiation and morphogenesis in a bacterium.

1. Spore formation is a tale of two cells

Principal stages of spore formation (sporulation)

- Sporulation is triggered by nutrient limitation
- At the onset of sporulation, a single rod-shaped bacterial cell (termed the predivisional sporangium) divides asymmetrically, forming two cells: the small forespore (destined to become the spore) and large mother cell (nurtures the forespore).
- Initially the two cells lie side-by-side. Subsequently the mother cell “swallows” the forespore in a process called engulfment that is similar to phagocytosis. During engulfment the mother cell membranes migrate around the forespore and fuse, pinching it off as a cell-within-a-cell.

How does asymmetric cell division (septation) occur?

- Bacteria divide through the action of the tubulin-like protein FtsZ, which forms a cytokinetic ring termed a Z-ring. Interestingly, actin (not tubulin) plays this role in eukaryotic cells.
- During vegetative growth, the Z-ring forms in the middle of the rod shaped bacterial cell, marking the future site of cell division and resulting in two equal daughter cells.
- During sporulation, two Z-rings form, one near each pole. Only one Z-ring is converted into a division septum, the other is disassembled. The result is two daughter cells of unequal size.

How are chromosomes segregated during asymmetric septation?

Ordinarily, chromosome segregation occurs prior to cell division. During sporulation, however, chromosome segregation takes place by a unique mechanism before, during, and after asymmetric cell division.

- First, the two chromosomes (often referred to as nucleoids) present in the predivisional sporangium are rearranged into an elongated structure termed the axial filament, with the two origins of replication anchored at opposite poles.
- The proteins RacA and DivIVA are important for positioning of the origins at the cell poles. RacA binds to the chromosome, especially near the origin, and in turn interacts with DivIVA, which is anchored at the cell poles.

- Given this positioning of the chromosomes, only a fraction of the forespore chromosome is present in the forespore itself following asymmetric septation. The remainder of the chromosome is pumped from the mother cell into the forespore through the septum. An ATP-dependent DNA translocase located in the division septum mediates this process.

What are events that occur during the final stages of sporulation?

- Remodeling of forespore chromosome
- Formation of a protective layer of cell wall material called the cortex
- Formation of a thick protein coat
- Lysis of the mother cell to release the mature spore

The mature spore can survive for years, but is also capable of resuming normal vegetative growth upon the return of favorable environmental conditions.

2. Cell-specific transcription factors drive gene expression

Transcription factors that act in cell-specific manners orchestrate the events of sporulation (including asymmetric septation, forespore engulfment, cortex and coat assembly) and drive the distinct cell fates of the mother cell and forespore.

- Spo0A = Master regulator of sporulation. Is active in the predivisional sporangium in response to nutrient limitation.
- σF = first forespore-specific transcription factor, active following asymmetric septation
- σE = first mother cell-specific transcription factor
- σG = replaces σF in the forespore following engulfment
- σK = replaces σE in the mother cell at late times during sporulation

The σF, σE, σG, and σK factors belong to a family of transcription factors in bacteria known as RNA polymerase sigma (σ) factors. σ factors bind to RNA polymerase and direct it to specific promoter sequences on the chromosome.

The activity of transcription factors is often measured and/or visualized by a reporter gene. One popular reporter gene is that encoding the Green Fluorescent Protein (GFP), which can be easily monitored by fluorescence microscopy. When the gene for GFP is

fused to a promoter controlled by σE, green fluorescence is observed exclusively in the mother cell. In contrast, a fusion of this reporter gene to a promoter controlled by σG results in forespore-specific fluorescence.

A major focus of work in the Losick laboratory has been to understand the mechanisms by which these transcription factors are activated in the correct cell at the correct time during development.

As an example, the known mechanisms of σF activation are presented:

- The σF protein is produced in the predivisional sporangium (under the control of Spo0A), but only becomes active in the forespore following asymmetric septation.
- An anti-σ factor called AB binds and inactivates σF prior to asymmetric septation and in the mother cell after asymmetric septation.
- However, σF escapes AB in the forespore. How? An anti-anti-σ factor called AA disrupts the AB•σF interaction, thus releasing active σF.
- AA itself is regulated by phosphorylation. AA~P is inactive and unable to disrupt the AB•σF interaction.
- A phosphatase called E converts AA~P σ AA, thus activating AA, which in turn disrupts the AB•σF interaction, releasing active σF.
- How does this happen only in the forespore? We don't know the full answer to this, but an important clue is that the E phosphatase is situated in the asymmetric septum that separates the mother cell and forespore. It is likely that this localization allows E to act preferentially on the forespore side of the septum to dephosphorylate and activate AA, in turn activating σF.

3. The two cells talk to each other!

Three intercellular signaling pathways link gene expression and development in the forespore and mother cell:

i. First, σF activity in the forespore initiates a signal transduction pathway that leads to σE activation in the mother cell (σF σ σE)

ii. Next, σE activity in the mother cell causes σG activation in the forespore (σE σ σG)

iii. Finally, σG activity in the forespore initiates a signal transduction pathway that directs σK activation in the mother cell (σG σ σK)

These are two-way conversations, moving from the forespore to the mother cell, the mother cell to the forespore, and back again!

Listening in on one conversation: How does forespore σG direct activation of mother cell σKσ

- σK is synthesized in the mother cell as an inert pro-protein, called pro-σK. The pro-σK protein has an N-terminal extension (~20 amino acids) that anchors the protein in the mother cell membrane, thus rendering it inactive. To be activated, a protease must cleave pro-σK, liberating a soluble and active form of σK.
- Mature σK is not detected in sporangia lacking σG, indicating that the forespore sigma factor is required for proteolysis and activation of σK in the mother cell.
- How does σG control pro-σK proteolysis in the adjacent cell? The σK protease, which is itself a membrane protein, is held inactive by two inhibitory proteins in the mother cell membrane. However, this inhibitory complex can be disrupted by a signaling protein produced in the forespore under the control of σG. (Secretion of the signaling protein across the forespore membrane allows it to come in contact with the pro-σK protease and its inhibitors in the mother cell membrane.) Upon disruption of this complex, the protease cleaves pro-σK into its mature, active form.

Interestingly, the mode of σK activation and the pro-σK protease have been highly conserved through evolution. Pro-σK processing is an example of regulated intramembrane proteolysis, given that the active site of the pro-σK protease is located within its membrane-spanning regions of the protein. Studies in diverse cell types have revealed that regulated intramembrane proteolysis is a widely-conserved mechanism for activating membrane-bound regulatory proteins, including the human sterol response element binding protein (SREBP), a key transcription factor that regulates cholesterol metabolism. Moreover, the pro-σK protease is itself a member of a widely conserved protease family that participates in pathways of regulated intramembrane proteolysis in many cell types, including human cells. In fact, one of the proteases responsible for activating SREBP in human cells shares many features, including catalytic residues and mechanism, with the pro-σK protease!

Future research challenge: How do the many hundreds of proteins produced by Spo0A, σF, σE, σG, and σK collaborate to direct morphogenesis and ultimately produce a mature spore?

3. Recommended Reading

Textbook: *Molecular Biology of the Gene*, sixth edition. Watson et al. (Person/Benjamin Cummings). Chapter 20.

Three general reviews of spore formation by *B. subtilis*:

- Rudner DZ and Losick R (2001) Morphological coupling in development: lessons from prokaryotes. Dev Cell 1: 733-742.
- Piggot PJ and Hilbert DW (2004) Sporulation of Bacillus subtilis. Curr Opin Microbiol. 7: 579-586.
- Hilbert DW and Piggot PJ (2004) Compartmentalization of gene expression during *Bacillus subtilis* spore formation. Microbiol Mol Biol Rev. 68: 234-262.

4. Review Questions

1. What eukaryotic cytoskeletal protein is FtsZ related to?
2. Compare and contrast cell division during vegetative growth vs. sporulation
   Similarities = Z-ring formation by FtsZ
3. Cells of all kinds, including growing cells of B. subtilis, segregate their chromosomes prior to cytokinesis. What is unique about sporulation in this regard?
4. True or False: Bacteria are too simple to undergo developmental processes.
5. True or False: Chromosome segregation occurs before cell division during *B. subtilis* sporulation.
6. True or False: The forespore and the mother cell have a full complement of the genetic material at all times.

7. True or False: σ Factors are bacterial transcription factors that bind to and direct RNA polymerase to specific promoter sequences on the chromosome.

8. Define an anti-σ factor and an anti-anti-σ factor.

9. Why is the gene encoding Green Fluorescent Protein (GFP) a popular reporter gene?

10. Indicate the cell in which GFP would be observed if its gene were under the control of (A) Spo0A, (B) a σF promoter or (C) a σK promoter.

11. Why is it important for the forespore and mother cell to communicate during sporulation?

12. What do *B. subtlis* σK and the human protein SREBP have in common?

5. Answers to Review Questions

1. Tubulin

2. Differences = only 1 Z-ring at mid-cell during vegetative growth; 2 Z-rings form during sporulation. Symmetric cell division (binary fission) during vegetative growth; asymmetric cell division during sporulation.

3. During sporulation, septation, which takes near a cell pole, occurs before a chromosome is fully translocated into the forespore.

4. False. *B. subtilis* sporulation is a very complex developmental pathway! Many other bacteria undergo interesting morphological and developmental processes, including *Caulobacter crescentus*, *Streptomyces coelicolor*, and *Myxococcus xanthus*.

5. False. After asymmetric septation, only part of the forespore chromosome is trapped in the forespore. An ATP-dependent DNA translocase is required to pump the remainder of the chromosome into the forespore.

6. False. Immediately after asymmetric division the forespore contains only a portion of a chromosome.

7. True. σ factors confer specificity to RNA polymerase.

8. An anti-σ factor blocks the activity of a σ factor by binding directly to the σ factor. An anti-anti-σ factor binds to and disarms an anti-σ factor, thereby disrupting the interaction between the anti-σ factor and its target σ factor. Thus, an anti-anti-σ factor indirectly activates the σ factor.

9. Because GFP is easily visualized by fluorescence microscopy in living cells.

10. (A) predivisional sporangium; (B) forespore; (C) mother cell

11. To synchronize their programs of gene expression and morphological progression.

12. These two proteins are both transcription factors that are produced as inactive pro-proteins that are anchored in cellular membranes. Both proteins require regulated intramembrane proteolysis to become soluble and active. The protease responsible for processing σK is related to one of the proteases responsible for cleaving SREBP.

6. Discussion Questions

1. Once engulfed, how many membranes surround the forespore? Indicate the origin of each membrane.
2. FM4-64 is a red fluorescent dye that binds to, but cannot cross, cellular membranes (i.e. it is non-membrane permeable). In contrast, MitoTracker Green is a green fluorescent dye that binds to and crosses lipid bilayers (i.e. membrane permeable). Once inserted into a membrane, both dyes are able to freely diffuse in the lateral plane of the membrane. Explain how you could use these two dyes to distinguish sporangia that are in the process of forespore engulfment versus those have completed forespore engulfment.
3. How could localization of the anti-anti-σF factor E to the asymmetric septum cause preferential activation of σF in the forespore?

4. The DNA translocase that pumps the forespore chromsome into the forespore is also present during vegetative growth of *B. subtilis.* Why might that be?

5. Gene regulation during B. subtilis sporulation has often been described as “criss-cross” regulation. Why?

7. Answers to Discussion Questions

1. The answer to this question is not provided in the lecture, but should be easily deduced by drawing the process of engulfment by hand.

The forespore is immediately surrounded by two membranes, an inner membrane derived from the original forespore membrane, and an outer membrane derived from the engulfing mother cell membranes. The space between these inner and outer membranes can be considered equivalent to the extracellular space. The mother cell is in turn surrounded by its own membrane. (Although upon lysis of the mother cell, only two membranes are associated with the mature spore.)

2. During forespore engulfment, all membranes (forespore membrane and mother cell membrane) will be labeled by both FM4-64 and MitoTracker Green, given that both membranes are accessible from the medium. (Late in engulfment the forespore membrane may only be exposed a small amount, but still enough to allow intercalation of the dyes.) As such, during engulfment, all membranes would both be outlined in red AND green.

Following engulfment, however, the inner and outer forespore membranes (see above question) are pinched off from the extracellular space, and would only be accessible to a dye that could cross the (outer) mother cell membrane (i.e. MitoTracker Green). As such, engulfed forespore membranes would ONLY be labeled green (the outer mother cell membrane would be red AND green).

This assay has in fact been utilized in studies of forespore engulfment, see: Sharp MD and Pogliano K (1999) An in vivo membrane fusion assay implicates SpoIIIE in the final stages of engulfment during Bacillus subtilis sporulation. Proc Natl Acad Sci 96: 14553-14558.

3. Possible answers are not discussed in the lecture and will require some thought and brainstorming.

One possible model is that E is preferentially localized to the forespore side of the septum during cell division. The resolution of fluorescence microscopy, such as that used to generate the image of E-GFP shown in the lecture, is not sufficient to distinguish localization of this protein to one or the other face of the septum. This model would require an unknown mechanism to restrict E to the forespore face of the septum (or, for example, to preferentially degrade E on the mother cell face).

A second model is that the higher concentration of E in the forespore (due to the small size of the forespore) relative to the other σF regulators (AA, AB) is enough to tip the balance in favor of σF activation in the forespore. This model relies on the fact that E is a membrane protein and is bound preferentially to the septum. If one assumes an equal number of E molecules bound to the forespore and mother cell faces of the septum, then the concentration of E (and thus its specific phosphatase activity) in the forespore would be higher in the forespore due to its small volume. In contrast, other σF regulators (such as AA, AB), which are soluble, should have equivalent concentrations in both compartments after septation.

4. During vegetative growth, bacterial chromosomes are segregated prior to cell division. However, this is not a perfect process. Sometimes the division septum may trap part of the chromosome intended for one daughter cell in the other. Under this circumstance, the DNA translocase pumps the chromosome into the appropriate compartment. Interestingly, the DNA translocase also helps coordinate other events associated with segregating bacterial chromosomes, including resolution of chromosome concatamers (formed by homologous recombination between two chromosomes) and interlocked chromosomes (a result of chromosome replication).

   Given the availability of fusions of the gene for the Green Fluorescent Protein to promoters under the control of σF, how could you test the proposition that only a portion of the chromosome is initially present in the forespore after asymmetric division? (Clue: σF is active immediately following asymmetric septation.)

   You would create a collection of strains in which the fusion constructs are located at diverse positions around the chromosome and carry out time-lapse microscopy to determine the time at which green fluorescence appears in the forespore. The prediction is that promoter fusions located near the origin of replication should give

rise to fluorescence earlier than fusions located distal to the origin. Further, a mutant of the DNA translocase should be blocked in the expression of origin-distal fusions but not origin-proximal fusions.

5. Because the intercellular signaling pathways controlling σ factor activity move from the forespore to the mother cell (σF σE), from the mother cell to the forespore (σE σG), and back again from the forespore to the mother cell (σG σK).

   Upon nutrient depravation, only ~50% of B. subtilis cells commence sporulation. Speculate as to why this might be an advantageous strategy.

   Spore formation is a wise decision for cells only if nutrients will not be available for a long period of time. If nutrients are only transiently limited, sporulation can be an unnecessary, time-consuming, and energy-intensive process. By maintaining a subpopulation that does not enter sporulation, the collective *B. subtilis* population ensures that it will survive long periods of time without nutrients (i.e. via the sporulating subpopulation) or will quickly take advantage of nutrients should they reappear in the short term (i.e. via the non-sporulating subpopulation).

8. Explain or Teach These Concepts to a Friend

1. Draw the morphological stages of *B. subtilis* sporulation.
2. Explain how a chromosome is segregated into the forespore.
3. Explain how σF is activated in the forespore even though it is synthesized before asymmetric division.
4. Explain how the forespore becomes topologically isolated from the mother cell.
5. Explain why the forespore can be likened to a germ cell and the mother cell to a somatic cell.

9. Research the Literature on Your Own

1. Compare and contrast the intercellular signaling pathways that connect σF σ σE and σG σ σK. (While an overview of the σG σ σK signaling pathway is given in the lecture, more details of both pathways will be needed to give a complete answer.)
2. Recent research indicates that the σE σ σG pathway is not a conventional signal transduction pathway. Explain.
3. Sporulating cells do not become committed to spore formation until they reach the stage of asymmetric division. What is developmental commitment and how would you demonstrate it experimentally?
4. Under conditions that trigger sporulation, only some cells in the population begin sporulation. Remarkably, these early sporulating cells produce toxins that kill sibling cells that have not yet started to sporulate. This phenomenon is known as cannibalism. How does it work and why might it be a fitness advantage to kill one's siblings?
5. Compare and contrast the SpoIIIE DNA translocase with other motors that transport DNA across membranes, such as those involved in conjugation and competence.