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COMMENTARY

Protein Synthesis during Germination: Shedding New Light on a Classical Question

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Despite over a century of research into the mystery of bacterial spore dormancy and germination, a key question remains unresolved: is protein synthesis required for germination? The development of more sophisticated techniques for assessing and preventing protein synthesis has renewed interest in this long-standing question in recent years. In this issue, Korza et al. (G. Korza, B. Setlow, L. Rao, Q. Li, and P. Setlow, J. Bacteriol 198:3254–3264, 2016, http://dx.doi.org/10.1128/JB.00583-16) address this with a novel approach. We discuss their results in the context of recently published data.

Bacterial spores were discovered by Cohn and Koch in the species *Bacillus subtilis* and *Bacillus anthracis* and described in two jointly published papers in the year 1876 (1). Among the most striking of their observations was that spores are metabolically dormant and highly resistant. In spite of these features, however, spores can break dormancy extremely rapidly; within minutes of sensing the appropriate stimulus (typically, a small molecule such as an amino acid or a sugar) the spore converts to a vegetative cell and resumes growth and division (2). During the first step in this revival process, known as germination, the dormant spore sheds its protective outer layers, takes up water, and swells. In the second step, called outgrowth, the spore begins active production of new cellular macromolecules. Outgrowth is complete when the cell fully converts to a rod shape; the now fully restored vegetative cell is poised to begin division. This rapid reawakening from the dead (or the near dead) raises a number of intriguing and very deep questions, including (i) how can metabolism be reactivated so quickly after the completion of germination and (ii) do spores really lack all metabolic activity? Or, to phrase it another way, just how dead are spores, anyway?

Elucidating the mechanistic basis of germination and outgrowth is one of the longest-running challenges in microbiology; despite nearly 150 years of research, there are still major gaps in our understanding of these processes. Nonetheless, since germination was first observed, a large body of evidence has accumulated documenting that, throughout the course of germination, up until the start of outgrowth, spores are, indeed, quite dormant; no metabolic or biosynthetic processes (until quite recently) have been detected. While these data appeared to exclude significant levels of metabolic activity during germination, an outstanding question has always lingered: could germinating spores possess some degree of physiologically important metabolic activity that is simply too low to detect or for some other reason inaccessible to measurement by the methods used so far?

This issue was addressed in a landmark study in 2015 by Sinai et al. (3). These authors showed, first, that protein synthesis occurs prior to the completion of germination in two ways: (i) by a novel biochemical approach (bio-orthogonal noncanonical amino acid tagging [BONCAT]) that specifically identifies newly synthesized proteins and (ii) by monitoring the timing, during germination, of the appearance of fluorescently tagged versions of some of the proteins discovered by BONCAT. Second, and more directly relevant to our discussion here, these authors argued that protein synthesis is required for successful germination by demonstrating that (i) protein synthesis inhibitors (specifically, the antibiotics tetracycline and lincomycin) prevent germination (cleverly overcoming the limitations of previous experiments attempting to use antibiotics in a similar manner, by increasing spore permeability during antibiotic administration) and (ii) deletion of genes encoding two translation factors (which they identified by BONCAT) delays germination. These striking results appear to overturn a major view in the field and suggest that protein synthesis not only takes place during germination but is actually required for germination.

Clearly, there is more to germination than the previous literature suggested. Since the work of Sinai et al., it is well recognized in the field that the general question of what, if any, cellular processes transpire during dormancy or germination needs further investigation. In the present issue, Korza et al. (4) do exactly this by addressing the question of whether protein synthesis is required for germination in a different way than Sinai et al. Korza et al. inhibited protein synthesis during germination by depleting spores of rRNA. They found that rRNA depletion did not significantly reduce the rate of germination, strongly suggesting that protein synthesis is not required for germination. The differing conclusions of these two studies bear careful analysis. To do this, we turn first to a very brief review of spore ultrastructure and the process of germination.

The major spore structures are organized as a set of concentric shells that are readily visualized by transmission electron microscopy. The most striking of these layers are the outermost, a set of biochemically and morphological complex shells whose number and composition vary among species (5, 6). However, in all of the members of the order *Bacillales* that form spores, the spore pos-

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sesses a protein layer called the coat that, among other functions, protects the spore from diverse environmental stresses. In many species (and in *B. subtilis* in particular) the coat is the outermost layer. Underneath the coat is the second major protective layer, a thick peptidoglycan layer called the cortex. Below the cortex is a biochemically distinct peptidoglycan layer (the germ cell wall) that becomes the vegetative cell wall after germination has completed. Underneath this layer is a lipid membrane (the inner membrane) that encases the interiormost compartment, the core, which houses the spore's DNA.

Germination is a remarkable process in its own right. It provides a mechanism for breaking dormancy that is highly sensitive to even low levels of specific small molecules and yet is very effectively prevented when those molecules are absent. Once germination starts, it is both rapid and exquisitely orchestrated. Germination begins when small molecules, often amino acids or sugars (referred to as germinants) in the environment, are recognized by specialized germinant receptors within the spore. Receptor engagement triggers the release of a complex of calcium (Ca) and dipicolinic acid (DPA) that is stored at a high concentration within the core of the dormant spore. As the Ca-DPA complex moves outward through the porous outer layers, it activates cortex-lytic enzymes that break down the cortex, which relieves the constraint on core volume imposed by the cortex. This, in turn, allows a major influx of water into the core, thereby causing it to swell and to rehydrate sufficiently to support metabolism. Concomitantly, the coat (as well as any of the other, more exotic outer layers that are present in many species [5]) is shed by processes that remain poorly understood (7). Disassembly of these layers reveals the germ cell wall, which serves as the cell wall of a newly emerging vegetative cell. In the classical picture of germination, none of these steps require metabolism and no metabolic activity takes place. In particular, there is no protein synthesis. No experiments over the past decades (until very recently) have suggested otherwise (2, 9, 10).

The conclusions of Korza et al. and Sinai et al. rely on differing methods to detect and/or inactivate protein synthesis. Reliably inactivating protein synthesis without damaging the spore is highly challenging, and no approach is free of caveats. Korza et al. depleted spores of rRNA through prolonged heat treatment. They documented that rRNA levels were decreased by 97 to 99%, a very significant amount that should severely reduce the number of functional ribosomes that could potentially produce proteins during germination. However, a limitation of this approach is that, since rRNA is not completely eliminated, a low level of protein synthesis might still occur. While Korza et al. made every practical effort to mitigate this limitation by reducing rRNA levels to nearly the limit of detection, this caveat cannot be entirely avoided. Nonetheless, because the potential for protein synthesis should be severely reduced, if protein synthesis is required for germination, there should be at least a significant reduction in germination efficiency after rRNA depletion. Instead, Korza et al. found that germination is not greatly delayed in rRNA-depleted spores. While these experiments cannot definitively rule out a requirement for protein synthesis in germination, they suggest that if protein synthesis is needed, the amount is very small.

Sinai et al. addressed the role of protein synthesis during germination in two ways. In the first of these, they applied two protein synthesis-inhibiting antibiotics, lincomycin and tetracycline. This allowed them to make a strong case for a major role for protein synthesis in germination. Nonetheless, it is difficult to entirely exclude the possibility that the antibiotics had unintended biochemical effects impacting germination. The modes of action of many commonly used antibiotics can be very complex, with hundreds of genes either expressed or repressed in response to antibiotic application, suggesting that many antibiotics may have more than one target (8). Although Sinai et al. appropriately used two structurally unrelated antibiotics to mitigate the risk of confounding off-target effects, this caveat cannot be entirely excluded. Importantly, however, Sinai et al. also demonstrated an important role for protein synthesis by a second method: analysis of germination after deletion of the translation factor genes *tig* and *rpmE*, the products of which they identified as proteins synthesized during germination. Mutations in *tig*, *rpmE*, or both result in delays in germination but do not obviously impair sporulation efficiency, germinant receptor levels, or coat integrity. Therefore, the authors reasonably ascribe the effects of these mutations to the roles of the corresponding proteins during germination rather than to defects in protein synthesis during spore formation. However, minor defects in the spore specifically affecting germination could have remained unnoticed in these assays.

Meaningfully comparing the two studies also requires considering the differing methods they used to measure germination. In both studies, germination was monitored by measuring the release of DPA which, as already discussed, occurs early in germination. Sinai et al. noted that antibiotic-treated spores released DPA to a similar degree as wild-type spores. Similarly, Korza et al. found that using heat treatment to deplete rRNA did not prevent DPA release. Therefore, the two studies agree that protein synthesis is not required for the initiation of germination.

Sinai et al. measured germination at later times during the process by monitoring changes in the refractility of the core during germination, both by microscopy and by changes in optical density (OD) (which largely reflects the degree of refractility under the experimental conditions these authors used). Spore rehydration during germination causes a drop in refractility (a change in the core's appearance from bright to dark), which is readily measured by phase-contrast light microscopy or OD. The OD also is influenced by changes in cell shape as cells begin to increase in volume. Sinai et al. found that the expected decrease in spore refractility was inhibited by the application of antibiotics and also by mutations in *rpmE* and *tig*, strong evidence for the view that protein synthesis is important for normal germination.

Korza et al. also assessed germination by measuring changes in OD during germination (but with the inclusion of nutrient in the germination medium, conditions under which outgrowth will occur). They did detect an effect of heat treatment on the OD during germination. Because this effect was not dramatic and was possibly confounded by concurrent outgrowth, Korza et al. did not interpret their OD measurements as evidence of a significant delay in germination. Therefore, there is disagreement on the question of whether protein synthesis is required for germination.

Reconciling these differing conclusions is challenging because of both the complexity of germination and the technical difficulty of interrogating this system. Nonetheless, a detailed understanding of germination is a critical goal for both the field of spore biology and the microbiology community in general, which has treated sporulation and germination as foundational model systems for over 125 years. We suggest that a productive path forward would be an analysis combining the methodologies employed in

both studies in a single study. Not only could such a study resolve a long-standing question of deep significance, but it would likely reveal deeper levels of fascinating mechanistic complexity that both Korza et al. and Sinai et al. suggest remain unplumbed.

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