The Nature and Application of Biocontrol Microbes: *Bacillus* spp.The *Bacillus* Spore Coat

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ABSTRACT

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Bacilli, which are abundant in the soil, form highly resistant dormant cell types, called spores, in response to starvation. The spore is organized into a series of concentrically arranged structures, each of which contribute in a different way to resistance against environmental stress. In certain bacteria, including *Bacillus subtilis*, the outermost of these structures is a multilayered protein shell, called the coat. The coat is both an armor plating and, almost certainly, possesses enzymatic activities, allowing it to have active roles as well. Assembly of the proteins comprising the coat is carefully controlled during spore assembly, resulting in

a distinct pattern of layers, seen in cross section, and a discreet pattern of ridges on the surface. Although our understanding of spore coat composition and assembly is deepening, we still know little about the roles of the coat in interactions between spores and other organisms, particularly in the soil. Critical future directions for spore coat research include continued identification of the proteins that comprise the coat surface, characterization of the global chemical characteristics of this surface, and elucidation of how these features impact on other organisms in the soil.

Additional keyword: exosporium.

Gram-positive bacteria, in particular *Bacillus subtilis* and a few of its relatives, are among the best-studied experimental systems in bacteriology. Research in *B. subtilis* is remarkably diverse, spanning genetics, biochemistry, cell biology, and ecology, and as a result, has had an enormous impact on both basic and applied biology (25). One of the best-studied features of *Bacilli* is their ability to form a dormant, highly resistant cell type, called a spore, which allows these organisms to survive the direst conditions of starvation. Spores can withstand a wide variety of extreme stresses, including most or all of those found in the soil, a common niche (20,21). The process by which cells make spores and the molecular determinants of spore resistance are questions that have been intensely studied since sporulation was first observed, over 125 years ago, in an effort to understand the infectious nature of the disease anthrax (15). Since then, it has become clear that *Bacilli* survive in many diverse environments, often with extreme variations in temperature, nutrient, and other stresses. It is not surprising, therefore, that these organisms have mastered the art of hunkering down in a dormant form to await better times.

The fundamental job of the spore appears to be survival during periods of extreme stress. Spores are encased in layers of tough shells, have reduced water content, and are metabolically dormant. These features are essential to survival of the cell and resistance to environmental stresses normally encountered in field soils. However, the inert spore must be able to return to active growth when nutrient is again available. This is possible because, although dormant, spores are not unalert; they continuously monitor the environment for the renewed presence of nutrient. Once nutrient is detected, a process known as germination initiates and, within a few minutes, the spore will have returned to vegetative growth.

Bacterial spores are marvels of engineering. Their apparently simple design conceals an impressive array of protective strategies that permit survival in essentially any terrestrial environment for indefinite periods of time. Thin-section electron microscopy reveals they are constructed of several concentric shells (29) (Fig. 1A). The spore interior, called the core, houses the chromosome, and is filled with small acid-soluble proteins (called SASP) that saturate the DNA and help to protect it (8). Surrounding this is a lipid membrane and then a thick layer of peptidoglycan. The major portion of this layer, called the cortex, differs from non-spore peptidoglycan both in cross-linking and composition (22). The cortex keeps the spore core water activity relatively low, which in turn, is essential for spore resistance. Surrounding the cortex is a complex multilayered structure called the coat. The coat plays roles in spore resistance, germination, and apparently possesses enzymatic functions that may possibly permit interactions with other organisms in the environment (6,8). A number of species, including *B. anthracis* and *B. thuringiensis*, but not *B. subtilis*, possess an additional layer as well, called the exosporium (Fig. 1B) (6). This structure does not sit directly on the coat but is separated from it by a substantial gap. Consistent with a loose connection between the coat and exosporium, its shape varies from spore to spore. Its function is unknown.

The coat is the most biochemically complex structure in the spore, being composed of about 60 proteins in *B. subtilis*, *B. anthracis*, and probably most other species (R. Giorno and A. Driks, *unpublished data*; 16,17). In *B. subtilis*, the coat is morphologically complex as well, possessing several layers that are readily identified in thin-section electron microscopy, and which are organized into two sets of layers, called the inner and outer coats. The layers vary in precise number and thickness from spore to spore and around the circumference of a single spore. Therefore, the rules that govern their assembly are likely to differ from those that control formation of more crystalline structures, like phage.

The intriguing structure of the coat, as well as its biochemical complexity and the experimental tractability of *B. subtilis*, has

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made it an attractive system for studies of macromolecular assembly, particularly those with some degree of disorder. It is known that only a subset of coat proteins control the deposition of coat proteins other than themselves; these are referred to as mor-

Fig. 1. Line drawing illustrating the appearance of spores in thin-section electron microscopy. Ridges are not shown for simplicity. The core is white, the cortex stippled, and the inner and outer coat layers are in gray and black, respectively. The irregular line encircling the spore in panel **B** is the exosporium, which is not present in *Bacillus subtilis*. **C** shows just two quadrants of spores. The spore on the left shows the position of CotE (white dots) at the inner coat-outer coat interface. The spore on the right shows the effect of a *cotE* mutation, in which the outer coat is not assembled.

phogenetic proteins (6,8,13,25,26). For example, the coat protein CotE (30) directs the assembly (but not the synthesis) of at least 10 additional coat proteins, including most or all the outer coat proteins (Fig. 1C). CotE is modular; N- and C-terminal regions direct coat protein assembly and an internal region guides CotE itself to the interface between the inner and outer coat layers (1,9,18). Other morphogenetic proteins direct assembly of CotE-independent proteins or work in conjunction with CotE to control subsets of the CotE-controlled proteins.

Interestingly, while the functions of the morphogenetic proteins are becoming clearer, the specific roles of most of the remaining structural proteins remain obscure. These proteins probably act together as a sieve to exclude large potentially harmful molecules, but the biochemical complexity of the coat suggests it houses additional functions associated with the structural proteins. A clue to the nature of these functions is the finding that several coat proteins are homologues of oxidases (17). Notably, one of these, called CotA (5), has been shown to be a multi-copper oxidase in vitro (14,19). A related enzyme, MnxG, is found in the exosporium of the marine *Bacillus* SG-1. This extraordinary organism is able to surround itself in a crust of manganese, which it harvests from the environment as a result of MnxG activity (11,12,27,28). Clearly, enzymatic activities built into the spore surface can have potent effects on the environment, potentially including plants.

The *B. anthracis* genome encodes some of the same coat proteins as *B. subtilis*, including several of the major morphogenetic proteins, such as CotE. This raises the possibility that coat assembly in the two organisms is similar (7). This is likely to be true to a certain extent, but at least the role of the major morphogen CotE differs between *B. anthracis* and *B. subtilis* (R. Giorno, unpublished data). Instead of directing assembly of a large set of coat proteins and the formation of the outer coat layer, the *B. anthracis* CotE homologue controls deposition of only a single coat protein detectable by gel electrophoresis. Nonetheless, CotE has a critical role in *B. anthracis*; it is required for proper assembly of the exosporium. How CotE does this remains to be discovered.

The structure of the spore surface deserves as much attention as the layers seen by thin-section electron microscopy. Previous studies using scanning electron microscopy documented ridges that run along the spore coat, which are also visible in thin-section microscopy, in many cases (2,4). Recent studies using atomic force microscopy (AFM) support these results and extend them to unfixed samples (3). AFM analysis of spore coat mutants further showed that two previously identified coat proteins, CotA and CotB, have novel roles in spore surface morphology. Deletion of either gene alters but does not prevent ridge formation. Therefore, these two genes can be regarded as spore coat surface morphogens.

Several lines of evidence, in addition to the AFM analysis cited above, argue that the coat is a dynamic structure that can expand and contract during spore formation and during germination. Especially important in this regard is the observation that during germination, spores swell and the ridges in the coat disappear (3,24). From this perspective, the coat is both an armor plating and a flexible structure.

Studies so far have focused on how the coat affords protection to the spore and its role in germination. A major challenge for the future will be understanding the ecological impact of the coat. Given the complex interactions between bacteria and other organisms in the environment (10,23), it is only to be expected that the coat plays a pivotal role.

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