The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat

Peter T. McKenney[1], *Adam Driks*[2] *and Patrick Eichenberger*[1]

Abstract | Sporulation in *Bacillus subtilis* involves an asymmetric cell division followed by differentiation into two cell types, the endospore and the mother cell. The endospore coat is a multilayered shell that protects the bacterial genome during stress conditions and is composed of dozens of proteins. Recently, fluorescence microscopy coupled with high-resolution image analysis has been applied to the dynamic process of coat assembly and has shown that the coat is organized into at least four distinct layers. In this Review, we provide a brief summary of *B. subtilis* sporulation, describe the function of the spore surface layers and discuss the recent progress that has improved our understanding of the structure of the endospore coat and the mechanisms of coat assembly.

Sporulation
The developmental process of spore formation.

Endospores
Metabolically dormant cells composed of a partially dehydrated central core (containing the genome) surrounded by several concentrically arranged protective layers. An endospore develops inside a mother cell.

[1]Center for Genomics and Systems Biology, Department of Biology, New York University, New York, New York 10003, USA.
[2]Department of Microbiology and Immunology, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois 60153, USA.
Correspondence to P.E.
e-mail: pe19@nyu.edu
doi:10.1038/nrmicro2921
Published online
3 December 2012

Bacteria have many strategies for surviving environmental challenges. These strategies frequently involve rapid changes in gene expression that temporarily alter the phenotype of a cell and allow it to survive. A more sophisticated and prolonged example of stress response is spore formation, or sporulation, wherein the bacterial genome is sequestered in a safe place (the spore) until environmental conditions improve, upon which the spore quickly germinates and returns to the vegetative state. Endospores are formed and nurtured completely within a mother cell, which must lyse to release the spore into the environment. Unrelated mechanisms of bacterial sporulation[1] include the formation of exospores, myxospores and akinetes (BOX 1).

Endospores (hereafter referred to as spores) exhibit extraordinary resistance properties and have served as textbook examples of long-term cell survival[2–4]. The exact mechanism of spore persistence is unknown, but in addition to being resistant to ultraviolet (UV) radiation, chemicals (such as peroxide and hypochlorite), extreme heat and other stresses, spores are metabolically dormant and partially dehydrated, which is likely to allow their survival in nutrient-free and harsh environments. Although the upper limit of viability is unknown, some researchers have reported the revival of spores from samples ranging in age from decades to several thousands of years[5], a claim that is difficult to prove unambiguously. Nevertheless, viable spores have been isolated from specimens such as dried plant samples dating from 1640 onwards[6] and a flask of *Bacillus anthracis* spores sealed in Louis Pasteur's laboratory for 60 years[7].

Two taxa within the phylum Firmicutes — the aerobic Bacillaceae and the anaerobic Clostridia — form spores via an evolutionarily conserved mechanism[8,9] (see below). These bacteria include the pathogens *B. anthracis* and *Clostridium difficile*, and the model organism *Bacillus subtilis*. Sporulation is a simple example of differentiation: a cell replicates its DNA, divides asymmetrically and places copies of its genome in both compartments. Over the next 8 to 10 hours, the smaller of the two compartments, the forespore (or prespore), develops into a mature spore capable of protecting the genome. When the spore is released into the surrounding environment it can persist or germinate to re-enter the vegetative cycle. A variety of spore-forming species are routinely found in soil samples[10], but they have also been isolated from ecosystems as diverse as hot springs[11], arctic sediments[12] and the mammalian gastrointestinal tract[13].

Our focus here will be the spore coat, a structure present on the exterior of all spores that is crucial for conferring resistance to environmental stress. The paradigm for coat architecture is provided by *B. subtilis*, which uses at least 70 different proteins to build this multilayered structure. In this Review, we provide a brief overview of sporulation in *B. subtilis* with a focus on the assembly and function of the coat. We discuss recent investigations combining genetic, biochemical and cytological approaches that have led to a better

© 2013 Macmillan Publishers Limited. All rights reserved

Box 1 | Resting cells: endospores, exospores, myxospores and akinetes

A variety of metabolically dormant bacterial cells, called resting cells, have been found in nature[1]. Endospores form inside a mother cell after the separation of the sporangium into distinct forespore and mother cell compartments; however, endospore formation is not limited to the production of a single spore per mother cell. *Metabacterium polyspora*, which is found in the gastrointestinal microbiota of guinea pigs, forms endospores at both poles of the mother cell. *M. polyspora* endospores also appear to have the capacity to divide further by binary fission, enabling the production of more than three spores per cell[108]. Genetic manipulation of *Bacillus subtilis* can also result in the formation of two viable endospores in a single mother cell[109].

Various other resting cell types that form 'outside' the original vegetative cell have also been described. *Streptomyces* species sporulate by forming a multinucleate sporogenic cell at the leading tip of an aerial hypha filament. Divisomes are assembled along the length of the sporogenic cell to partition each chromosome into an individual compartment that will eventually become an exospore[110]. During myxospore formation in *Myxococcus* species and akinete formation in heterocyst-forming species of Cyanobacteria, an entire individual cell transforms its morphology to form a resting cell in the absence of division[1]. To our knowledge, there has been no comprehensive parallel study of the relative resistance properties of the known types of resting cells; however, meta-analysis suggests that all resting cell types appear to be resistant to desiccation, whereas resistance to other types of stress such as extreme heat and predation by protozoa is variable[111]. The spore coat of myxospores has been investigated to some extent[112], but it appears to be unrelated to the spore coat of endospores, both in terms of composition (it is made essentially of exopolysaccharides) and mechanism of assembly, which requires a dedicated protein machinery.

Mother cell
The larger of the two compartments formed by asymmetric division of the sporulating cell, it synthesizes most of the building blocks required to assemble the endospore protective structures and lyses at the end of sporulation, releasing the spore into the environment.

Forespore
The smaller of the two compartments that are formed by asymmetric division of the sporulating cell. It matures into an endospore.

Coat
A spore protective structure, which is made up of dozens of proteins. It is usually multilayered, consisting of inner and outer layers.

Sporangium
A vessel in which spores are formed. In endospore formation it refers to a cell that has entered sporulation by dividing asymmetrically.

Engulfment
The morphological transition in sporulation during which the mother cell swallows the forespore in a phagocytosis-like process involving membrane migration. After engulfment is complete, the forespore becomes a cell within the mother cell cytoplasm.

understanding of coat morphogenesis. Knowledge about the molecular mechanisms behind the formation of this complex structure should reveal novel principles underlying the assembly of multiprotein structures in various organisms.

Sporulation in *Bacillus subtilis*

In laboratory cultures, sporulation begins at the onset of stationary phase when nutrients are depleted; however, the ultimate 'molecular signal' of sporulation, if it exists, remains elusive. Sporulation is triggered by the activation of histidine sensor kinases (including KinA, KinB and KinC), which shuttle phosphate through an extended phosphorelay, resulting in phosphorylation of the master regulator of sporulation, the transcription factor Spo0A. Simply upregulating the expression of KinA appears to be sufficient to trigger sporulation, regardless of nutrient status[14]. Phosphorylated Spo0A controls a large regulon of genes, including those involved in asymmetric cell division and those involved in activation of the sporulation-specific sigma factors[15]. An asymmetric division of the sporulating cell creates a sporangium composed of two compartments, the larger mother cell and the smaller forespore, which ultimately becomes the spore (FIG. 1). The next stage is the engulfment of the forespore by the mother cell in a process resembling phagocytosis. After the completion of engulfment, the forespore is a double membrane-bound cell within the mother cell. Engulfment is coordinated with the building of two external protective structures: the cortex is composed of peptidoglycan[16] and is assembled between the inner and outer forespore membranes, and the proteinaceous coat comprises the outermost layer of the spore[17,18]. Spore peptidoglycan precursors are synthesized in the mother cell[19] and are flipped across the outer forespore membrane into the intermembrane space by an as yet incompletely understood mechanism[20]. The coat is composed of at least 70 individual proteins that are produced by the mother cell and begin to localize to the spore surface during engulfment[21].

Structure of the spore

Since the initial application of electron microscopy to sporulating bacteria, researchers have been captivated by the concentrically arranged layers making up the spore external structure[22,23] (FIG. 2). The genome is contained in the partially dehydrated central core, in which most of the water has been replaced with Ca^{2+}-dipicolinic acid. The core is surrounded, from innermost to outermost, by the inner spore membrane, germ cell wall (a thin layer of vegetative cell wall-like peptidoglycan that is not depicted in the figures), cortex, outer spore membrane and coat. Most of these structures are rapidly lost upon germination: the coat is shed and the cortex is degraded by cell wall hydrolases. The germ cell wall is retained to serve as a primer for vegetative cell wall assembly. The inner spore membrane becomes the plasma membrane, whereas the fate of the outer spore membrane is less clear. It may be lost during spore maturation, but there is at least one organism (*Acetonema longum*) in which it persists after germination, thus generating a double membrane vegetative cell akin to Gram-negative bacteria[24].

Three layers of the *B. subtilis* spore coat are observed in thin-section electron microscopy: a lamellar inner coat, a more coarsely layered outer coat and a recently identified layer named the crust[23,25] (FIG. 2a,b). In some species, such as *B. anthracis*, a different type of outermost layer exists, called the exosporium[22]. The exosporium is a distinct glycoprotein layer that surrounds the spore and is separated by a gap called the interspace[26–29] (FIG. 2c). In *Bacillus cereus* and its close relatives *B. anthracis* and *Bacillus thuringensis*, the exosporium consists of a basal layer adorned with hair-like projections. Recent structural studies have documented the crystalline two-dimensional architecture of the basal layer[30]. In isolation, or in combination with the exosporium, the coat fulfils several functions other than protection of the genome (see below).

Between species, the spore surface differs most obviously by the presence or absence of an exosporium, but there is also considerable diversity in the individual coat proteins. Analysis of existing genome sequences suggests that about half of the known *B. subtilis* coat protein genes have recognizable orthologues in other *Bacillus* species, whereas the other half do not appear to be conserved at all. In the genomes of *Clostridium* spp., conservation of *B. subtilis* coat genes is even more limited[8,9,17,31]. Considering that the spore surface represents the interface between spores and the environment, diversity in coat composition may have a crucial role in defining the ecological niches of spore-forming bacteria (see below).

Functions of the spore coat

Protection from environmental stress. The resistance of spores to extreme environmental stresses is likely to be an emergent property of spore architecture, making it

© 2013 Macmillan Publishers Limited. All rights reserved

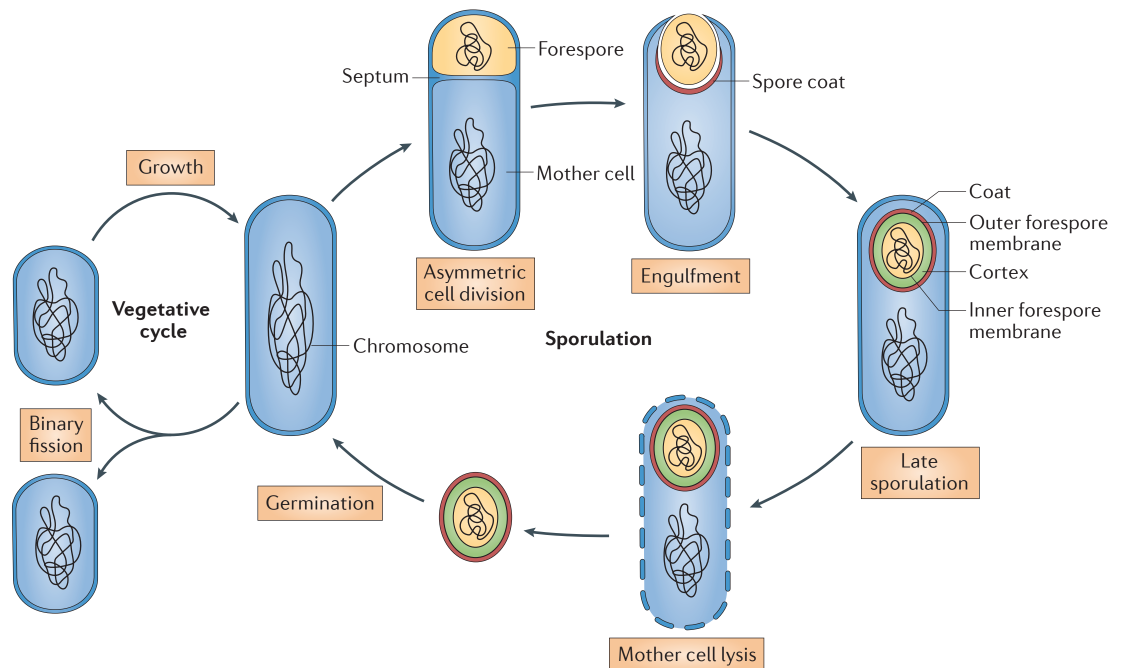

Figure 1 | **The sporulation and germination cycle in *Bacillus subtilis*.** Sporulation begins when a sporangium divides asymmetrically to produce two compartments: the mother cell and the forespore, which are separated by a septum. Next, the mother cell engulfs the forespore, and following membrane fission at the opposite pole of the sporangium, a double-membrane bound forespore is formed. Coat assembly begins just after the initiation of engulfment and continues throughout sporulation. The peptidoglycan cortex between the inner and outer forespore membranes is assembled during late sporulation. In the final step, the mother cell lyses to release a mature spore into the environment. Spores are capable of quickly germinating and resuming vegetative growth in response to nutrients.

difficult to describe causal connections between individual stresses and resistance mechanisms. For example, resistance to extreme heat requires partial dehydration of the spore core, a functional cortex, RecA-dependent DNA repair machinery and an array of small acid-soluble proteins, which bind to the genome in the spore core[32]. Similarly, spores may be resistant to desiccation because the spore core itself is partially dehydrated. Late in sporulation, most of the water in the spore core is replaced with Ca^{2+}-dipicolinic acid, and mutants deficient in Ca^{2+}-dipicolinic acid production do not survive desiccation.

Spores of *B. subtilis* are also more resistant to UV irradiation and peroxide than vegetative cells[32]. CotA, a copper-dependent laccase found in the outer coat, contributes to protection against UV radiation and peroxide by generating a pigment that is structurally similar to melanin[33]. Melanin confers resistance to reactive oxygen species in a number of different microorganisms[34], and deactivation of reactive oxygen species may be a common mediator of resistance to both UV light and peroxide. Melanin has also been shown to interfere with phagocytosis of fungal pathogens such as *Cryptococcus neoformans* by human macrophages, and it is necessary for yeast survival within macrophages[35]. It is currently unknown whether CotA has a similar effect on the phagocytosis and survival of spores.

The major known function of the coat is spore protection and, indirectly, preservation of the spore genome. Remarkably, *B. subtilis* spores can survive ingestion by bacteriovores as different as the protozoan *Tetrahymena thermophila* and the nematode *Caenorhabditis elegans*[36–38]. After being fed wild-type spores of *B. subtilis*, electron microscopy revealed that *T. thermophila* phagosomes were full of intact, undigested spores; however, mutations that disrupt the coat result in rapid digestion of the spore cortex and core. Thus, in the natural environment, the coat is likely to provide physical protection from predators. Although the exact molecular mechanism of this protection is unknown, the genomes of both *T. thermophila* and *C. elegans* contain multiple homologues of putative peptidoglycan hydrolases, suggesting that the coat may have an important role in limiting access of these enzymes to the peptidoglycan-containing cortex.

Regulation of germination. For dormancy to be a viable survival strategy, spores must be able to germinate rapidly when nutrients become available again. Spores continually monitor their surroundings using an array of germination receptors embedded in the inner spore membrane. Germinants of *B. subtilis* include sugars, amino acids, peptidoglycan fragments and ions[39,40]. As germinants will reach their receptors by traversing the spore coat and cortex, the coat must ultimately be both protective and permeable. The coat can be conceptualized as a molecular sieve that excludes large molecules, such as lysozyme, while allowing the passage of small-molecule germinants[22,41].

Cortex
A spore protective structure composed of peptidoglycan. It is assembled between the inner and outer forespore membranes.

Crust
The outermost layer of the coat in *Bacillus subtilis*. It closely follows the contours of the outer coat.

Exosporium
The outermost structure of the spore in several species. It is a protein (and, in at least some cases, a glycoprotein) layer separated from the outer coat by a large gap of irregular width referred to as the interspace.

Bacteriovores
Free-living heterotrophs that feed on bacteria.

© 2013 Macmillan Publishers Limited. All rights reserved

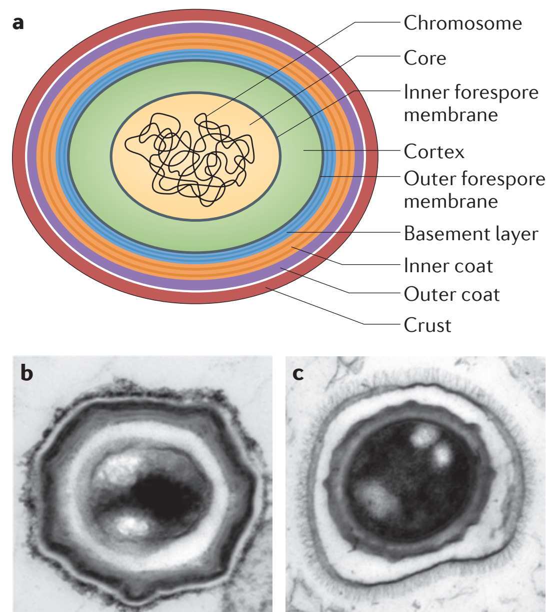

Figure 2 | **Spore ultrastructure. a** | Cartoon of a typical *Bacillus subtilis* spore. The multiple layers of the spore serve to protect the genome, which is housed in the partially dehydrated central core. The inner forespore membrane (grey) is visible in electron micrographs of sectioned spores, but the outer forespore membrane is not usually visible. The core is protected by the cortex (green) and the spore coat, which consists of four layers: the basement layer (blue), inner coat (orange), outer coat (purple) and crust (red), which is only visible after ruthenium red staining. The concentric rings of the basement layer and inner coat reflect the lamellar appearance of the inner coat in electron micrographs. It is unknown how these lamellae form or whether they are composed of specific proteins. **b** | Thin section transmission electron micrograph of a *B. subtilis* spore stained with ruthenium red. **c** | Thin section transmission electron micrograph of a *Bacillus anthracis* spore stained with osmium tetroxide. The exosporium is the outermost protective layer of the spore and it contains hair-like projections. It is separated from the rest of the spore by a large interspace. Part **b** is reproduced, with permission, from REF. 25 © (2010) Elsevier.

Although the effect of the coat on the initiation of germination may be strictly passive, the coat and the exosporium also have the potential to enzymatically affect germination. An intrinsic property of spore surfaces is the ability to convert L-alanine to D-alanine using an alanine racemase [42]. Because L-alanine is a potent germinant, conversion of L-alanine to D-alanine by the coat could suppress germination. Recently, an alanine racemase enzyme (Alr) from the exosporium of *B. anthracis* spores was characterized[43,44]. During sporulation of *B. anthracis* cells lacking Alr, forespores germinate prematurely within the mother cell, suggesting that Alr insulates the developing forespore from free L-alanine in the mother cell cytoplasm[45]. An orthologue of *alr* in *B. subtilis* (*yncD*) encodes an alanine racemase that localizes to the outer coat layer[25,46]; however, deletion of *yncD* does not result in premature forespore germination[47].

The coat also influences germination by sequestering enzymes required for degradation of the cortex peptidoglycan. In particular, the *B. subtilis* inner coat contains at least two peptidoglycan hydrolases, CwlJ and YaaH (SleL)[21,48–51]. Furthermore, peptidoglycan-binding domains, such as the LysM domain[52], are present in several coat proteins (including YaaH), but their exact functions have not yet been determined in the context of the sporulation–germination cycle. It is possible that the impact of the coat on peptidoglycan hydrolysis is more complex than currently suspected. The functional interdependency between the coat and cortex layers is emphasized further by the recent discovery of CmpA, a mother cell-expressed protein that appears to be involved in a checkpoint ensuring that cortex synthesis is delayed until coat assembly successfully initiates[53].

After the initiation of germination, the coat must be opened and ultimately shed to allow the vegetative cell to grow out[54]. In *B. subtilis*, it is unclear whether outgrowth is a polarized process, wherein one pole of the spore is favoured over another. In *B. anthracis*, outgrowth appears to be biased towards the mother cell proximal (MCP) pole, which is the initial localization site of the spore coat and exosporium proteins[55].

Defining an ecological niche? For all bacteria, the properties of the cell surface define the range of interactions that the bacterium may establish with other organisms and surfaces. The diversity of spore surface structures (see, for example, REFS 22,27,56–58) and the conservation of only a subset of coat proteins[8,9,17], which includes the coat morphogenetic proteins[17], suggests that spore surface properties are diverse and may contribute to the wide dispersal of spore-forming bacteria. The available data suggest that, between species, spore surfaces can display varying degrees of hydrophobic or hydrophilic characteristics[29,59,60].

If the function of the coat is to resist ubiquitous environmental stresses, then why is coat structure so variable? In contrast to the coat, the core and cortex do not usually display interspecies differences that are distinguishable by electron microscopy. Given the diversity in coat structure among species, it seems reasonable to speculate that this diversity is driven by adaptation to a variety of niches. However, as any specific niche harbouring bacterial spores typically possesses diverse species (see, for example, REF. 61), addressing this question is extremely complex[62]. The functions of the coat and the exosporium might have evolved in an exquisitely fine-tuned manner to allow spores to thrive in diverse habitats, both natural and man-made. For example, to cause disease in a hospital setting, a *C. difficile* spore needs to interact with abiotic surfaces, such as stainless steel, as well as the varied microenvironments within a host. During their transit between niches, it is likely that the level and type of stresses vary considerably, and the surfaces to which spores need to adhere represent another important source of variation. Thus, spore transmission in complex environments, where the ability to adhere to and survive on variable surface structures is crucial, could be a key factor that promotes diversity

© 2013 Macmillan Publishers Limited. All rights reserved

in coat structure and composition. Moreover, the ability of a spore to attach to or avoid specific surfaces could facilitate future growth after germination. Elucidation of the physical and chemical properties of spores, in conjunction with their variability in different ecological conditions, is an important goal for future research and should help to identify specific environmental features driving coat variation.

Spore coat assembly

The first coat proteins were identified by reverse genetic approaches. Specifically, extracts of coat material were subjected to peptide sequencing, the resulting sequences were cloned and mutants were constructed to determine the role of each protein in coat assembly, which was examined by electron microscopy[63–65] (TABLE 1). Although electron microscopy offers unparalleled resolution of subcellular structures, the lack of systems for high-sensitivity labelling of individual proteins has limited its application. More recently, proteomics approaches and global transcriptional profiling of the mother cell compartment led to the discovery of several novel spore proteins[66–70]. Candidate coat proteins can then be verified by fluorescence microscopy, which involves examining the localization of green fluorescent protein (GFP)-tagged candidate coat proteins in mutants with disrupted coat assembly[71,72]. Moreover, the combination of a GFP fusion protein with a fluorescent membrane stain allows the kinetics of coat protein localization relative to the membrane dynamics of engulfment to be observed[21].

Coat morphogenetic proteins. The first description of spore coat structure came from thin-section electron microscopy[23] (FIG. 2). Distinct inner and outer coat layers were made visible using osmium tetroxide staining: the outer coat was darkly stained, whereas the inner coat was stained more lightly and contained multiple concentric dark lamellae. Initial forward genetic screens aimed at identifying the genes required for sporulation in *B. subtilis* revealed two loci that have a major effect on coat structure: *spoIVA*[73] and *spoVM*[74]. Electron microscopy of *spoIVA* mutants showed that coat material was present, but instead of encircling the spore, the coat assembled in aggregates floating in the mother cell cytoplasm[73,75]. Interestingly, the darkly and lightly stained layers of the coat were still present, suggesting that even in the absence of spore-surface localization, some self-assembly was preserved. These data are consistent with a role for SpoIVA in anchoring the coat to the spore surface. By contrast, a transposon insertion in *spoVM* resulted in a spore coat that was localized to the spore surface but was only partially attached and significantly disorganized[74].

Among the genes identified using reverse genetics approaches were *safA*, *cotE* and *spoVID*[64,65,76,77]. Electron microscopy of mutant strains revealed that *safA* mutant spores lack an inner coat but retain a seemingly unaffected outer coat[76], whereas *cotE* mutant spores retain an inner coat but lack an outer coat[65]. By contrast, the phenotype of *spoVID* mutant spores resembled that of the *spoVM* mutant spores[64]; in both cases the two coat layers could be distinguished, but the coat did not form a shell of protein around the forespore and was often found detached from the forespore surface. Subsequent work showed that *spoVID*, along with *spoVM*, is required for a later stage in coat assembly, spore encasement (see below). Collectively, the electron microscopy data suggested that assembly of the two layers occurs independently: SafA is necessary for assembly of the inner coat, whereas CotE is necessary for assembly of the outer coat. We now know that these two proteins, together with SpoIVA, form the core of a modular network responsible for assembly of a scaffold cap of the spore coat on the MCP pole of the forespore[25] (FIG. 3). By contrast, SpoVM and SpoVID are crucial for the transition from a single cap to a full spherical shell that encases the spore. These key proteins (SpoIVA, SpoVM, SpoVID, SafA and CotE) were named morphogenetic proteins as they have a major role in coat morphogenesis but do not affect gene expression in the mother cell.

The spore coat genetic interaction network. The application of immuno-electron microscopy to *B. subtilis* spores revealed the subcellular localization of the morphogenetic proteins and provided initial clues as to how these proteins direct spore coat protein assembly: SpoIVA was detected in close proximity to the membrane, SafA in the inner coat[77] and CotE at the interface of the inner and outer coats[78]. By using the localization data of these three proteins and the phenotypes of the corresponding mutants, it was possible to draw the skeleton of an interaction network (FIG. 3a). Our laboratories and others took a genetic approach to further characterize this network[25,69,79]. Spore coat proteins were defined by a failure to localize to the spore surface in the absence of SpoIVA, and catalogued as inner or outer coat proteins based on their inability to localize to the spore surface in mutants of *safA* and *cotE,* respectively (TABLE 1). To date, 41 GFP-tagged spore coat proteins have been examined for genetic interactions with *spoIVA*, *cotE* and *safA*[25,69].

These investigations revealed a network of genetic interactions with three nearly independent modules: a SafA-dependent subnetwork, a CotE-dependent subnetwork and a third module consisting of proteins that are independent of both SafA and CotE, and primarily dependent on SpoIVA (FIG. 3a; TABLE 1). Spatial information was inferred from the genetic dependencies. For example, because SpoIVA is necessary to anchor the coat to the spore surface, the subnetwork around SpoIVA should localize closest to the spore membrane.

To test whether the genetic interaction network accurately predicted the localization of individual coat proteins, high-resolution image analysis was used to circumvent the theoretical limit of light microscopy, which overlaps with the maximal width of the spore coat (~200 nm). The distances between the spore membrane and 17 individual coat protein fusions were measured. When these data were then sorted by the mean distance between the spore membrane and each fusion protein, coat proteins grouped according to the interactions predicted by the genetic interaction network: CotE-independent proteins were closest to the membrane and CotE-dependent proteins were furthest

Lamellae
The characteristic alternating dark and light rings of the inner coat that are visible by electron microscopy.

Encasement
The morphological transition in spore coat assembly from a cap of coat proteins on the mother cell proximal pole of the forespore to a symmetric distribution around the circumference of the spore.

© 2013 Macmillan Publishers Limited. All rights reserved

Table 1 | **Table of representative coat proteins from *Bacillus subtilis***

Coat protein	EM phenotype of null mutant	Dependent coat proteins	Domains*	Conservation‡	Refs
Basement layer proteins (SpoIVA-dependent)					
SpoIVA	No coat attached, no cortex	All tested	Walker A ATPase	All endospore formers	73,75,86,93
SpoVM	No cortex, encasement blocked	All tested	Amphipathic α-helix	All members of the Bacillaceae and most *Clostridium* spp.	74,82,83,90,118
SpoVID	Encasement blocked	Most tested proteins	LysM	Most members of the Bacillaceae and no *Clostridium* spp.	64,78,83,91,92
LipC (also known as YcsK)	ND	ND	Lipase	All members of the Bacillaceae and most *Clostridium* spp.	122
YhaX	ND	ND	HAD (haloacid dehydrogenase) family	All members of the Bacillaceae and most *Clostridium* spp.	25,72
YheD	ND	ND	–	Some members of the Bacillaceae and no *Clostridium* spp.	88
Inner coat proteins (SafA-dependent)					
SafA	No inner coat	17	LysM	Most members of the Bacillaceae and no *Clostridium* spp.	76,77,85
CotD	ND	ND	–	Most members of the Bacillaceae and no *Clostridium* spp.	25,51,63
CotT	Thinner inner coat	ND	–	Only found in *Bacillus subtilis*	25, 51,123
CwlJ	ND	ND	Peptidoglycan hydrolase	All endospore formers	21,48,49
OxdD (also known as YoaN)	ND	ND	Oxalate decarboxylase	Most members of the Bacillaceae and some *Clostridium* spp.	124
YaaH (also known as SleL)	ND	ND	LysM (×2)§, *N*-acetyl-glucosaminidase	All members of the Bacillaceae and most *Clostridium* spp.	25,48,50,51
YuzC	ND	ND	–	Some members of the Bacillaceae and no *Clostridium* spp.	25,72
Outer coat proteins (CotE-dependent)					
CotE	No outer coat	16	–	All members of the Bacillaceae and no *Clostridium* spp.	25,65,71,78,94
CotA	No phenotype	ND	Multi-copper oxidase	Most members of the Bacillaceae and some *Clostridium* spp.	25,33,51,63
CotM	Modest outer coat defect	ND	α-crystallin family of stress proteins	Most members of the Bacillaceae and some *Clostridium* spp.	25,125
CotO (also known as YjbX)	Outer coat defect	ND	–	Most members of the Bacillaceae and no *Clostridium* spp.	25,72,102
CotS	No phenotype	ND	Bacterial spore kinase	Most members of the Bacillaceae and some *Clostridium* spp.	25,69,126,127
YncD	ND	ND	Alanine racemase	All endospore formers	25,46
YtxO	ND	ND	–	Most members of the Bacillaceae and no *Clostridium* spp.	25,51,69
Crust proteins (CotX/Y/Z-dependent)					
CotX/CotY/CotZ‖	No crust attached	1	–	Most members of the Bacillaceae and no *Clostridium* spp.	25,79,97
CotW	ND	ND	Coiled coil	Some members of the Bacillaceae and no *Clostridium* spp.	25,97

EM, electron microscopy; ND, not determined. *'–' indicates that no homology to a previously defined domain was identified for this protein. ‡Data from REF. 9. §Two copies of the LysM domain. ‖The conservation information refers only to CotY and CotZ.

© 2013 Macmillan Publishers Limited. All rights reserved

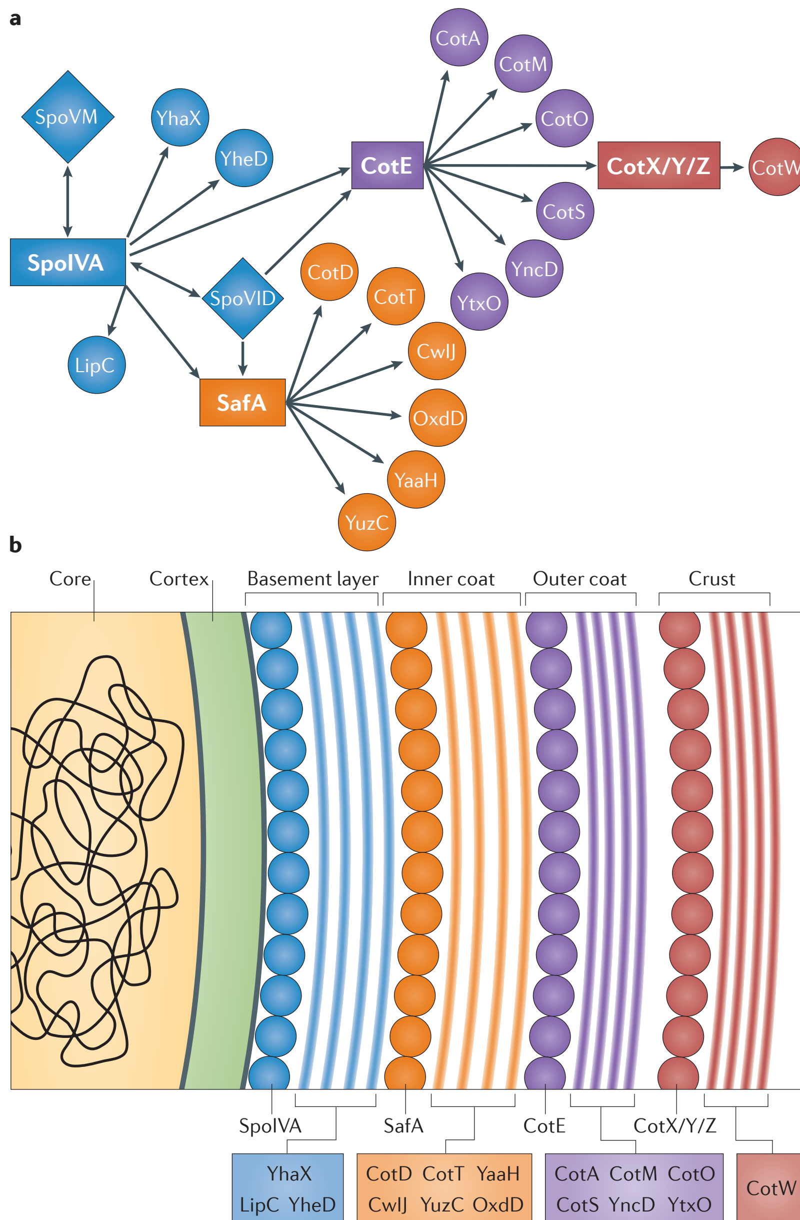

Figure 3 | **The coat genetic interaction network.** **a** | Recognition of the spatially scaled genetic interaction network led to the proposal of a coat made up of four layers (the basement layer (blue), inner coat (orange), outer coat (purple) and crust (red)), each with its own independent morphogenetic protein (SpoIVA, SafA, CotE and CotX/CotY/CotZ, respectively). The morphogenetic proteins SpoVM and SpoVID are required for spore encasement (FIG. 4). **b** | Assembly of each layer may be driven by the multimerization of the underlying morphogenetic protein. Polymerization of a morphogenetic protein would, presumably, create the necessary binding sites for each of the individual coat proteins that make up each layer. It remains to be determined whether and how the proteins of the individual layers interact with adjacent layers and how adjacent layers interact to form the mature spore coat.

from the membrane[25]. Unexpectedly, a group of proteins that are significantly separated from CotE was also identified, suggesting the possibility of an additional layer and perhaps a fourth genetic interaction module controlled by *cotX*, *cotY* and *cotZ*[25]. Notably, a homologue of CotY had been identified in extracts of the exosporium of *B. anthracis*[80], but as mentioned above, *B. subtilis* spores do not have an exosporium. Electron microscopy in combination with conventional staining indicated that the outer coat is the outermost layer in *B. subtilis*. However, more recent analysis using staining with ruthenium red showed an additional electron-dense layer assembled over the outer coat[81], which we have named the crust[25]. Because the contours of the outer coat and crust follow each other very closely, we propose that these layers are in direct contact, in spite of the electron-translucent region seen by transmission electron microscopy. The crust was absent in Δ*cotXYZ* mutant spores, arguing that CotX, CotY and CotZ, individually or in combination, are morphogenetic proteins involved in crust assembly[25,79].

Encasement: the second step in coat assembly. The morphogenetic proteins discussed thus far have similar mutant phenotypes in that they appear to be involved in localizing spore coat proteins to the spore surface. Another distinct phenotypic class also exists: in these mutants, coat proteins localize to the spore surface, but the coat fails to form a full shell around the spore. Two examples are mutants of *spoVID* and *spoVM*[64,82,83] (TABLE 1). In these two mutants, most of the 41 GFP-tagged coat proteins localize to the MCP pole of the forespore but fail to transition to complete concentric rings of fluorescence[78,83–87]. This phenomenon was named 'spore encasement' to distinguish it from the localization of coat proteins to the spore surface, which is controlled by SpoIVA, SafA, CotE, CotX, CotY and CotZ.

Morphogenesis of the spore coat in real time

The genetic interaction network was assembled using data from mature spores; however, it has been clear from the first observations of the localization of individual coat proteins that coat morphogenesis is dynamic and coordinated with spore development[78,88] (FIG. 4). For example, SpoIVA and SpoVM were observed tracking along with the engulfing forespore membrane[78,86,89,90], whereas CotE appears to localize in two distinct steps: first forming a cap on the MCP pole of the spore and later forming a second cap on the mother cell distal (MCD) pole of the spore after the completion of engulfment[71,83,89].

Recently, the dynamics of spore coat protein localization over the course of sporulation were examined by fluorescence microscopy of the library of 41 GFP-tagged coat proteins[21]. The proteins could be divided into two roughly equal groups based on whether initial localization on the forespore surface occurred during or after engulfment. The proteins that localized during engulfment did so simultaneously to the forespore MCP pole and included the morphogenetic proteins SpoIVA, SpoVM and SpoVID. These three proteins tracked along the membrane, forming a full shell of fluorescence upon the completion of engulfment (FIG. 4). Thus, their presence in the earliest kinetic class links the genetically uncoupled phenomena of coat protein localization and encasement. Consistent with these observations, these proteins interact physically in biochemical assays[83,87,91]. Surprisingly, additional waves of encasement could be

© 2013 Macmillan Publishers Limited. All rights reserved

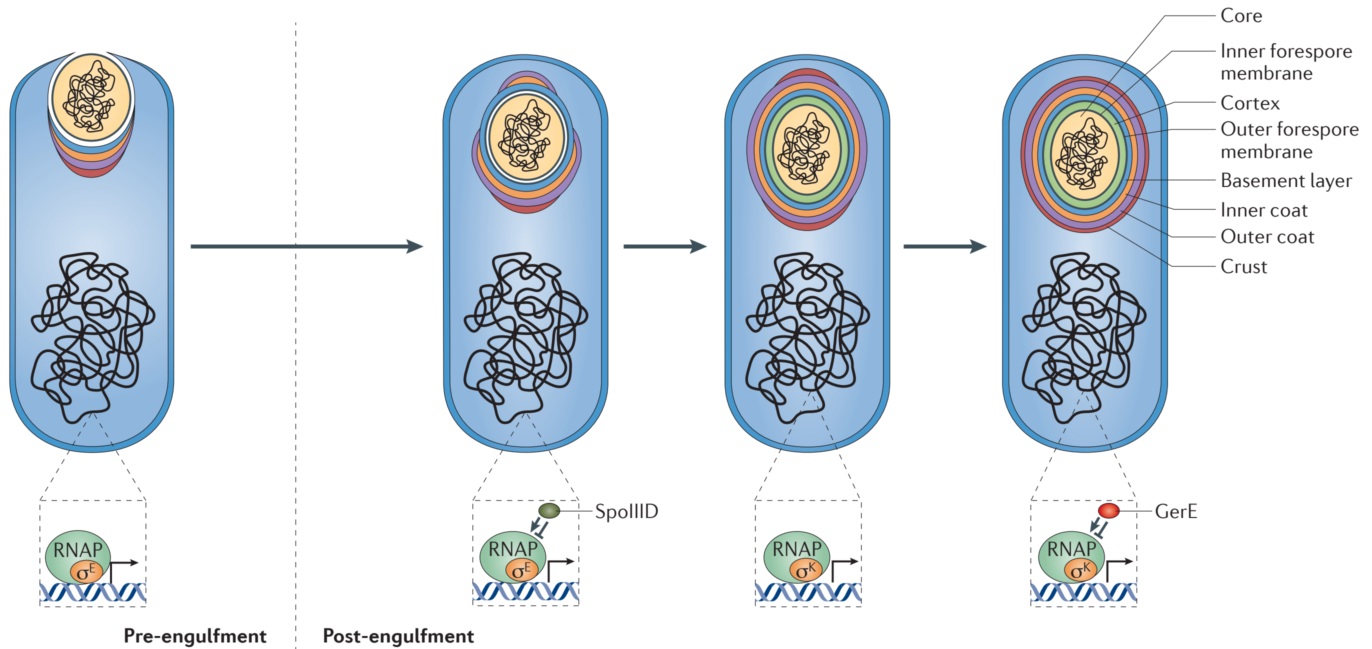

Figure 4 | **A model for coat morphogenesis: successive waves of spore encasement.** Coat morphogenesis begins with the assembly of a scaffold containing half of all coat proteins on the mother cell proximal (MCP) pole of the forespore. Assembly of the basement layer (blue), which consists of proteins that are solely under the control of the sigma factor σ^E for transcription, is dependent on SpoIVA. Encasement by SpoIVA is likely to be driven by multimerization and happens concomitantly with engulfment. Assembly of the inner (orange) and outer (purple) coat layers is dependent on SafA and CotE, respectively. Proteins of the inner and outer coat appear to have delayed encasement kinetics in comparison to the basement layer, and although they begin to assemble on the MCP pole during engulfment, they only form a cap on the mother cell distal (MCD) pole of the forespore after engulfment is complete. Most proteins in this class show dependency on combinations of σ^E and σ^K, as well as the transcription factor SpoIIID for expression. CotZ, a key component of the crust (red) belongs to the last encasement class and is dependent on σ^E, σ^K and the transcription factor GerE for expression. All four layers contain late-expressed σ^K-dependent proteins that are not part of the initial scaffold. These proteins presumably diffuse through the permeable coat matrix to reach their final location within the coat. Transcriptional regulation in the mother cell controls the kinetics of spore encasement, in particular the two sigma factors — σ^E (for the control of early gene expression during engulfment) and σ^K (for the control of late gene expression post-engulfment) — and two mother cell-specific transcription factors — SpoIIID (which is turned on by σ^E and modulates the σ^E regulon) and GerE (which is turned on by σ^K and modulates the σ^K regulon). RNAP, RNA polymerase.

readily distinguished. Among the early-localizing coat proteins, another wave of encasement occurred only after completion of engulfment and the establishment of the MCD pole of the forespore, arguing that proteins in this class, including CotE, require the MCD pole for encasement (FIG. 4). Importantly, this later wave is dependent on SpoVM and SpoVID, as highlighted by the observation that a direct interaction between CotE and the amino-terminal domain of SpoVID is crucial for encasement of the outer coat[92]. The final wave of encasement among the early-localizing coat proteins, which includes the crust protein CotZ, occurs even later (FIG. 4). In summary, encasement by the morphogenetic proteins proceeds in a distinct order from basement layer to outer coat to crust. As such, the timing of encasement (which is subject to transcriptional control, see below) may represent the primary organizational phenomenon of coat morphogenesis[21].

Encasement. The molecular mechanism driving encasement is unknown; however, the sequential encasement of the spore by the morphogenetic proteins may offer some clues. For example, multimerization of all or some of the morphogenetic proteins is a plausible model of coat assembly (FIG. 3b). Purified SpoIVA polymerizes in an ATP-dependent manner and forms cables of varying diameter[93], and the morphogenetic proteins SafA, CotE and CotZ have all been shown to self-interact biochemically[84,94,95].

Because polymerization is limited by the availability of polymer subunits, one way to control the timing of encasement would be to limit the availability of coat protein monomers so that they are only expressed when they are needed. Gene expression in the mother cell is organized into a series of feedforward loops that result in successive waves of gene transcription and protein synthesis[68,96]. This hierarchical organization of gene expression is the result of transcriptional control of promoters by compartment-specific sigma factors and transcription factors (FIG. 4). The sigma factor σ^E is activated shortly after the completion of asymmetric division and regulates early gene expression in the mother cell. After the completion of engulfment, a second sigma factor, σ^K, replaces σ^E and regulates late gene expression in the

© 2013 Macmillan Publishers Limited. All rights reserved

mother cell. Among the >250 genes in the σ^E regulon is the gene encoding the transcription factor SpoIIID[68,96]. SpoIIID regulates subsets of the σ^E regulon, both positively and negatively. This results in three waves of gene expression: an initial wave that is σ^E-dependent and later repressed by SpoIIID, a second wave under the control of σ^E alone and unaffected by SpoIIID, and a third wave that is σ^E-dependent and further upregulated by SpoIIID[68]. Similarly, a late mother cell-specific transcription factor, GerE, positively and negatively regulates subsets of the σ^K regulon, also subdividing it into three waves[68].

The genes encoding the coat morphogenetic proteins belong to four temporally distinct classes of promoter regulation. Although clusters of sporulation genes can be found in the genome, genes encoding coat proteins appear to be randomly distributed around the chromosome and are rarely organized into operons (one exception being the *cotXYZ* cluster of genes that is required for crust formation[97,98]). SpoIVA is expressed in a short pulse under the control of σ^E, and its synthesis is later repressed by SpoIIID[68,75]. SafA is upregulated by σ^E alone and is unaffected by SpoIIID[76], whereas CotE is expressed in two pulses, first by σ^E and then by σ^K, before being repressed by GerE[68,99]. Expression of the crust protein operon, *cotYZ*, is activated by σ^E, σ^K and GerE[68,98]. The transcriptional data correspond well to the localization kinetics of these proteins (as discussed earlier) and support the notion that the major coat morphogenetic proteins are expressed in ordered waves. SpoIVA is a member of the first kinetic class of fusion proteins that finish encasement at the end of engulfment[86]. The localization kinetics of SafA are unknown; however, it is either a member of the first kinetic class (considering that it interacts directly with SpoVID[85]) or, along with CotE, it could belong to a second kinetic class. Finally, encasement by CotZ begins only after the spore becomes visible by phase contrast microscopy as a dark sphere in the mother cell cytoplasm. Although it is initially transcribed at a modest level under the control of σ^E, *cotYZ* is only transcribed at a high level during late sporulation.

Summary of coat assembly. The large number of proteins that localize simultaneously to the MCP pole indicate that the coat may be spatially organized into layers from the beginning of the assembly process, as previously suggested[78] (FIG. 4). Time-resolved measurements of individual protein fusions during engulfment confirmed that proteins are organized into four distinct layers early in morphogenesis and may form an organized scaffold on the MCP pole[21]. Encasement then occurs in successive waves, beginning with proteins of the basement layer, followed by the outer coat and finally the crust. This establishes each layer of the scaffold on both poles and around the circumference of the spore. Intuitively, the easiest way to continue coat assembly for the late-expressed coat proteins would be to build additional layers on top of the pre-existing scaffold. The presence of some inner coat proteins among the latest-expressed coat gene promoters suggests that the coat remains permeable late in morphogenesis, even to protein fusions as large as 70 kDa[21]. Thus, with all coat layers present at the beginning of coat morphogenesis, we expect that the major mechanism of addition of late-expressed coat proteins is by diffusion through the permeable coat. Crosslinking among proteins may limit this permeability. The one example of crosslinking among coat proteins that has been described in detail, the Tgl-mediated crosslinking of GerQ, appears to occur only after mother cell lysis and the release of spores into the environment[100].

Injectisomes
In Gram-negative bacteria, a family of secretion systems that have a molecular architecture homologous to flagella.

Outstanding questions and concluding remarks

Many important questions regarding the mechanism of spore coat assembly have not yet been addressed. In addition to CotE, other coat morphogenetic proteins such as CotO and CotH appear to be necessary for the recruitment of proteins to the outer coat[101–103]. This raises the possibility of hierarchical interactions among individual proteins within a single coat layer. It also remains to be determined whether the current genetic interaction network accurately predicts physical interactions among proteins. For example, does CotE exclusively interact with predicted outer coat proteins and not with inner coat proteins? Various physical interactions between coat proteins have been described[77,83–85,87,91,92,95,104], but this has not yet been comprehensively investigated with, for example, a high-throughput two-hybrid technique. A systematic test of all possible physical interactions among coat proteins would also address the extent of hierarchical organization within coat layers.

Another potentially interesting question is how the thickness of each layer is determined. A possible clue is provided by a study that analysed *B. subtilis* spores with mutations in the spore coat protein genes *cotH* and *cotO*[102]. Individually, mutation in either gene results in a disorganized outer coat. The double mutation has an intriguing additional phenotype: outer coat proteins form aggregates that bulge out significantly from the spore surface, resulting in a thicker outer coat. It is possible that proteins such as CotH and CotO act synergistically to enforce a specific width on the outer coat, analogous to the ‘molecular rulers’ of flagella and injectisomes[105].

The coat morphogenetic proteins may be important targets for evolutionary adaptation across species, providing a pathway for rapid modification of coat architecture. Thus, an especially interesting area of future research will be to compare the coat protein assembly network of *B. subtilis* with networks in other species. *B. anthracis* possesses orthologues of several *B. subtilis* coat morphogenetic proteins, so it is tempting to speculate that *B. anthracis* uses some of the same proteins as *B. subtilis* to direct assembly of its coat[27]. For example, SpoIVA is necessary to anchor the coat to the spore surface in both species. By contrast, deletion of *cotE* in *B. anthracis* has only a modest effect on coat assembly; however, it is necessary for proper assembly of the exosporium.

The dynamics of spore encasement may also be largely conserved among spore-forming bacteria. In early electron micrographs, assembly of the exosporium in *B. anthracis* was observed to progress from an initial cap

© 2013 Macmillan Publishers Limited. All rights reserved

Box 2 | **Applications of spore surfaces**

An understanding of spore coat organization may benefit the development of microbial cell surface display technologies, which have been used to produce biocatalysts, biosorbents and vaccines[113]. *Bacillus subtilis* is edible and has been used in Japan for centuries to ferment soy beans for the production of natto. Spores are also naturally heat-stable, so spore-based therapies may not require refrigeration, eliminating a major cost of vaccine distribution to developing nations[114]. Several groups have attempted to generate vaccines against *Clostridium tetani* by engineering the surface of *B. subtilis* spores to display *C. tetani* antigens, and this has shown some promise[115,116]. However, the antigens were fused to the outer coat proteins CotB and CotC, which are covered by the crust layer in *B. subtilis*. Antigen display on the crust or preparations of crust-free spores, such as the Δ*cotXYZ* strain, may prove to be more successful strategies for vaccine development. In order to avoid using protein fusions to coat proteins, attempts have been made to use heterologous proteins that can be adsorbed to the spore surface. Nevertheless, this approach would also require a detailed characterization of the spore surface to determine how the different coat layers influence adsorption properties. For example, a recent study indicated that the efficiency of adsorption of β-galactosidase is improved in spores that lack the crust or the outer coat[117].

Another avenue to explore in surface-display technologies is the generation of synthetic vesicles encased by selected coat proteins that could be tailor-made to display specific properties. Eukaryotic vesicles are readily produced with purified components *in vitro*, so it should also be possible to produce vesicles coated with spore coat proteins. A first step in that direction was accomplished with the coat morphogenetic protein SpoVM fused to green fluorescent protein (SpoVM–GFP), which appears to bind preferentially to sites of positive membrane curvature and displays affinity for vesicles close to the size of *B. subtilis* spores (<2 μm)[118]. By changing a proline to alanine, potentially straightening the SpoVM helix, it was possible to expand the size range of vesicles bound by SpoVM–GFP. Our understanding of the mechanisms of coat assembly in bacteria can be integrated with what is known about coated vesicle formation in eukaryotes. The first step in both events is the localization of proteins to a site of membrane curvature[119,120]. A common mediator of curvature recognition among the domains of life may be amphipathic α-helices. This feature is common to both SpoVM and the GTPases, along with BAR (Bin–Amphiphysin–Rvs) domain-containing proteins that are involved in the early steps of eukaryotic vesicle formation[121]. Second, a scaffold of coat material is assembled at the initial site of localization, and protein polymerization proceeds around the circumference of the developing vesicle. Ultimately, the process terminates with membrane fission at the opposite pole of the vesicle.

on the MCP pole of the spore to a full shell[106]. These electron microscopy observations appear to agree with the localization dynamics of individual exosporium proteins, which form an initial cap on the MCP pole before transitioning to a complete shell of protein that surrounds the spore[107]. In cells deleted of *exsY*, a homologue of *B. subtilis cotZ*, the exosporium assembles as a cap on the MCP pole of the forespore but fails to encase the spore[26]. This phenotype is reminiscent of the spore encasement phenotypes of the *spoVM* and *spoVID* mutants of *B. subtilis*[83].

Through a combination of genetic, biochemical and microscopy studies, much progress has been made in understanding how the spore protective layers are assembled in *B. subtilis*. By examining how spores interact with various surfaces, biofilms and microbial consortia, it should be possible to connect what has been learned in the laboratory to what occurs in nature. Future studies that assess how well-conserved the proteins and assembly mechanisms are should help us to determine whether the phenomena observed in *B. subtilis* coat assembly are applicable to other spore formers. These future goals also bring to mind a key gap in our knowledge: we currently know very little about the diversity in spore coat structure and composition among species. Progress in addressing some of these outstanding questions could be substantially facilitated by analysis of species beyond the familiar models. Importantly, knowledge of the coat assembly mechanisms could also be exploited in various applications with public health implications, such as vaccine development (BOX 2).

Natto
A Japanese dish of cooked soy beans fermented by a strain of *Bacillus subtilis*. It has a pungent aroma and a unique texture.

1. Brun, Y. & Shimkets, L. J. *Prokaryotic Development* (ASM Press, 2000).
2. Cohn, F. Untersuchungen über Bacterien. IV. Beiträge zur Biologie der Bacillen. *Beiträge Biol. Pflanzen* **7**, 249–276 (1877).
Along with Koch (below), this paper contains the first (and highly prescient) description of sporulation.
3. Koch, R. Untersuchungen über Bacterien V: Die Aetiologie der Milzbrand-Krankheit, begründet auf die Entwicklungsgeschichte der *Bacillus anthracis*. *Beiträge Biol. Pflanzen* **7**, 277–308 (1877).
4. Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J. & Setlow, P. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol. Mol. Biol. Rev.* **64**, 548–572 (2000).
5. Kennedy, M. J., Reader, S. L. & Swierczynski, L. M. Preservation records of micro-organisms: evidence of the tenacity of life. *Microbiology* **140**, 2513–2529 (1994).
6. Sneath, P. H. Longevity of micro-organisms. *Nature* **195**, 643–646 (1962).
7. Jacotot, H. & Virat, B. La longévité des spores de *B. anthracis* (premier vaccin de Pasteur). *Ann. Inst. Pasteur* **87**, 215–217 (1954).
8. de Hoon, M. J., Eichenberger, P. & Vitkup, D. Hierarchical evolution of the bacterial sporulation network. *Curr. Biol.* **20**, R735–R745 (2010).
9. Galperin, M. Y. *et al.* Genomic determinants of sporulation in Bacilli and Clostridia: towards the minimal set of sporulation-specific genes. *Environ. Microbiol.* **14**, 2870–2890 (2012).
A comprehensive study of sporulation gene conservation among endospore formers.
10. Earl, A. M., Losick, R. & Kolter, R. Ecology and genomics of *Bacillus subtilis*. *Trends Microbiol.* **16**, 269–275 (2008).
11. Wu, M. *et al.* Life in hot carbon monoxide: the complete genome sequence of *Carboxydothermus hydrogenoformans* Z-2901. *PLoS Genet.* **1**, e65 (2005).
12. Hubert, C. *et al.* A constant flux of diverse thermophilic bacteria into the cold Arctic seabed. *Science* **325**, 1541–1544 (2009).
13. Eckburg, P. B. *et al.* Diversity of the human intestinal microbial flora. *Science* **308**, 1635–1638 (2005).
14. Fujita, M. & Losick, R. 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 (2005).
15. Molle, V. *et al.* The Spo0A regulon of *Bacillus subtilis*. *Mol. Microbiol.* **50**, 1683–1701 (2003).
16. Popham, D. L. Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox. *Cell. Mol. Life Sci.* **59**, 426–433 (2002).
17. Henriques, A. O. & Moran, C. P. Jr. Structure, assembly, and function of the spore surface layers. *Ann. Rev. Microbiol.* **61**, 555–588 (2007).
A thorough review that includes a comprehensive list of coat proteins in *B. subtilis* and *B. anthracis*.
18. Driks, A. The *Bacillus anthracis* spore. *Mol. Aspects Med.* **30**, 368–373 (2009).
19. Vasudevan, P., Weaver, A., Reichert, E. D., Linnstaedt, S. D. & Popham, D. L. Spore cortex formation in *Bacillus subtilis* is regulated by accumulation of peptidoglycan precursors under the control of sigma K. *Mol. Microbiol.* **65**, 1582–1594 (2007).

© 2013 Macmillan Publishers Limited. All rights reserved

20. Fay, A. & Dworkin, J. *Bacillus subtilis* homologs of MviN (MurJ), the putative *Escherichia coli* lipid II flippase, are not essential for growth. *J. Bacteriol.* **191**, 6020–6028 (2009).
21. McKenney, P. T. & Eichenberger, P. Dynamics of spore coat morphogenesis in *Bacillus subtilis*. *Mol. Microbiol.* **83**, 245–260 (2012).
This study ties the phenomenon of spore encasement to the regulation of expression of individual spore coat genes.
22. Aronson, A. I. & Fitz-James, P. Structure and morphogenesis of the bacterial spore coat. *Bacteriol. Rev.* **40**, 360–402 (1976).
23. Warth, A. D., Ohye, D. F. & Murrell, W. G. The composition and structure of bacterial spores. *J. Cell Biol.* **16**, 579–592 (1963).
24. Tocheva, E. I. *et al.* Peptidoglycan remodeling and conversion of an inner membrane into an outer membrane during sporulation. *Cell* **146**, 799–812 (2011).
25. McKenney, P. T. *et al.* A distance-weighted interaction map reveals a previously uncharacterized layer of the *Bacillus subtilis* spore coat. *Curr. Biol.* **20**, 934–938 (2010).
26. Boydston, J. A., Yue, L., Kearney, J. F. & Turnbough, C. L. Jr. The ExsY protein is required for complete formation of the exosporium of *Bacillus anthracis*. *J. Bacteriol.* **188**, 7440–7448 (2006).
27. Giorno, R. *et al.* Morphogenesis of the *Bacillus anthracis* spore. *J. Bacteriol.* **189**, 691–705 (2007).
28. Bozue, J. *et al. Bacillus anthracis* spores of the *bclA* mutant exhibit increased adherence to epithelial cells, fibroblasts, and endothelial cells but not to macrophages. *Infect. Immun.* **75**, 4498–4505 (2007).
29. Chen, G., Driks, A., Tawfiq, K., Mallozzi, M. & Patil, S. *Bacillus anthracis* and *Bacillus subtilis* spore surface properties and transport. *Colloids Surf. B Biointerfaces* **76**, 512–518 (2010).
30. Kailas, L. *et al.* Surface architecture of endospores of the *Bacillus cereus/anthracis/thuringiensis* family at the subnanometer scale. *Proc. Natl Acad. Sci. USA* **108**, 16014–16019 (2011).
This study provides a high-resolution characterization of the exosporium structure, revealing a crystalline layer made of a honeycomb-like array of cups.
31. Permpoonpattana, P. *et al.* Surface layers of *Clostridium difficile* endospores. *J. Bacteriol.* **193**, 6461–6470 (2011).
32. Setlow, P. I will survive: DNA protection in bacterial spores. *Trends Microbiol.* **15**, 172–180 (2007).
33. Hullo, M. F., Moszer, I., Danchin, A. & Martin-Verstraete, I. CotA of *Bacillus subtilis* is a copper-dependent laccase. *J. Bacteriol.* **183**, 5426–5430 (2001).
34. Liu, G. Y. & Nizet, V. Color me bad: microbial pigments as virulence factors. *Trends Microbiol.* **17**, 406–413 (2009).
35. Eisenman, H. C. & Casadevall, A. Synthesis and assembly of fungal melanin. *Appl. Microbiol. Biotechnol.* **93**, 931–940 (2012).
36. Klobutcher, L. A., Ragkousi, K. & Setlow, P. The *Bacillus subtilis* spore coat provides “eat resistance” during phagocytic predation by the protozoan *Tetrahymena thermophila*. *Proc. Natl Acad. Sci. USA* **103**, 165–170 (2006).
37. Laaberki, M. H. & Dworkin, J. Role of spore coat proteins in the resistance of *Bacillus subtilis* spores to *Caenorhabditis elegans* predation. *J. Bacteriol.* **190**, 6197–6203 (2008).
38. Carroll, A. M., Plomp, M., Malkin, A. J. & Setlow, P. Protozoal digestion of coat-defective *Bacillus subtilis* spores produces “rinds” composed of insoluble coat protein. *Appl. Environ. Microbiol.* **74**, 5875–5881 (2008).
39. Paredes-Sabja, D., Setlow, P. & Sarker, M. R. Germination of spores of *Bacillales* and *Clostridiales* species: mechanisms and proteins involved. *Trends Microbiol.* **19**, 85–94 (2011).
40. Shah, I. M., Laaberki, M. H., Popham, D. L. & Dworkin, J. A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. *Cell* **135**, 486–496 (2008).
41. Driks, A. *Bacillus subtilis* spore coat. *Microbiol. Mol. Biol. Rev.* **63**, 1–20 (1999).
42. Stewart, B. T. & Halvorson, H. O. Studies on the spores of aerobic bacteria. I. The occurrence of alanine racemase. *J. Bacteriol.* **65**, 160–166 (1953).
43. Steichen, C., Chen, P., Kearney, J. F. & Turnbough, C. L. Jr. Identification of the immunodominant protein and other proteins of the *Bacillus anthracis* exosporium. *J. Bacteriol.* **185**, 1903–1910 (2003).
44. Todd, S. J., Moir, A. J., Johnson, M. J. & Moir, A. Genes of *Bacillus cereus* and *Bacillus anthracis* encoding proteins of the exosporium. *J. Bacteriol.* **185**, 3373–3378 (2003).
45. Chesnokova, O. N., McPherson, S. A., Steichen, C. T. & Turnbough, C. L. Jr. The spore-specific alanine racemase of *Bacillus anthracis* and its role in suppressing germination during spore development. *J. Bacteriol.* **191**, 1303–1310 (2009).
46. Pierce, K. J., Salifu, S. P. & Tangney, M. Gene cloning and characterization of a second alanine racemase from *Bacillus subtilis* encoded by *yncD*. *FEMS Microbiol. Lett.* **283**, 69–74 (2008).
47. Butzin, X. Y. *et al.* Analysis of the effects of a *gerP* mutation on the germination of spores of *Bacillus subtilis*. *J. Bacteriol.* **194**, 5749–5758 (2012).
48. Chirakkal, H., O’Rourke, M., Atrih, A., Foster, S. J. & Moir, A. Analysis of spore cortex lytic enzymes and related proteins in *Bacillus subtilis* endospore germination. *Microbiology* **148**, 2383–2392 (2002).
49. Bagyan, I. & Setlow, P. Localization of the cortex lytic enzyme CwlJ in spores of *Bacillus subtilis*. *J. Bacteriol.* **184**, 1219–1224 (2002).
50. Lambert, E. A. & Popham, D. L. The *Bacillus anthracis* SleL (YaaH) protein is an N-acetylglucosaminidase involved in spore cortex depolymerization. *J. Bacteriol.* **190**, 7601–7607 (2008).
51. Imamura, D., Kuwana, R., Takamatsu, H. & Watabe, K. Localization of proteins to different layers and regions of *Bacillus subtilis* spore coats. *J. Bacteriol.* **192**, 518–524 (2010).
52. Buist, G., Steen, A., Kok, J. & Kuipers, O. P. LysM, a widely distributed protein motif for binding to (peptido)glycans. *Mol. Microbiol.* **68**, 838–847 (2008).
53. Ebmeier, S. E., Tan, I. S., Clapham, K. R. & Ramamurthi, K. S. Small proteins link coat and cortex assembly during sporulation in *Bacillus subtilis*. *Mol. Microbiol.* **84**, 682–696 (2012).
54. Santo, L. Y. & Doi, R. H. Ultrastructural analysis during germination and outgrowth of *Bacillus subtilis* spores. *J. Bacteriol.* **120**, 475–481 (1974).
55. Steichen, C. T., Kearney, J. F. & Turnbough, C. L. Jr. Non-uniform assembly of the *Bacillus anthracis* exosporium and a bottle cap model for spore germination and outgrowth. *Mol. Microbiol.* **64**, 359–367 (2007).
A description of the polarity of the spore envelope and a model for spore germination and outgrowth.
56. Holt, S. C. & Leadbetter, E. R. Comparative ultrastructure of selected aerobic spore-forming bacteria: a freeze-etching study. *Bacteriol. Rev.* **33**, 346–378 (1969).
57. Traag, B. A. *et al.* Do mycobacteria produce endospores? *Proc. Natl Acad. Sci. USA* **107**, 878–881 (2010).
58. Walker, J. R. *et al. Clostridium taeniosporum* spore ribbon-like appendage structure, composition and genes. *Mol. Microbiol.* **63**, 629–643 (2007).
59. Lequette, Y. *et al.* Role played by exosporium glycoproteins in the surface properties of *Bacillus cereus* spores and in their adhesion to stainless steel. *Appl. Environ. Microbiol.* **77**, 4905–4911 (2011).
60. Buhr, T. L., Young, A. A., Minter, Z. A., Wells, C. M. & Shegogue, D. A. Decontamination of a hard surface contaminated with *Bacillus anthracis*∆Sterne and *B. anthracis* Ames spores using electrochemically generated liquid-phase chlorine dioxide ($eClO_2$). *J. Appl. Microbiol.* **111**, 1057–1064 (2011).
61. Siala, A., Hill, I. R. & Gray, T. R. G. Populations of spore-forming bacteria in an acid forest soil, with special reference to *Bacillus subtilis*. *J. Gen. Microbiol.* **8**, 183–190 (1974).
62. Nicholson, W. L. Roles of *Bacillus* endospores in the environment. *Cell. Mol. Life Sci.* **59**, 410–416 (2002).
63. Donovan, W., Zheng, L. B., Sandman, K. & Losick, R. Genes encoding spore coat polypeptides from *Bacillus subtilis*. *J. Mol. Biol.* **196**, 1–10 (1987).
64. Beall, B., Driks, A., Losick, R. & Moran, C. P. Jr. Cloning and characterization of a gene required for assembly of the *Bacillus subtilis* spore coat. *J. Bacteriol.* **175**, 1705–1716 (1993).
65. Zheng, L. B., Donovan, W. P., Fitz-James, P. C. & Losick, R. Gene encoding a morphogenic protein required in the assembly of the outer coat of the *Bacillus subtilis* endospore. *Genes Dev.* **2**, 1047–1054 (1988).
66. Lai, E. M. *et al.* Proteomic analysis of the spore coats of *Bacillus subtilis* and *Bacillus anthracis*. *J. Bacteriol.* **185**, 1443–1454 (2003).
67. Kuwana, R. *et al.* Proteomics characterization of novel spore proteins of *Bacillus subtilis*. *Microbiology* **148**, 3971–3982 (2002).
68. Eichenberger, P. *et al.* The program of gene transcription for a single differentiating cell type during sporulation in *Bacillus subtilis*. *PLoS Biol.* **2**, e328 (2004).
69. Kim, H. *et al.* The *Bacillus subtilis* spore coat protein interaction network. *Mol. Microbiol.* **59**, 487–502 (2006).
70. Abhyankar, W. *et al.* Gel-free proteomic identification of the *Bacillus subtilis* insoluble spore coat protein fraction. *Proteomics* **11**, 4541–4550 (2011).
71. Webb, C. D., Decatur, A., Teleman, A. & Losick, R. Use of green fluorescent protein for visualization of cell-specific gene expression and subcellular protein localization during sporulation in *Bacillus subtilis*. *J. Bacteriol.* **177**, 5906–5911 (1995).
72. Eichenberger, P. *et al.* The σ^E regulon and the identification of additional sporulation genes in *Bacillus subtilis*. *J. Mol. Biol.* **327**, 945–972 (2003).
73. Piggot, P. J. & Coote, J. G. Genetic aspects of bacterial endospore formation. *Bacteriol. Rev.* **40**, 908–962 (1976).
74. Levin, P. A. *et al.* An unusually small gene required for sporulation by *Bacillus subtilis*. *Mol. Microbiol.* **9**, 761–771 (1993).
75. Roels, S., Driks, A. & Losick, R. Characterization of *spoIVA*, a sporulation gene involved in coat morphogenesis in *Bacillus subtilis*. *J. Bacteriol.* **174**, 575–585 (1992).
76. Takamatsu, H., Kodama, T., Nakayama, T. & Watabe, K. Characterization of the *yrbA* gene of *Bacillus subtilis*, involved in resistance and germination of spores. *J. Bacteriol.* **181**, 4986–4994 (1999).
77. Ozin, A. J., Henriques, A. O., Yi, H. & Moran, C. P. Jr. Morphogenetic proteins SpoVID and SafA form a complex during assembly of the *Bacillus subtilis* spore coat. *J. Bacteriol.* **182**, 1828–1833 (2000).
78. Driks, A., Roels, S., Beall, B., Moran, C. P. Jr & Losick, R. Subcellular localization of proteins involved in the assembly of the spore coat of *Bacillus subtilis*. *Genes Dev.* **8**, 234–244 (1994).
This article proposes a seminal model for coat assembly.
79. Imamura, D., Kuwana, R., Takamatsu, H. & Watabe, K. Proteins involved in formation of the outermost layer of *Bacillus subtilis* spores. *J. Bacteriol.* **193**, 4075–4080 (2011).
80. Redmond, C., Baillie, L. W., Hibbs, S., Moir, A. J. & Moir, A. Identification of proteins in the exosporium of *Bacillus anthracis*. *Microbiology* **150**, 355–363 (2004).
81. Waller, L. N., Fox, N., Fox, K. F., Fox, A. & Price, R. L. Ruthenium red staining for ultrastructural visualization of a glycoprotein layer surrounding the spore of *Bacillus anthracis* and *Bacillus subtilis*. *J. Microbiol. Methods* **58**, 23–30 (2004).
This study introduces the use of ruthenium red staining to analyse the outermost layer of the spore.
82. Cutting, S. *et al.* SpoVM, a small protein essential to development in *Bacillus subtilis*, interacts with the ATP-dependent protease FtsH. *J. Bacteriol.* **179**, 5534–5542 (1997).
83. Wang, K. H. *et al.* The coat morphogenetic protein SpoVID is necessary for spore encasement in *Bacillus subtilis*. *Mol. Microbiol.* **74**, 634–649 (2009).
84. Ozin, A. J., Samford, C. S., Henriques, A. O. & Moran, C. P. Jr. SpoVID guides SafA to the spore coat in *Bacillus subtilis*. *J. Bacteriol.* **183**, 3041–3049 (2001).
85. Costa, T., Isidro, A. L., Moran, C. P. Jr & Henriques, A. O. Interaction between coat morphogenetic proteins SafA and SpoVID. *J. Bacteriol.* **188**, 7731–7741 (2006).
86. Price, K. D. & Losick, R. A four-dimensional view of assembly of a morphogenetic protein during sporulation in *Bacillus subtilis*. *J. Bacteriol.* **181**, 781–790 (1999).
87. Ramamurthi, K. S., Clapham, K. R. & Losick, R. Peptide anchoring spore coat assembly to the outer forespore membrane in *Bacillus subtilis*. *Mol. Microbiol.* **62**, 1547–1557 (2006).
88. van Ooij, C., Eichenberger, P. & Losick, R. Dynamic patterns of subcellular protein localization during spore coat morphogenesis in *Bacillus subtilis*. *J. Bacteriol.* **186**, 4441–4448 (2004).
89. Pogliano, K., Harry, E. & Losick, R. Visualization of the subcellular location of sporulation proteins in *Bacillus subtilis* using immunofluorescence microscopy. *Mol. Microbiol.* **18**, 459–470 (1995).
90. van Ooij, C. & Losick, R. Subcellular localization of a small sporulation protein in *Bacillus subtilis*. *J. Bacteriol.* **185**, 1391–1398 (2003).

© 2013 Macmillan Publishers Limited. All rights reserved

91. Mullerova, D., Krajcikova, D. & Barak, I. Interactions between *Bacillus subtilis* early spore coat morphogenetic proteins. *FEMS Microbiol. Lett.* **299**, 74–85 (2009).
92. de Francesco, M. *et al.* Physical interaction between coat morphogenetic proteins SpoVID and CotE is necessary for spore encasement in *Bacillus subtilis*. *J. Bacteriol.* **194**, 4941–4950 (2012).
93. Ramamurthi, K. S. & Losick, R. ATP-driven self-assembly of a morphogenetic protein in *Bacillus subtilis*. *Mol. Cell* **31**, 406–414 (2008).
94. Little, S. & Driks, A. Functional analysis of the *Bacillus subtilis* morphogenetic spore coat protein CotE. *Mol. Microbiol.* **42**, 1107–1120 (2001).
95. Krajcikova, D., Lukacova, M., Mullerova, D., Cutting, S. M. & Barak, I. Searching for protein-protein interactions within the *Bacillus subtilis* spore coat. *J. Bacteriol.* **191**, 3212–3219 (2009).
96. Kroos, L., Kunkel, B. & Losick, R. Switch protein alters specificity of RNA polymerase containing a compartment-specific sigma factor. *Science* **243**, 526–529 (1989).
97. Zhang, J., Fitz-James, P. C. & Aronson, A. I. Cloning and characterization of a cluster of genes encoding polypeptides present in the insoluble fraction of the spore coat of *Bacillus subtilis*. *J. Bacteriol.* **175**, 3757–3766 (1993).
98. Zhang, J., Ichikawa, H., Halberg, R., Kroos, L. & Aronson, A. I. Regulation of the transcription of a cluster of *Bacillus subtilis* spore coat genes. *J. Mol. Biol.* **240**, 405–415 (1994).
99. Zheng, L. B. & Losick, R. Cascade regulation of spore coat gene expression in *Bacillus subtilis*. *J. Mol. Biol.* **212**, 645–660 (1990).
100. Ragkousi, K. & Setlow, P. Transglutaminase-mediated cross-linking of GerQ in the coats of *Bacillus subtilis* spores. *J. Bacteriol.* **186**, 5567–5575 (2004).
101. Naclerio, G., Baccigalupi, L., Zilhao, R., De Felice, M. & Ricca, E. *Bacillus subtilis* spore coat assembly requires *cotH* gene expression. *J. Bacteriol.* **178**, 4375–4380 (1996).
102. McPherson, D. C. *et al.* Characterization of the *Bacillus subtilis* spore morphogenetic coat protein CotO. *J. Bacteriol.* **187**, 8278–8290 (2005).
103. Giglio, R. *et al.* Organization and evolution of the *cotG* and *cotH* genes of *Bacillus subtilis*. *J. Bacteriol.* **193**, 6664–6673 (2011).
104. Isticato, R. *et al.* CotC-CotU heterodimerization during assembly of the *Bacillus subtilis* spore coat. *J. Bacteriol.* **190**, 1267–1275 (2008).
105. Erhardt, M., Namba, K. & Hughes, K. T. Bacterial nanomachines: the flagellum and type III injectisome. *Cold Spring Harb. Perspect. Biol.* **2**, a000299 (2010).
106. Ohye, D. F. & Murrell, W. G. Exosporium and spore coat formation in *Bacillus cereus* T. *J. Bacteriol.* **115**, 1179–1190 (1973).
107. Thompson, B. M. & Stewart, G. C. Targeting of the BclA and BclB proteins to the *Bacillus anthracis* spore surface. *Mol. Microbiol.* **70**, 421–434 (2008).
108. Angert, E. R. Alternatives to binary fission in bacteria. *Nature Rev. Microbiol.* **3**, 214–224 (2005).
109. Eldar, A. *et al.* Partial penetrance facilitates developmental evolution in bacteria. *Nature* **460**, 510–514 (2009).
110. Flardh, K. & Buttner, M. J. *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium. *Nature Rev. Microbiol.* **7**, 36–49 (2009).
111. Sudo, S. Z. & Dworkin, M. Comparative biology of prokaryotic resting cells. *Adv. Microb. Physiol.* **9**, 153–224 (1973).
112. Muller, F. D., Schink, C. W., Hoiczyk, E., Cserti, E. & Higgs, P. I. Spore formation in *Myxococcus xanthus* is tied to cytoskeleton functions and polysaccharide spore coat deposition. *Mol. Microbiol.* **83**, 486–505 (2012).
113. Wu, C. H., Mulchandani, A. & Chen, W. Versatile microbial surface-display for environmental remediation and biofuels production. *Trends Microbiol.* **16**, 181–188 (2008).
114. Cutting, S. M., Hong, H. A., Baccigalupi, L. & Ricca, E. Oral vaccine delivery by recombinant spore probiotics. *Int. Rev. Immunol.* **28**, 487–505 (2009).
115. Permpoonpattana, P. *et al.* Immunization with *Bacillus* spores expressing toxin A peptide repeats protects against infection with *Clostridium difficile* strains producing toxins A and B. *Infect. Immun.* **79**, 2295–2302 (2011).
116. Amuguni, H. *et al.* Sublingual immunization with an engineered *Bacillus subtilis* strain expressing tetanus toxin fragment C induces systemic and mucosal immune responses in piglets. *Microbes Infect.* **14**, 447–456 (2012).
117. Sirec, T. *et al.* Adsorption of beta-galactosidase of *Alicyclobacillus acidocaldarius* on wild type and mutants spores of *Bacillus subtilis*. *Microb. Cell Fact.* **11**, 100 (2012).
118. Ramamurthi, K. S., Lecuyer, S., Stone, H. A. & Losick, R. Geometric cue for protein localization in a bacterium. *Science* **323**, 1354–1357 (2009).
This study reveals that the coat morphogenetic protein SpoVM has the ability to recognize positive membrane curvature.
119. Huang, K. C. & Ramamurthi, K. S. Macromolecules that prefer their membranes curvy. *Mol. Microbiol.* **76**, 822–832 (2010).
120. Pucadyil, T. J. & Schmid, S. L. Conserved functions of membrane active GTPases in coated vesicle formation. *Science* **325**, 1217–1220 (2009).
121. Bhatia, V. K., Hatzakis, N. S. & Stamou, D. A unifying mechanism accounts for sensing of membrane curvature by BAR domains, amphipathic helices and membrane-anchored proteins. *Semin. Cell Dev. Biol.* **21**, 381–390 (2010).
122. Masayama, A. *et al.* A novel lipolytic enzyme, YcsK (LipC), located in the spore coat of *Bacillus subtilis*, is involved in spore germination. *J. Bacteriol.* **189**, 2369–2375 (2007).
123. Bourne, N., FitzJames, P. C. & Aronson, A. I. Structural and germination defects of *Bacillus subtilis* spores with altered contents of a spore coat protein. *J. Bacteriol.* **173**, 6618–6625 (1991).
124. Costa, T., Steil, L., Martins, L. O., Volker, U. & Henriques, A. O. Assembly of an oxalate decarboxylase produced under σ^K control into the *Bacillus subtilis* spore coat. *J. Bacteriol.* **186**, 1462–1474 (2004).
125. Henriques, A. O., Beall, B. W. & Moran, C. P. Jr. CotM of *Bacillus subtilis*, a member of the α-crystallin family of stress proteins, is induced during development and participates in spore outer coat formation. *J. Bacteriol.* **179**, 1887–1897 (1997).
126. Takamatsu, H. *et al.* A spore coat protein, CotS, of *Bacillus subtilis* is synthesized under the regulation of σ^K and GerE during development and is located in the inner coat layer of spores. *J. Bacteriol.* **180**, 2968–2974 (1998).
127. Scheeff, E. D. *et al.* Genomics, evolution, and crystal structure of a new family of bacterial spore kinases. *Proteins* **78**, 1470–1482 (2010).

Acknowledgements
We apologize to colleagues whose work could not be cited in full owing to space limitations. Work in P.E.'s laboratory is supported by grant GM081571 from the US National Institutes of Health (NIH). Work in A.D.'s laboratory is supported by NIH grants R21AI097934 and R01AI093493, and HDTRA1-11-1-0051 from the US Department of Defense.

Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION
Patrick Eichenberger's homepage:
http://www.nyu.edu/projects/eichenberger/index.htm

ALL LINKS ARE ACTIVE IN THE ONLINE PDF

© 2013 Macmillan Publishers Limited. All rights reserved