

Sporulation in Bacteria: Beyond the Standard Model

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ABSTRACT Endospore formation follows a complex, highly regulated developmental pathway that occurs in a broad range of *Firmicutes*. Although *Bacillus subtilis* has served as a powerful model system to study the morphological, biochemical, and genetic determinants of sporulation, fundamental aspects of the program remain mysterious for other genera. For example, it is entirely unknown how most lineages within the *Firmicutes* regulate entry into sporulation. Additionally, little is known about how the sporulation pathway has evolved novel spore forms and reproductive schemes. Here, we describe endospore and internal offspring development in diverse *Firmicutes* and outline progress in characterizing these programs. Moreover, comparative genomics studies are identifying highly conserved sporulation genes, and predictions of sporulation potential in new isolates and uncultured bacteria can be made from these data. One surprising outcome of these comparative studies is that core regulatory and some structural aspects of the program appear to be universally conserved. This suggests that a robust and sophisticated developmental framework was already in place in the last common ancestor of all extant *Firmicutes* that produce internal offspring or endospores. The study of sporulation in model systems beyond *B. subtilis* will continue to provide key information on the flexibility of the program and provide insights into how changes in this developmental course may confer advantages to cells in diverse environments.

AN INTRODUCTION TO ENDOSPORE FORMATION

Bacteria thrive in amazingly diverse ecosystems and often tolerate large fluctuations within a particular environment. One highly successful strategy that allows a cell or population to escape life-threatening conditions is the production of spores. Bacterial endospores, for example, have been described as the most durable cells

in nature (1). These highly resistant, dormant cells can withstand a variety of stresses, including exposure to temperature extremes, DNA-damaging agents, and hydrolytic enzymes (2). The ability to form endospores appears restricted to the *Firmicutes* (3), one of the earliest branching bacterial phyla (4). Endospore formation is broadly distributed within the phylum. Spore-forming species are represented in most classes, including the *Bacilli*, the *Clostridia*, the *Erysipelotrichi*, and the *Negativicutes* (although compelling evidence to demote this class has been presented [5]). To the best of our knowledge endospores have not been observed in members of the *Thermolithobacteria*, a class that contains only a few species that have been isolated and studied. Thus, sporulation is likely an ancient trait, established early in evolution but later lost in many lineages within the *Firmicutes* (4, 6).

Endospores occur most commonly in rod-shaped bacteria (Fig. 1), but also appear in filamentous cells and in cocci (7–11). Many endospores have been observed only in samples from nature. For instance, large, morphologically diverse helical bacteria (40 to 100 μm long), named *Sporospirillum* spp., produce one or two endospore-like

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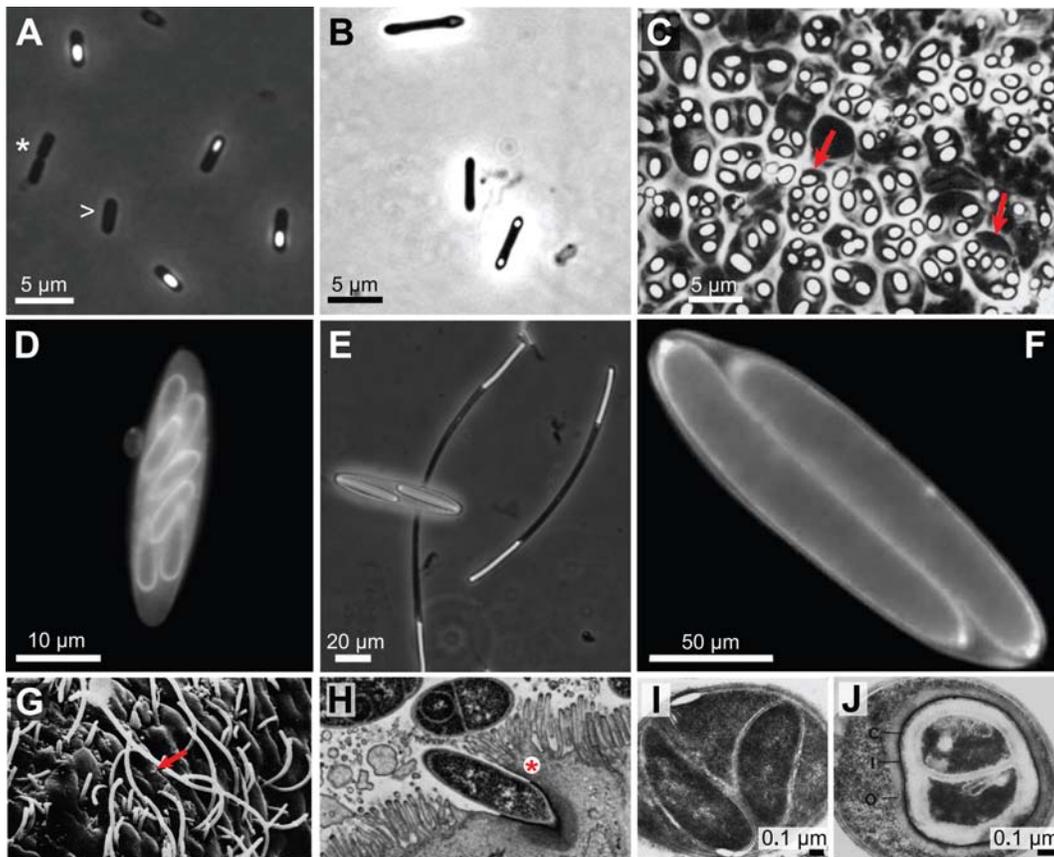


FIGURE 1 Bacteria that produce endospores or intracellular offspring exhibit a wide variety of morphological phenotypes. **(A)** Phase-contrast microscopy is often used to identify mature endospores (A to C and E) as these highly mineralized cells appear phase-bright. In this image of *B. subtilis*, the caret (>) indicates a cell that is not dividing or sporulating and the asterisk (*) indicates a cell undergoing binary fission. All other cells in the image contain a phase-bright endospore. **(B)** *Clostridium oceanicum* frequently produces phase-bright endospores at both ends of the cell. Image courtesy of Avigdor Eldar and Michael Elowitz, California Institute of Technology. **(C)** In this image of *Anaerobacter polyendosporus*, the arrows indicate cells with seven endospores. **(D)** The fluorescence micrograph of *Metabacterium polyspora* outlines cell membranes and spore coats stained with FM1-43. **(E)** *Epulopiscium*-like type C (cigar-shaped cell) and type J (elongated cells), each containing two phase-bright endospores. **(F)** *Epulopiscium* sp. type B with two internal daughter cells, stained with DAPI. Cellular DNA is located at the periphery of the cytoplasm in the mother cell and each offspring. **(G)** Scanning electron micrograph (SEM) of the ileum lining from a rat reveals the epithelial surface densely populated with SFB. Arrow indicates a holdfast cell that has not yet elongated into a filament. **(H)** Transmission electron micrograph (TEM) of a thin section through the gut wall reveals the structure of the SFB holdfast cell (indicated by an asterisk). **(I to J)** TEMs illustrate the two possible fates for developing intracellular SFB: (I) two holdfast cells or (J) two endospores that are encased in a common coat (C), inner (I) and outer (O) cortex. Panel C reproduced from Siunov et al. (47) with permission from Society for General Microbiology. Panel E reproduced from Flint et al. (33) with permission from ASM Press. Panel F reproduced from Mendell et al. (93) with permission from the National Academy of Sciences, USA. Panels G and H reproduced from Erlandsen and Chase (69) with permission from the American Society for Nutrition. Panels I and J reproduced from Ferguson and Birch-Andersen (74) with permission from John Wiley and Sons. [doi:10.1128/microbiolspec.TBS-0013-2012.f1](https://doi.org/10.1128/microbiolspec.TBS-0013-2012.f1)

structures (12, 13). These bacteria have been found in the gut of batrachian tadpoles, although their affiliation within the *Firmicutes* has not been established. The diversity of endospore-producing bacteria and their varied lifestyles suggest that the sporulation pathway is finely tuned to life in a particular environment, and is an advantageous means of cellular survival, dispersal, and, in some cases, reproduction.

The basic and most familiar mode of sporulation (Fig. 2A) involves an asymmetrical cell division that leads to the formation of a mother cell and a smaller forespore (14, 15). Unique transcriptional programs within these cells result in distinct fates for the forespore and the mother cell. The initiation of sporulation in *Bacillus subtilis* is triggered by a lack of nutrients and by high cell density (2, 15). The decision to sporulate is tightly regulated, because this energy-intensive process serves as a last resort for these starving cells. In the early stages of sporulation, gene regulation mainly depends on the stationary-phase sigma factor σ^H and the master transcriptional regulator Spo0A (16, 17). Activation of Spo0A in *B. subtilis* is governed by a phosphorelay system involving several kinases, each of which transmits information about cell condition and environmental stimuli to determine the phosphorylation state of the intracellular pool of Spo0A (18). Prior to asymmetric cell division, the chromosome replicates, and each replication origin rapidly migrates to a different pole of the cell (19). Subsequently, the origin-proximal regions become tethered to opposite poles and the chromosomal DNA stretches from one pole to the other to form an axial filament (20, 21). During division, only ~30% of the origin-proximal portion of one chromosome is trapped within the forespore, and the rest is translocated into the forespore by SpoIII ϵ , a DNA transporter protein (17, 22). The other chromosome copy remains in the mother cell.

Differential activation of sporulation-specific sigma factors in the mother cell and forespore manages the fate of each cell (14). First, σ^F is activated exclusively in the forespore (17). Shortly thereafter, a signal is sent to the mother cell to process and hence activate σ^E . Both early sigma factors promote the expression of genes necessary for forespore engulfment, as well as genes needed for the production and activation of the late sporulation sigma factors (17, 23). Remodeling of septal peptidoglycan allows migration of the mother-cell membrane around the forespore (2, 17, 24, 25). Eventually, the leading edge of the migrating mother-cell membrane meets, and fission establishes the double-membrane-bound forespore within the mother cell. Completion of forespore

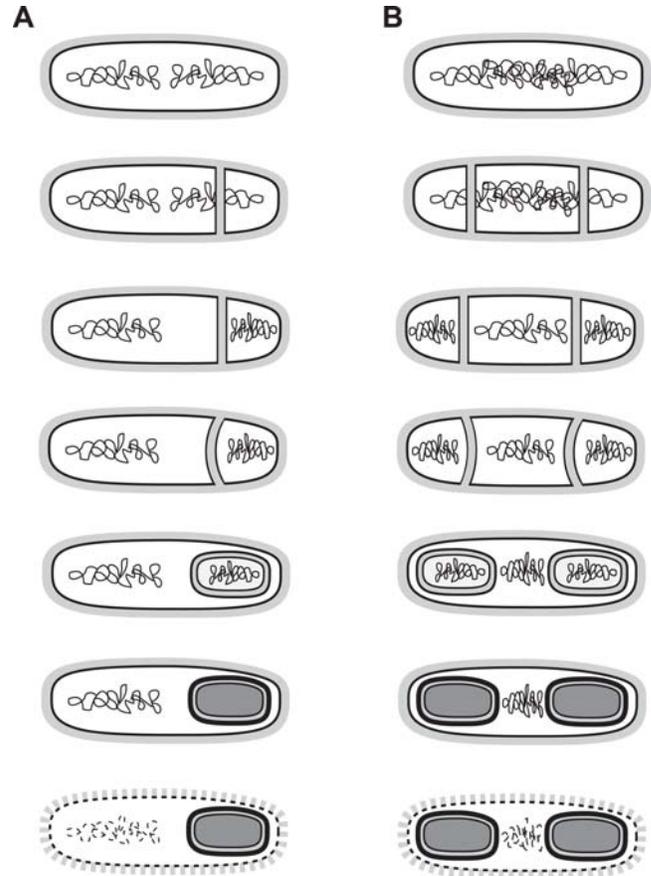


FIGURE 2 Endospore development. In monosporic bacteria, complete division occurs at only one end of the developing sporangium (A), while bacteria that produce two endospores generally divide at both poles (B). In some lineages, such as the SFB and *M. polyspora*, engulfed forespores undergo division (not shown). Note that at least three chromosome copies are required to produce two viable endospores. Following endospore engulfment, cortex and coat layers develop, and upon endospore maturation, the mother cell lyses, releasing one (A) or two (B) endospores. [doi:10.1128/microbiolspec.TBS-0013-2012.f2](https://doi.org/10.1128/microbiolspec.TBS-0013-2012.f2)

engulfment, combined with further intercellular signaling, allows activation of σ^G in the forespore and the subsequent activation of σ^K in the mother cell. These sigma factors regulate the genes necessary for spore maturation and germination (2, 17). Ultimately, the mother cell undergoes programmed cell death and lysis, which releases the mature endospore (26, 27).

TWIN ENDSPORE FORMATION IN *B. SUBTILIS* AND TWINS PRODUCED IN NATURE

Although sporogenesis in *B. subtilis* typically culminates in the production of a single endospore, simple

mutations can vary the outcome of the program and lead to the production of two viable, mature spores (28). This is due in part to the normal assembly of functional division apparatus at both ends of the cell even if only one is used. Null mutations that block activation or expression of σ^E will arrest sporulation after asymmetric division. These mutants produce abortive disporics where the developing sporangium divides sequentially at both poles and chromosome copies are transferred into each of the polar forespores, leaving the mother cell devoid of a chromosome. Forespore-specific expression of *spoIIR* is necessary for intercellular signaling to activate σ^E in the mother cell. Eldar et al. found that, by manipulating the expression of *spoIIR*, a small percentage of cells “escape” sporulation, resume chromosome replication, and then undergo division at both poles to produce viable and UV-resistant “twin endospores.” When combined with mutations that increase chromosome copy number, such as those that prevent expression of the replication inhibitor *yabA*, the frequency of twins in the population elevates, provided that the mother cell retains a copy of the chromosome (28). Data from Eldar et al. and studies of other sporulation systems (discussed later) suggest that natural mutations that increase ploidy and promote bipolar division could gradually increase the occurrence of this alternative developmental outcome, thus leading to twin endospore formation as a means of reproduction (28, 29).

Several bacterial lineages naturally produce these “fraternal” twin endospores. The marine anaerobe *Clostridium oceanicum* is a rod-shaped bacterium that typically produces two endospores (Fig. 1B), depending on the temperature or medium composition (30). The DNA replication and septation events (Fig. 2B) leading to twin endospore formation in *C. oceanicum* closely resemble those of twin endospore formation in *B. subtilis* in the mutant strains described above (28). Although rare, twin endospores naturally occur in *Bacillus thuringiensis* as well (31). Other twin endosporeformers, such as the large, rod-shaped spore-forming bacteria from the intestinal tract of batrachian tadpoles and rodents (12, 13, 32, 33), have been observed, but many of these have not been phylogenetically characterized. Finally, the regular production of twin endospores has been described in *Epulopiscium*-like cells (34). Twin endospore-forming bacteria are frequently observed in the gastrointestinal tract, which suggests that these nutrient-rich ecosystems may better support increased ploidy (35), a requisite for the production of more than one endospore.

Epulopiscium spp. and their close relatives, known as “epulos,” are intestinal inhabitants of certain species

of surgeonfish (36). All morphotypes characterized to date are exceptionally large, with some reaching 600 μm (37–39). Due to their large size, *Epulopiscium* spp. were originally classified as protists (36, 39–41), but further ultrastructural and molecular phylogenetic analyses proved that these symbionts are bacteria (37, 38). Phylogenetically, epulos group within the clostridial cluster XIVb in the *Lachnospiraceae* (34, 37, 42, 43). A survey of surgeonfish intestinal communities provided a first assessment of the distribution of epulos among host species and classified these diverse symbionts into ten morphotypes (A to J) based on their cellular and reproductive characteristics (36).

These surgeonfish symbionts exhibit a variety of novel reproductive patterns (44). Only the two largest morphotypes, A and B, are referred to as *Epulopiscium* spp., and these lineages appear to reproduce solely by the formation of multiple, nondormant intracellular offspring (39, 40, 45), which will be described below. Some of the smaller epulo morphotypes undergo binary fission, and many have the ability to produce phase-bright endospores (34, 36, 39, 40, 45). The generation of intracellular offspring in *Epulopiscium* spp. or of endospores in smaller epulo morphotypes is similar to endosporulation in other *Firmicutes*, and developmental progression can be highly synchronized in naturally occurring populations (34, 39, 45, 46).

The phase-bright endospores of the epulo C and J morphotypes from the surgeonfish *Naso lituratus* have been described in the most detail (34). Type C cells are typically cigar shaped, 40 to 130 μm long, and do not undergo binary fission, while type J cells are thin filaments, 40 to 400 μm long, and capable of binary fission (Fig. 1E). Endospore maturation in type C and type J epulos occurs nocturnally in a highly synchronized manner, with 95 to 100% of the cells in these populations producing spores. Endospores are not seen in fish during daylight hours, suggesting that epulos have evolved a mechanism to regulate endospore development and germination in a diurnal fashion (34). Formation of endospores may promote offspring survival by entering a period of dormancy when nutrients in the gut become depleted, as the host fish sleeps. These endospores may be more resilient than an actively growing epulo, and could aid in transfer to a new host, although the importance of spores in transmission has yet to be fully evaluated (34). Thus, type C and J epulos have modified their sporulation program to produce “fraternal” twin endospores and coordinate this developmental program with regular fluctuations in their environment.

SPORULATION PROGRAMS THAT CAN PRODUCE MORE THAN TWO ENDOSPORES

While the bacteria discussed above have the ability to form twin endospores, others have evolved the means of producing more than two endospores per cell. Endospore formation is generally considered a survival strategy, but the study of these multiple endospore-forming bacteria could provide insight into its use as a reproductive strategy, which may be better suited to some bacterial lifestyles than binary fission alone.

Anaerobacter polyendosporus was first isolated in 1985 from rice paddy soil (47). Depending on growth conditions, *A. polyendosporus* can produce up to seven endospores per cell (Fig. 1C). Cultures of *A. polyendosporus* are pleomorphic (47, 48), and the varying cell types appear related to the metabolic transitions that lead cells to sporulate, although this has not been the subject of targeted studies. Thick rods with rounded ends predominate cultures in exponential growth. Cells become wider as the culture ages and eventually thick, phase-bright rods and football-shaped cells appear. All of these forms undergo binary fission (A. M. Johnson and E. R. Angert, unpublished data). Each football-shaped sporangium generally produces one or two endospores. Under certain conditions, such as growth on potato agar or in a liquid medium containing galactose, cells with more than two endospores are observed (47). Twin endospores are produced by division at both cell poles (Johnson and Angert, unpublished). Since *A. polyendosporus* is a member of the cluster I clostridia (43), some of the cell forms observed in sporulating cultures may be homologous to the phase-bright, spindle-shaped clostridial form observed in others of this group such as *Clostridium acetobutylicum*

and *Clostridium perfringens* (49, 50). Little research has been conducted on *A. polyendosporus*, and many questions remain regarding its ability to produce multiple endospores, including the role of morphological transitions in sporulation and the factors that lead to the production of more than two endospores.

Metabacterium polyspora (Fig. 1D), an inhabitant of the intestinal tract of guinea pigs, has been studied in some detail, revealing insights into how and why these cells produce multiple endospores (29). Cells of *M. polyspora* pass through the digestive system and rely on the coprophagous character of guinea pigs for cycling back into its original host and for transmission to new hosts (51). The *M. polyspora* cell would not last long outside the host, and only mature endospores appear to survive transit through the mouth and stomach. Germination occurs after the passage of spores into the small intestine. While these cells have the ability to reproduce by binary fission, not all cells use this process. In those that do, binary fission occurs during a short period of time following germination (Fig. 3). After germination, the cells quickly transition to sporulation. In fact, cells with polar septa are often observed emerging from the soon-to-be discarded spore coat. The production of multiple endospores, up to nine per cell (52), allows *M. polyspora* to produce offspring that are prepared for conditions outside the host (29, 51). Considering the rapid passage of material through the gut and possibly the limited time *M. polyspora* spends inside the host, we speculate that reproduction by the instant formation of multiple endospores is advantageous to the symbiotic lifestyle of *M. polyspora* and has allowed it to move away from a reliance on binary fission.

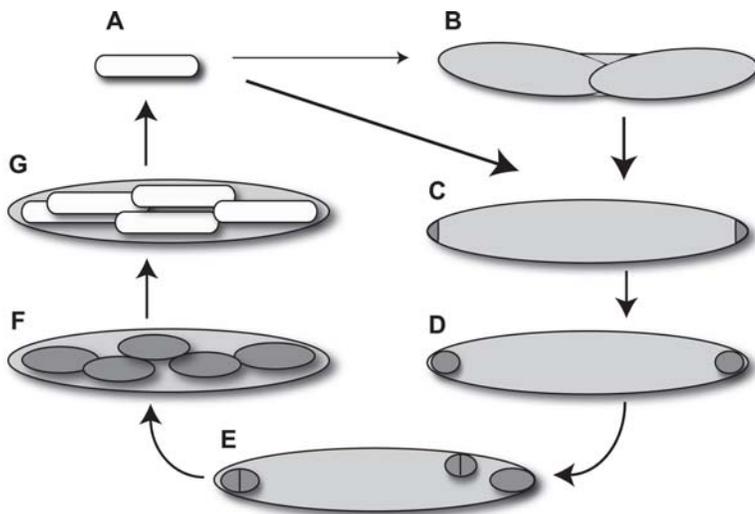


FIGURE 3 Life cycle of *Metabacterium polyspora*. Endospores germinate (A) and, during outgrowth, a cell may undergo binary fission (B) or immediately begin to sporulate by dividing at the poles (C). The forespores are engulfed (D), and the forespores may undergo binary fission to produce additional forespores (E). Forespores then elongate (F) and develop into mature endospores (G). Figure reproduced from Ward and Angert (52) with permission from John Wiley and Sons. doi:10.1128/microbiolspec.TBS-0013-2012.f3

To produce multiple endospores, cells of *M. polyspora*, like the twin endosporeformers described above, divide at both poles (Fig. 3) (17, 51). Each of the forespores receives at least one copy of the chromosome and another copy (or copies) is retained in the mother cell (53). Normally, in *B. subtilis*, DNA replication occurs once, early in sporulation, and any additional rounds of initiation are inhibited during sporulation (54, 55). In contrast, DNA replication in *M. polyspora* occurs throughout development, even after forespore engulfment (53). To form more than two spores, the fully engulfed forespore(s) divide (51). Additionally, DNA replication within forespores loads the endospores with multiple chromosomes, allowing cells to enter sporulation immediately after germination without the requisite of binary fission or chromosome replication seen in *B. subtilis*. Bacteria that have the ability to produce two or more endospores, like *M. polyspora*, have been reported in other coprophagous rodents (33). For intestinal symbionts, it appears that spore formation not only provides protection from the harsh external environment and to the host's natural barriers to infection, but also the process may be modified to provide a consistent means of cellular propagation.

MODIFICATION OF THE SPORULATION PROGRAM FOR PRODUCTION OF NONDORMANT INTERNAL OFFSPRING

In some groups of bacteria, the sporulation program has evolved to produce multiple intracellular offspring, some of which no longer go through a dormancy period. Notably, members of the genus *Candidatus* Arthromitus, as well as members of the segmented filamentous bacteria (SFB) (also known as *Candidatus* Savagella), reproduce via filament segmentation and internal daughter cell production in addition to forming endospores (56). *Candidatus* Arthromitus and the SFB are Gram-positive, sometimes motile, endospore-forming bacteria found in the intestinal tract of a diverse array of organisms, ranging from mammals, to birds, to fish, to arthropods (56–60). The genus “Arthromitus” was first described and characterized by Joseph Leidy in the mid-1800s from his observations of filamentous bacteria in arthropods and other animals (61, 62). Phylogenetic analyses revealed that SFB (from rats, mice, chickens, and fish) form a distinct clade within the group I clostridia, while spore-forming filaments from arthropods constitute a distinct group within the *Lachnospiraceae* (56–59, 63–66). Isolates from different host species are distinct (56) and exhibit host specificity (67, 68).

As yet, none of the SFB from vertebrate hosts are available in pure culture, although the development of gnotobiotic mammalian hosts mono-associated with SFB has been successful for some lineages (69). Genome sequences derived from populations established in rodents revealed that these bacteria lack almost all biosynthetic pathways for amino acids, vitamins, cofactors, and nucleotides (63–65). The SFB likely live off simple sugars and other essential nutrients gleaned from the host and surrounding environment.

SFB can be abundant in the mammalian host (Fig. 1G) but are restricted to the distal ileum (70, 71). SFB filaments are predominantly attached to the ileal wall and localized to the Peyer's patches, specialized lymphoid follicles that function in antigen sampling and surveillance in the small intestine (72, 73). Close examination of the gut environment revealed that SFB are simultaneously present in various stages of their life cycle, including unattached teardrop-shaped cells in the inter-villar spaces, and long or short filaments attached to the ileal epithelium (71). The conical tip of the teardrop-shaped cell is referred to as the holdfast, which anchors the cell to the epithelium. Upon attachment of a holdfast cell, distinct morphological changes occur. The conical tip of the holdfast protrudes into, but does not penetrate, the membrane of the host epithelial cell (Fig. 1H) (70, 71, 74–76). In the host cell cytoplasm, the area adjacent to the SFB attachment site forms an electron-dense layer that comprises predominantly actin filaments (70, 77). Although some holdfast cells appear to be phagocytosed by the host, inflammation of the epithelial tissue at the attachment site does not occur (78).

Once attached, the holdfast cell begins to elongate and septate (Fig. 4A). SFB filaments are typically 50 to 80 μm , but can reach lengths up to 1,000 μm (70, 73). As a filament transitions into its developmental cycle, starting at the free end of the filament, the so-called primary cells of the filament divide symmetrically, producing two equivalent secondary segments (Fig. 4A, iii) (71, 75). These divisions establish an alternating orientation of cells in the filament, with respect to new and old cell poles, which in turn appears to dictate the pattern of asymmetric division of the secondary segments. After secondary segment division, the larger cell engulfs the smaller cell, which eventually forms a spherical body within the larger mother cell (Fig. 4A, iv). These events closely resemble the early stages of endospore formation (71, 75, 76). Within each SFB mother cell, the engulfed spherical body divides by first becoming crescent-shaped (Fig. 4A, v to vi) and then constricting at the midcell, leaving a pair of cells in each mother cell (71, 73, 75).

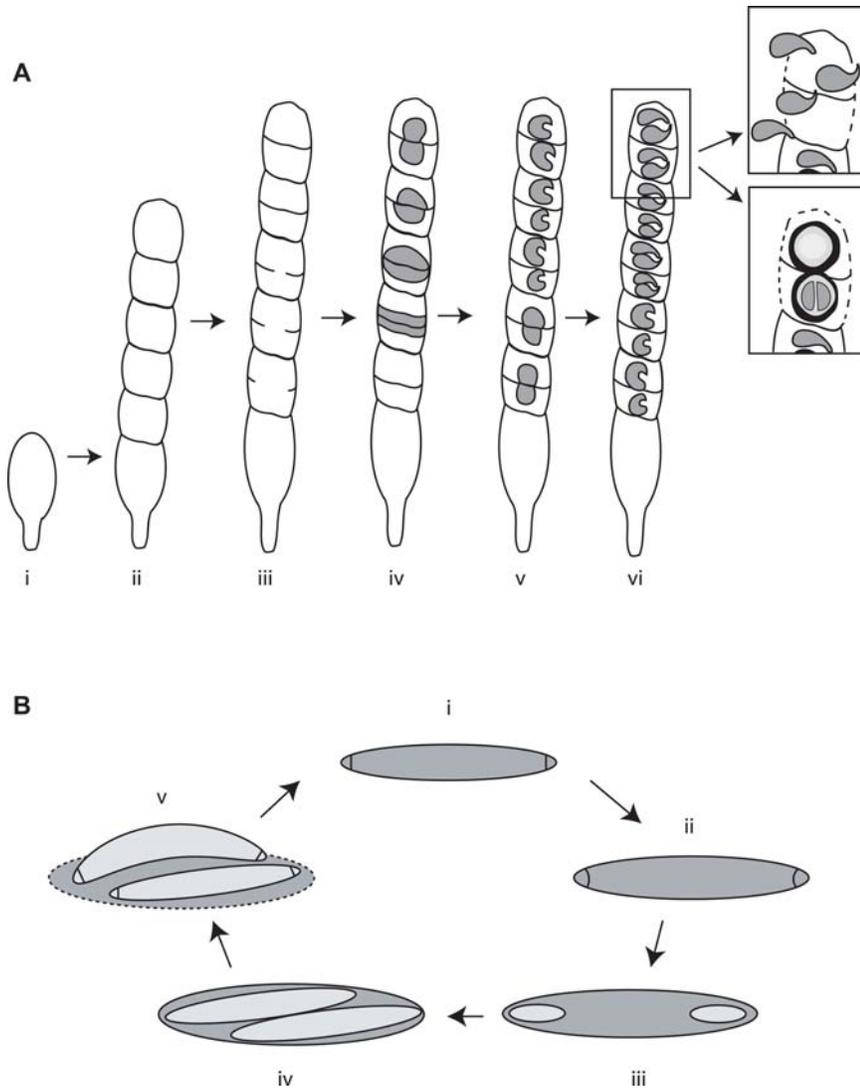


FIGURE 4 Life cycle of SFB and *Epulopiscium* sp. type B. **(A)** (i) The SFB life cycle begins with a holdfast cell that is anchored to the intestinal epithelia (not shown). (ii) Holdfast cells elongate and divide into primary segments as the filament grows. (iii) At the start of development, cells in the filament divide again to produce secondary segments. (iv) Next, secondary segments divide asymmetrically, and then engulfment of the smaller cell (in grey) occurs, in a manner similar to that of other endosporeformers. Development progresses from the free end of the filament toward the holdfast. (v) Each engulfed offspring cell then forms into a crescent shape (vi) and then divides to either form two holdfast offspring cells per segment (inset, top) or develop into an endospore via formation of a spore cortex and coat (inset, bottom). **(B)** (i) In *Epulopiscium* sp. type B, twin offspring form by division at both cell poles. Engulfment occurs (ii to iii) and offspring cells elongate (iv). The offspring cells begin to produce their own offspring before they are released from the mother cell (v). [doi:10.1128/microbiolspec.TBS-0013-2012.f4](https://doi.org/10.1128/microbiolspec.TBS-0013-2012.f4)

These “identical” twin offspring cells then differentiate into holdfasts. At this point, the two cells can follow one of two developmental pathways (Fig. 1I to J). In one, the cells can progress through sporulation, producing a cortex and two distinct coat layers. The emergent spores are encased in a common spore coat, a feature that appears to be unique to the SFB sporogenesis pathway. Eventually the mother cell deteriorates, releasing the spore carrying these two offspring. Alternatively, the holdfast cells are simply released upon mother cell lysis (71, 75). A free holdfast cell may establish a new filament within the host, while the spore is an effective dispersal vehicle capable of airborne infection of a naive host (73). Thus, SFB have modified their developmental program such that they can either produce two daughter holdfast cells or an endospore that contains two cells,

likely conferring an advantage to this organism in the dynamic environment of the gut and outside the host. It is unclear how these alternative developmental processes are instigated in a given filament or how different proportions of active or dormant cells impact population dynamics.

The genetics of sporulation have not yet been characterized in detail for SFB, but genome sequence data from these organisms suggest that many components of the sporulation pathway from *B. subtilis* and clostridial genomes are conserved. Approximately 60 to 70 putative sporulation genes have been identified in SFB genomes, including those coding for sporulation sigma factors, stage-specific transcriptional regulators, and spore germination proteins (63–65). Characterization of the kinases that influence phosphorylation of Spo0A

could provide insight into factors that control the decision to sporulate or produce daughter cells, but, like other clostridia, genes encoding the phosphorelay proteins in *B. subtilis* are absent in SFB (63, 64).

SFB have been adopted as a model for examining the effect of commensals on host immune system development and homeostasis. SFB have a broad range of immunostimulatory effects (79–82), and it has been suggested that SFB affect pathogen resistance and autoimmune disease susceptibility of their host (83–89). SFB are generally considered harmless to a healthy host, and may provide critical signals for immune development (90). However, because of their intimate association with host cells and potential to trigger an inflammatory response, the SFB may contribute to disease susceptibility, depending on the genetic background of the host and composition of its resident gut microbiota (83, 91).

As an aside, sporulation in a group of unattached, multicellular filamentous gut symbionts has been described in some morphological detail. *Oscillospira guilliermondi*, later called *Oscillospira guilliermondii*, is a Gram-positive gastrointestinal bacterium found in the cecum of guinea pigs and in the rumen of cattle, sheep, and reindeer (92, 93). These filaments or ovals (5 to 100 μm long) are composed of a stack of disc-shaped cells that in some ways resemble *Beggiatoa* spp., but are members of the *Ruminococcaceae* or clostridial cluster IV (92–94). Within a filament of *Oscillospira*, one or more sections may produce an endospore. While nothing is known about the genetics of sporulation in *Oscillospira*, ultrastructural images of filaments undergoing development have been published, and the process appears to have many of the hallmarks of endosporulation, including forespore engulfment and the production of a spore with a multilayered envelope of cortex and coat. Genome sequence from a non-spore-forming, closely related bacterium, *Oscillibacter valericigenes*, isolated from the gut of a clam, revealed some conservation of sporulation genes, particularly those involved in regulating early events (95, 96).

As with the SFB described above, *Epulopiscium* spp. type A and type B live successfully in the gut of a vertebrate host and exhibit an intracellular offspring developmental program. This process has been best studied in type B cells from the host fish *Naso tonganus* (36, 45). *Epulopiscium* sp. type B cells are very large, usually 100 to 300 μm long (Fig. 1F). Although some epulos, such as types C and J, reproduce via endospore production and/or binary fission, type B cells have never been observed to form endospores or undergo binary fission. Instead, *Epulopiscium* sp. type B typically produces 2 to

3 nondormant, intracellular offspring per mother cell; however, as many as 12 have been observed (29, 36).

To form offspring (Fig. 4B), *Epulopiscium* sp. type B cells undergo asymmetric cell division, much like that observed in classical endospore formation, but division occurs at both cell poles (45). A given type B cell contains tens of thousands of copies of its genome to accommodate its large size, and polar division traps only a small amount (<1%) of this DNA (45, 97). Next, the insipient offspring are engulfed and grow within the mother cell. Unlike endospore formation in *B. subtilis*, DNA replication continues in both the mother cell and offspring as the offspring grow (53). Upon completion of offspring growth, the mother cell undergoes a form of programmed cell death (45, 98). The entire developmental process occurs synchronously within a population. Given the close phylogenetic relationship of *Epulopiscium* sp. type B and other epulos to endospore-forming bacteria, as well as the morphological similarities in the early stages of daughter cell development to that of the early stages of endospore formation, it is likely that the ancestor of all epulos produced endospores, and, with time, the program was modified to function in intracellular offspring production in these viviparous *Firmicutes* (42).

The *Epulopiscium* sp. type B genome has homologs of the *B. subtilis* *spoIIE* gene and the *spoIIA* operon, which contains genes coding for σ^F and its regulators SpoIIAA and SpoIIAB (46). During sporulation in *B. subtilis*, SpoIIE has dual roles: the promotion of asymmetric cell division and the activation of σ^F . The pattern of *spoIIE* expression with respect to asymmetric division and offspring development in *Epulopiscium* sp. type B populations is similar to that of *B. subtilis*, except that *spoIIE* expression peaks slightly later in *B. subtilis* and stays elevated for a longer developmental interval. Differences in expression of *spoIIE* could be a consequence of differences in the role of SpoIIE in each organism. Also, it may reflect differences in population heterogeneity because endospore formation is a last resort in *B. subtilis* and cells delay entry into sporulation as long as possible (99, 100), while development in *Epulopiscium* is essential for reproduction.

Epulopiscium sp. type B has become a model for studies of cytoarchitecture and evolutionary potential. These massive microbes are extremely polyploid and maintain tens of thousands of genome copies throughout their life cycle (97). This adaptation appears essential for maintaining an active metabolism to support such a large cytoplasmic volume (35). Likewise, polyploidy naturally provides one of the prerequisites of

multiple internal offspring production. Studies of the *Epulopiscium* genome have revealed a tolerance for unstable genetic elements, which appears to be a feature shared with other polyploid symbionts (101). For *Epulopiscium* specifically, extreme polyploidy and the use of an endosporulation-derived reproduction have led to the establishment of a cell with chromosomes of differing fates (98). A small subset of chromosomes is inherited by offspring directly, and we consider these “germ line” chromosomes. Most chromosomal copies remain in the mother cell after offspring are formed, and, surprisingly, these chromosomes continue to replicate, despite the fact that they cannot be directly passed on to offspring. This suggests that replication of “somatic” chromosomes is necessary to support the metabolic needs of the mother cell and its growing offspring (98). Studies of this unconventional bacterium are providing fundamental insights into cellular biology and maintenance of genomic resources.

INSIGHTS FROM OTHER UNUSUAL NONMODEL ENDOSPOREFORMERS

Thus far, we have focused on modifications of the basic sporulation program to allow for the formation of multiple endospores or multiple nondormant, intracellular offspring. Here, we describe two other noteworthy and fruitful experimental systems that produce a single endospore per mother cell.

Pasteuria spp., parasites of nematodes and *Daphnia*, constitute another diverse group within the *Firmicutes* that forms endospores that function in a remarkable manner. Endospores of *Pasteuria* spp. consist of a spherical, opaque structure with several spore coat layers, and an additional exosporial fibrillar matrix layer that skirts the spore (102–105). This fibrillar matrix serves in host-specific attachment. The attached *Pasteuria* spore germinates and produces a germ tube that enters the host, where this obligate parasite grows and proliferates (102, 105). Sporogenesis of *Pasteuria* spp. has been characterized in microscopic detail, and a phylogenetic assessment of these members of the *Bacilli* has been carried out for the *spo0A* gene (106), yet the biology behind these unique spore structures and factors that regulate germination and host specificity have yet to be characterized fully. With the recent development of *in vitro* culturing methods by Syngenta and Pasteuria Bioscience, Inc., the structure-function relationship of this unusual spore-delivery system may soon be uncovered.

Although the term *Firmicutes* is thought of as synonymous with “low G+C Gram-positive bacteria,” some

members of the family *Veillonellaceae* have a Gram-negative cell envelope and can form endospores. Recently, the process of sporulation was characterized in stunning ultrastructural detail in one of these Gram-negative sporeformers, *Acetonema longum* (107). Using 3D electron cryotomographic imaging and immunodetection methods, Tocheva and colleagues show that, through engulfment, the inner and outer membranes of the *A. longum* mother cell become inverted. During outgrowth, the membrane that was previously part of the cytoplasmic membrane transforms, as outer membrane components such as lipopolysaccharide and porins assemble in this now-exposed surface of the cell envelope. The authors suggest that *A. longum* may provide insight into the mechanisms by which an outer membrane could evolve, thus providing a plausible link between early Gram-positive cell forms and the appearance of the Gram-negative envelope (107). Further, this analysis provided evidence to support a hypothesis concerning peptidoglycan dynamics in all endosporeformers. When the state of peptidoglycan of the developing spore was investigated, the authors found that, during engulfment, a thin layer of peptidoglycan is formed and this eventually becomes part of the Gram-negative periplasm (107). While analyses of this unusual Gram-negative endospore-forming bacterium aimed at elucidating unique features of this cell, its study provided additional evidence supporting a novel model of peptidoglycan remodeling in driving a key forespore developmental process, which was later confirmed in *B. subtilis* (107).

EVOLUTION OF SPORULATION FROM A COMPARATIVE GENOMICS PERSPECTIVE

Morphological comparisons between different species and early genetic work on sporulation suggested that this developmental pathway evolved only once in bacteria (6, 108–110). As complete genome sequences became available, comparative studies to look for conserved sporulation genes became feasible (108, 111–114). In one of the first extensive published surveys, Onyenwoke et al. queried a set of 52 bacterial and archaeal genomes using BLAST for 65 select *B. subtilis* sporulation genes covering all stages of sporulation (108). Genes were deemed part of the “core” sporulation pathway if they were absent in non-spore-forming lineages but present only in sporeformers or asporogenous strains (which have conserved sporulation genes but do not produce spores). With this approach, Onyenwoke et al. identified a set of 45 sporulation-specific genes (108). In addition,

they noted differences between sporulation gene content in *Clostridia* versus *Bacilli* genomes, and difficulties in accurately identifying clostridial sporulation genes using sequences from *B. subtilis*.

More recently, de Hoon et al. assessed the distribution of 307 *B. subtilis* genes that are directly regulated by the sigma factors σ^H , σ^F , σ^E , σ^G , and σ^K in 24 different species of spore-forming bacteria, using BLAST (6). The authors confirmed that genes coding for the master regulator of sporulation, *spo0A*, and the main sporulation sigma factors are conserved in all sporeformers examined. Genes involved in signaling between sporulation sigma factors are also well conserved, but those genes downstream in the signaling pathway (those that function in a nonregulatory capacity) are not as conserved among sporeformers.

In an effort to improve the annotation of sporulation genes and the ability to predict sporeformers from genomic data, Galperin et al. used a clusters of orthologous genes (COG)-based approach to identify a core set of sporulation genes (96). The authors analyzed almost 400 *Firmicutes* genomes and sorted them into spore-forming and non-spore-forming based on the presence of *spo0A*, *sspA*, and *dpaAB* genes, which were previously known to be fairly accurate predictors of sporulation (108). The authors then compiled a list of 651 known sporulation genes and compared their distribution in sporeformers versus asporogenous strains versus nonsporeformers. The authors presented a set of approximately 60 genes conserved in members of the *Bacilli* and *Clostridia*. Consistent with the idea that these 60 genes represent the minimum gene content for spore formation, the sporulation gene complement in SFB genomes (which were published after the comparative analysis by Galperin et al.) matches the predicted core set almost exactly. SFB genomes are quite small (1.5 to 1.6 Mb) and appear streamlined (63–65, 115); therefore, the SFB may represent a minimal, yet fully functional, sporulation program. Abecasis et al. used a bidirectional BLAST approach to identify 111 genes conserved in 90% of known sporeformers (116). The authors refined this further to a sporulation signature comprising 48 genes that they used to predict sporulation competency. With comparative genomics, the authors were able to distinguish bacteria that appeared to have recently lost the ability to sporulate. In addition, they identified 22 species that have not been observed to sporulate in culture, but yet appear to have the ability to sporulate based on the presence of complete sporulation signatures.

Another general finding of these studies is that some members of the *Firmicutes* have retained many sporu-

lation genes despite their apparent inability to form an endospore. As discussed previously in this review, *Epulopiscium* sp. type B forms multiple intracellular offspring cells using a process that is morphologically similar to sporulation. A recent study by Miller et al. used a BLAST-based approach to define and then compare the distribution of 147 highly conserved core sporulation genes in *Epulopiscium* sp. type B as well as the genome of its closest endospore-forming relative, *Cellulosilyticum lentocellum* (117). While the *C. lentocellum* genome contains 87 of the core genes, the *Epulopiscium* sp. type B genome contains 57. The conserved genes include homologs of *spo0A*, all sporulation sigma factors, and the central regulatory network that governs cell-specific transcriptional programs, as well as genes required for engulfment. Late-stage sporulation genes that confer resistance properties, such as the synthesis and forespore transport of dipicolinic acid and germinant receptors located in the *C. lentocellum* genome, were not found in *Epulopiscium* sp. type B. Surprisingly, genes that code for small acid-soluble proteins (SASPs) and their degradation, as well as cortex biosynthesis and cortex/coat scaffolds, were conserved in both *C. lentocellum* and *Epulopiscium* sp. type B. It appears that some of these late-stage functions may still be important for *Epulopiscium*. Since endospores have never been observed in *Epulopiscium* sp. type B, it is possible that the conserved cortex-associated genes may provide a specialized envelope to support the development and rapid growth of daughter cells. SASPs may be important for DNA protection or chromosome organization in developing offspring.

In general, comparative studies have confirmed that the regulatory kinase cascade upstream of Spo0A is not conserved (108), particularly not between *Bacilli* and *Clostridia*. However, Spo0A and the sporulation sigma factors (σ^H , σ^F , σ^E , σ^G , and σ^K) are universally conserved in sporeformers. In addition, regulators of these sigma factors, for example, *spoIIAA*, *spoIIAB*, and the *spoIIIA* operon, are conserved. This suggests that, despite the ways in which the sporulation pathway has diverged among different *Firmicutes* lineages, these core regulatory components are ancient and essential for development. Previous morphological observations suggested that engulfment, whether it is of a developing forespore or a nondormant offspring cell, proceeds in a very similar manner to that of *B. subtilis*, and indeed genes involved in engulfment, such as *spoIID*, *spoIIP*, *spoIIM*, and *spoIIIE*, are highly conserved among sporeformers. Finally, genes involved in spore coat production and germination are not well conserved among

endospore-forming bacteria, but this is not surprising given the size of some of these proteins and the wide range of environments in which sporeformers grow, sporulate, and germinate.

An additional outcome of these comparative genomics studies is the finding that asporogenous and nonsporeformers retain homologs to sporulation genes. As more of these strains are characterized with respect to sporulation, it will be interesting to see if these genes have retained functions similar to that of their sporulation homologs or if they have become functionally divergent. Among the nonmodel sporeformers, there are several species that can form more than two spores. Since much of the engulfment machinery is conserved, it is likely that these bacteria have found ways to either engulf forespores that then divide to produce multiple endospores (like *M. polyspora*), or to engulf at cellular locations other than at the poles, as sometimes occurs in *Epulopiscium* sp. type B cells (118). In the latter case, it is currently unknown how these cells regulate where, and how many, additional engulfment sites will occur. Comparative genomics approaches have provided a valuable framework with which to assess the potential to form a spore, and future work on nonmodel spore-forming organisms will provide insight into how sporulation genes evolve to function in diverse forms of bacterial reproduction and development.

THE VALUE OF COMPARATIVE APPROACHES

The sporulation pathway, as it has been classically characterized, results in a single, stress-resistant spore that allows a bacterium to survive unfavorable or even potentially lethal environmental conditions. However, bacteria have evolved and co-opted this pathway to produce a wide range of endospore phenotypes, including multiple endospores and nondormant intracellular offspring. Although it is clear that forming an endospore is advantageous for the survival of organisms in harsh environments, the environmental or developmental triggers that control endospore production in these more highly derived systems remain to be characterized fully. Of particular interest is how the production of more than two endospores in some bacteria, such as *M. polyspora* and *A. polyendosporus*, is regulated, especially since the number of spores produced varies within populations of cells. Furthermore, the nuances of why and how some bacteria alternate between multiple endospores or nondormant offspring have yet to be fully elucidated.

A common theme presented here is that many of these unusual developmental systems have been identified in anaerobic, gastrointestinal symbionts. Our work, for example, uses a comparative approach with closely related symbionts, and we have found that these systems provide informative contrasts when considering the impact of host-symbiont relations on the evolution of novel reproductive strategies (29, 34, 42). All of these intestinal symbionts are rather distant relatives of the *B. subtilis* model, and we know that *Clostridium* spp. use very different signals to trigger the onset of sporulation (109). Recent work on members of the *Clostridia* has reinforced previous observations that, while sporulation genes are conserved between *Clostridia* and *Bacilli*, frequently the regulation of these genes (including key sigma factors and their regulons) is different between these two groups of sporeformers (119–121). For example, in *B. subtilis*, σ^K functions exclusively late in the sporulation pathway; however, in *C. botulinum* (122) and *C. perfringens* (123), σ^K is required early in sporulation. In *C. acetobutylicum*, σ^K is active both early and late in development (124). In *C. difficile*, σ^K only has a late role in sporulation, and a *sigK* mutant in *C. difficile* can be oligosporogenous (119, 121). Together, these observations illustrate that the clean, sequential model of sigma factor activation described for *B. subtilis* does not fully represent patterns seen in the *Clostridia* (119–121). We would suggest that the deep analysis of additional spore-forming anaerobes, including genomic and transcriptomic data, would provide a more robust comparative system for generating hypotheses on triggers and modifications of the basic sporulation program.

The advent of high-throughput sequencing methods has greatly expanded the ability to characterize uncultured bacteria, novel isolates with no established system for genetic dissection, and mutations that affect development. Efforts to sequence diverse bacterial genomes are providing key insights into the conservation of genes involved in sporogenesis (6, 108). In addition, the application of high-resolution microscopy, including fluorescence and cryotomographic imaging, is providing unprecedented access to the cellular structures and processes associated with developmental progression. The application of transcriptomics, proteomics, and comparative genomics to these unconventional systems will provide insight into the initiation process and potentially identify triggers that determine alternative cell fates. Together, these efforts will provide a better understanding of the conditions that repurpose sporulation, as well as the potential diversity of form and function accommodated by this complex and ancient developmental program.

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