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Review

Compartmentalization during bacterial spore formation

Olga Iwańska, Przemysław Latoch and Agata L. Starosta

Here, we explore the recent advancements in understanding cellular compartmentalization during bacterial spore formation, primarily focusing on the model organism *Bacillus subtilis*. The hallmark of sporulation, asymmetric septation, physically separates the mother cell and forespore, enabling distinct developmental fates. We highlight the role of the asymmetric septum as an organizational hub coordinating diverse compartmentalized functions — from gene regulation to metabolism and protein synthesis machinery localization.

Asymmetric septation involves precise positioning of the division machinery, chromosome segregation, and septal pore formation. Recent studies have revealed detailed structure of the asymmetric septum and its role in maintaining compartment integrity, especially through interactions involving SpoIIE, SpoIIIE, peptidoglycan remodeling enzymes (like the SpoIIDMP complex), and the SpoIIIA-SpoIIQ channel. The asymmetric septum also plays a role in the spatiotemporal localization of ribosomes, with their entry into the forespore being coupled to septal peptidoglycan remodeling. This observation not only demonstrates translational compartmentalization during sporulation but also reveals the uncoupling of transcription and translation processes in *B. subtilis*. Moreover, the mother cell and forespore establish distinct metabolic roles, as the mother cell supplies essential metabolites to the forespore through the SpoIIIA-SpoIIQ feeding tube channel, supporting the synthesis of the spore structural components necessary for spore maturation.

Advanced imaging techniques and multi-omics approaches have significantly enhanced our understanding of compartmentalization during sporulation. We conclude by discussing future research directions, including the application of machine learning approaches, expansion of research to nonmodel bacterial species, and exploration of evolutionary aspects of compartmentalization, which may reveal universal mechanisms of microbial organization.

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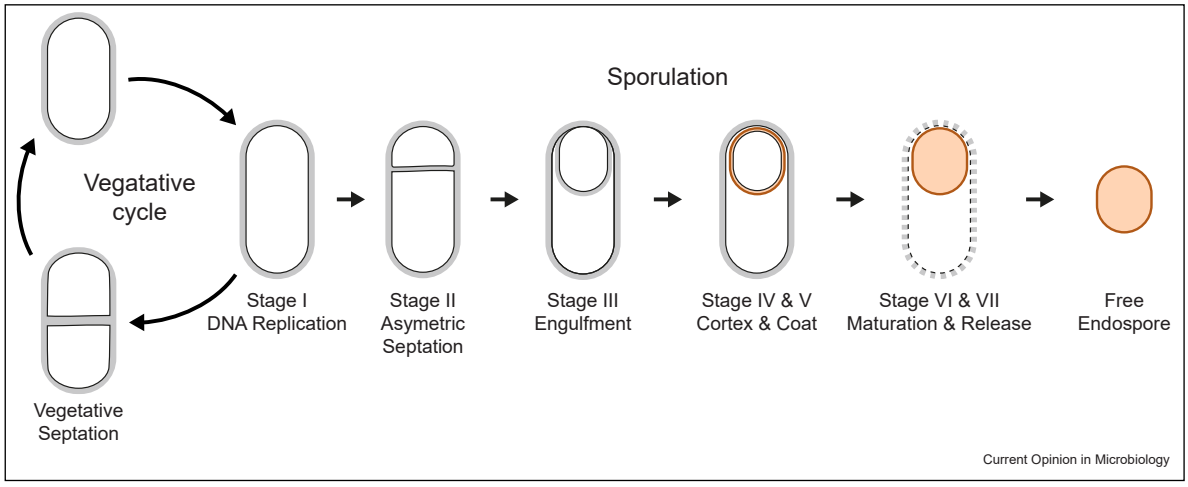
Introduction

Bacterial cells have been historically considered simply as cocktails of enzymes enclosed by rigid cell walls, with minimal, if any, subcellular organization. However, the first evidence of protein localization in a bacterial cell emerged in the early 1990s [1–3], and since then our understanding of bacterial cell organization has improved massively. Due to methodological advancements, especially in high-resolution imaging with examples, including structured illumination microscopy and cryo-electron tomography, research into the subcellular localization of protein complexes and spatiotemporal organization of prokaryotic machineries has been picking up pace since the 2000s (as described in [4–7]) and has yielded our current view of a bacterial cell as a complex, compartmentalized system capable of diverse cell differentiation/developmental programs [8,9].

One such example of bacterial cellular differentiation is sporulation. Briefly, in the process of sporulation, a vegetatively growing cell transforms into a dormant and resistant spore. Although sporulation is characteristic of several bacterial genera, including, among others, *Bacillus* [10], *Clostridium* [11], *Streptomyces* [12], and *Myxococcus* [13], it is by far the best understood in a model Gram-positive bacterium, *Bacillus subtilis*. Upon

Figure 1



The bacterial endospore formation. *Firmicutes*, including *Clostridium* and *Bacillus*, form internal endospores in response to adverse environmental conditions instead of following vegetative cell division. In both genera, sporulation is initiated by Spo0A phosphorelay regulator and comprises DNA replication (stage I), followed by asymmetric septation (stage II) that divides the cell into a larger mother cell and a smaller forespore. In stage III, engulfment, the mother cell membrane surrounds the forespore. Next, protective cortex and coat layers are synthesized around the developing spore during stages IV and V. The process culminates in stages VI & VII with the final maturation of the spore and its release through the lysis of the mother cell. The resulting dormant, highly resistant free endospore is indicated in orange.

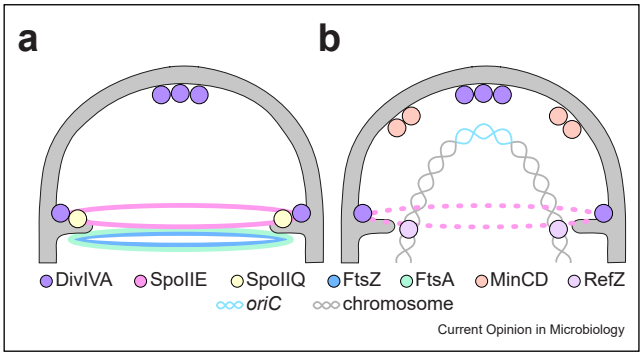
nutrient limitation, *B. subtilis* instead of undergoing binary fission can divide asymmetrically to produce a larger mother cell and a smaller forespore compartment. Next, the bacterial chromosome is translocated into the developing forespore, which is then engulfed by the mother cell to create an unusual cell-within-a-cell state — an endospore. Finally, the spore matures and is released by autolysis of the mother cell (Figure 1). This process is tightly controlled at the transcriptional level and highly trackable due to distinct morphological changes, rendering it an excellent model to study cell development and compartmentalization [10,14]. Although the process of sporulation proceeds rather differently in *Streptomyces* and *Myxococcus*, as it results in the production of exospores rather than endospores, the cause and effect remain very similar — in response to nutrient limitation both genera produce dormant and resistant spores that are produced as a consequence of initiation and maintenance of cellular compartmentalization [15,16].

Research on sporulation, especially in *B. subtilis*, has aided our understanding of bacterial cell organization. Hence, this review will focus on compartmentalization during spore formation in *B. subtilis*. Compartmentalization, which is intricately linked to the process of sporulation, serves three distinct functions in a bacterial cell and each is vital for spore formation: spatial coordination, physical separation, and segregation of function [6,10]. The first allows for directing biological processes to specific subcellular regions by establishing cellular addresses determined by protein machineries, for example, the polar localization of asymmetric division machinery guides segregation of the chromosomal DNA during sporulation. The establishment of physical boundaries through the formation of asymmetric septation is a defining feature in *B. subtilis* sporulation and enables compartment-specific gene expression programs essential for the spore formation and maturation. Finally, creating specific microenvironments, that is, a spore surrounded by proteinaceous layers, allows for establishing a dehydrated and Ca^{2+}-dipicolinic acid-rich spore with small, acid-soluble proteins protected chromosome and elaborate spore coat [17,18].

Establishing cellular addresses – divisome organization

Asymmetric septation is a hallmark of sporulation and, like any cell division in bacteria, requires cytokinesis, chromosome segregation, and membrane fission with peptidoglycan restructuring. These functions are coordinated and carried out by cell division proteins, the divisome, and chromosome translocation protein. As in the case of vegetative cell division, the sporulation division site is also marked by the Z-ring formed by the tubulin homolog FtsZ, and a localizing actin homolog FtsA, which is recognized by the remaining divisome proteins [19]. Thanks to the recent findings using cryo-FIB-ET and DNA-PAINT [20,21], the architecture of the sporulation septum has been described in much more detail, showcasing not only the structure but also the resulting function of the asymmetric septum, which is to establish two differing but connected cellular compartments — mother cell and forespore — rather than just serving as a

Figure 2



Models of divisome and chromosome segregation machineries organization in sporulation *B. subtilis*. **(a)** FtsAZ ring is localized to the mother cell side during sporulation, and SpoIIE is present exclusively on the forespore side and modulates septal thickness. DivIVA localizes to the negatively curved membranes and redeploys SpoIIE to the site of asymmetric septation. DivIVA together with SpoIIQ tether SpoIIE to the forespore membrane, ensuring septal stability and compartmentalization [20,26]. **(b)** DivIVA, MinCD, and RefZ assist in correct chromosome positioning and septum placement in the developing spore. RefZ binds to RefZ-binding motifs (RBM) on both chromosomal arms, localized near the *oriC*, and interacts with SpoIIE to indicate the position of Z-ring. DivIVA localizes the MinCD system, which inhibits aberrant Z-ring assembly too close to the cell pole [24].

divide to split the cells. The asymmetric septum is thinner than the vegetative one (approximately one-fourth of the thickness of the vegetative Z-ring) and exhibits a higher ratio of protein to membrane due to both division and sporulation-specific proteins [20,21]. The sporulation septum has also an intrinsic asymmetry to its structure resulting from the localization of septal proteins (Figure 2a). While in the vegetative septum, the FtsZ ring forms a uniform layer at the invaginating membrane, FtsZ filaments at the sporulation septum are localized only to the mother cell side. A sporulation-specific bifunctional protein SpoIIE, localized on the forespore side, regulates the septal thickness and positioning of the FtsAZ filaments to the mother cell side and interacts with FtsZ to form an E-ring [20]. SpoIIE also interacts with another Z-ring localizing protein and a divisome member, a tropomyosin homolog DivIVA [22,23] whose positioning in the sporulation septum was described in much detail recently [21]. The precise mechanism driving this segregation remains under investigation. Briefly, the proposed models include specific architecture of the developing spore — placement of Z-ring stabilizing proteins at the mother cell side and forespore geometry promoting higher DivIVA concentrations in the forespore compartment [21], and a molecular crowding mechanism suggested by Ref. [20] in which the FtsAZ filaments render DivIVA localization biased.

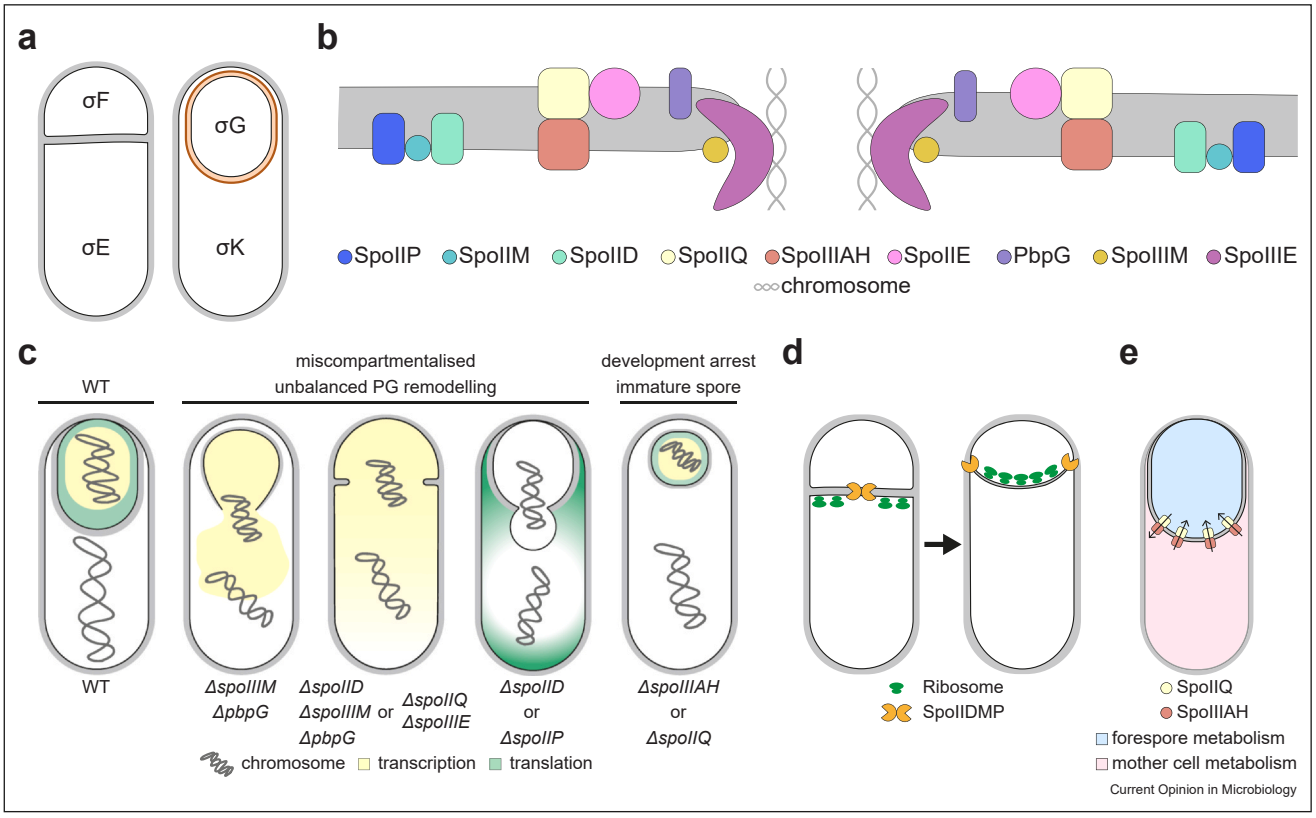
The site selection for asymmetric division has been somewhat elusive; however, recent research into sporulation septum localization revealed that two negative division regulators, RefZ and the MinCD system, play a role in precise septum positioning. RefZ, which directly interacts with SpoIIE and remains at the mother cell side, binds the DNA motifs on both chromosomal arms and determines the proportion of the chromosome that is trapped in the developing forespore (approximately one-third). By forming transient SpoIIE-RefZ-DNA complexes, RefZ appears to dictate the position of the Z-ring to one-sixth of the cell length since, in the deletion mutants, the septation is closer to the mid-cell. Once the septum position is determined by such transient complexes, RefZ dissociates and the septum forms [24]. The MinCD system, on the other hand, prevents asymmetric septation closer to the cell pole (at one-eighth and closer), which results in a small window for SpoIIE to establish its appropriate cellular address (Figure 2b) [25].

Shortly before the asymmetric septation, the chromosome must be replicated and segregated toward the two subcellular compartments. Recent research in *B. subtilis* revealed that chromatin remodeling into a so-called axial filament is accomplished by major redistribution of condensins homologs, structural maintenance of chromosomes (SMC) complexes, with the help of ParAB (Soj/Spo0J in *B. subtilis*) and upon ATP binding. The axial filament forms along the cell, with the two *oriC* regions positioned toward the cell poles, and the termini loci *ter* of the sister chromosomes colocalized together, in the mid-cell [27]. The *oriC* region can be bound by either the RacA protein or the Soj/Spo0J system, which shows redundancy, and is directed to the cell pole thanks to DivIVA, and also partly due to the driving force of the redistributed SMCs [27]. Since DivIVA is a negative membrane curvature-sensing protein and prelocalizes to the cell poles and the developing asymmetric septum, it can interact with the chromosome-binding proteins and anchor the segregating chromosomes to the opposite cell poles [28]. Once the axial filament is formed, the *oriC* delivered to the forespore pole and the Z-ring placed at the appropriate cellular address, the remaining 70% of the chromosome can be translocated into the developing spore across the septal pore. This is performed by a multifunctional, membrane-anchored DNA translocase SpoIIIE. The structure and mechanism of SpoIIIE mediated chromosome translocation and how it affects forespore geometry has been described extensively in Refs. [29–31], and summarized in Ref. [10]. However, recently SpoIIIE was also shown to maintain the septal pore after chromosome translocation, rendering it essential for cellular compartmentalization during sporulation [32], as discussed below.

Establishing physical boundary — septal remodeling-driven genetic compartmentalization

Transcription regulation during sporulation in *B. subtilis* has been described in great detail [10,14,33]. The cell-specific developmental programs are regulated by

Figure 3



Septal remodeling–driven compartmentalization of sporulating *B. subtilis*. **(a)** Compartmentalized programs of gene expression are under the control of compartment-specific RNA polymerase σ factors. The activation of each σ factor and thus, of the correct set of genes during different stages of sporulation, depends on the morphological changes to the cell. The early σ^F and σ^E become active at the time of asymmetric division, and the late σ^G and σ^K after the forespore engulfment. **(b)** Schematic of the protein machinery involved in maintenance of the asymmetric septum. SpoIIIE translocates both chromosomal arms into the developing forespore and stabilizes the septal pore together with the peptidoglycan (PG) remodeling protein SpoIIIM and PbpG. PbpG synthesizes PG, perhaps with additional PG synthesizing agents, and SpoIIIM and SpoIIIE reinforce the pore by protein–protein and protein–PG interactions [32]. SpoIIQ localizes SpoIIE to the engulfing membrane, which reinforces septal stability and is essential for compartmentalization at the onset of engulfment. The mechanism of septal maintenance by SpoIIE is hypothesized to be linked to the retention of PG synthetic machinery upon PG hydrolysis by the SpoIIDMP complex [26]. The forespore protein SpoIIQ and mother cell protein SpoIIIAH form a bridge that spans both membranes and holds them together, acting as a membrane zipper during engulfment. The PG hydrolases in the SpoIIDMP complex mediate forespore engulfment by partially degrading PG and allowing membrane migration [37]. **(c)** Phenotypes of miscompartmentalization during sporulation in *B. subtilis* resulting from deletions of different components of the asymmetric septum maintenance machinery. Upon deletion of PG synthesis machinery and septal pore stabilization proteins, the pore enlarges, which results in forespore chromosome efflux and transcriptional miscompartmentalization (yellow) [32]. When the PG hydrolyzing machinery is deleted, a thick asymmetric septum prevents membrane migration and engulfment and also, ribosome translocation into the developing spore resulting in translational miscompartmentalization (green). Upon deletion of the SpoIIIAH-SpoIIQ zipper, the cell retains both transcriptional and translational compartmentalization, however, the spore is at a developmental arrest and does not mature [38]. **(d)** SpoIIDMP-driven peptidoglycan rearrangement is crucial for ribosomes packing into the forespore. After the asymmetric septation, most of the ribosomes wait at the asymmetric septum at the mother cell side where they remain translationally active. After the chromosome translocation ribosomes are translocated into the developing spore and this transfer is hypothesised to be mediated by mother cell membrane or cell wall proteins, possibly via the septal pore [38]. **(e)** Differentiated metabolic compartments during sporulation — forespore (light blue) prioritizes the biosynthesis of structural components, whereas mother cell (pink) focuses on energy and amino acid production. The feeding tube channel comprised of SpoIIIAA-SpoIIIAH and SpoIIQ proteins functions in metabolic differentiation and nurtures the developing spore by providing small molecules needed for biosynthetic activity, including amino acids, NTPs and possibly high energy intermediates as source of ATP, while exporting low-energy products out of the forespore [39,40].

compartment-specific RNA polymerase sigma factors: early sporulation σ^F and σ^E in the forespore and mother cell, respectively, followed by σ^K and σ^G, which are switched on after engulfment and are responsible for expression of genes required for spore maturation and mother cell lysis (Figure 3a). Thus, each sigma factor regulon is expressed in a different cellular compartment at a different time during sporulation. The establishment of a physical boundary — the asymmetric septum — is a defining feature in this compartmentalized gene expression. The asymmetric septum functions as the organizational hub for sporulation machinery in both

cellular compartments as in the forespore membrane protein insertion occurs exclusively at the septal interface, while in the mother cell proteins undergo redistribution to the septum [34]. Recent research showed that the cell division and remodeling machineries take part in compartment specific gene expression regulation, demonstrating their function in maintaining cytoplasmic, genetic, and regulatory compartmentalization during sporulation and highlighting the role of the asymmetric septum as an organizational and regulatory hub. This leads to the emerging view that the septal proteins both in the mother cell and the forespore, rather than operating in isolation, form interconnected molecular mechanisms penetrating the thin peptidoglycan of the asymmetric septum, to ensure transcriptional and cytoplasmic compartmentalization during sporulation.

Beyond its structural function in asymmetric septum formation, SpoIIE plays an essential role in gene expression regulation due to its phosphatase activity. SpoIIE dephosphorylates the anti-anti-sigma factor SpoIIAA, which in turn disrupts binding of the anti-sigma factor SpoIIAB, consequently activating σ^F [14]. The preferential action of SpoIIE in the forespore compartment in early sporulation was recently shown to be associated with the scaffolding protein DivIVA. DivIVA, which localizes specifically to the cell pole and the forespore septal side, directly interacts with SpoIIE which results in SpoIIE tethering to the forespore septal membrane and compartment specific σ^F activation. Interestingly, a DivIVA variant, which failed to localize to the forespore, resulted in miscompartmentalized SpoIIE and hence, activation of σ^F in both the forespore and the mother cell [35]. Once the σ^F is activated, the engulfment commences, creating a membrane and peptidoglycan-bound forespore compartment in the mother cell. SpoIIE relocalizes to the engulfing membranes, with the aid of SpoIIQ, a forespore structural protein, and interacts with the forespore peptidoglycan synthesizing machinery. Thanks to this structural function, SpoIIE contributes to septal stabilization and compartmentalization during engulfment (Figure 3b) [26].

SpoIIIE interaction with peptidoglycan synthesis proteins recently described by Ref. [32] integrates chromosome translocation with peptidoglycan remodeling, which resulted in a proposal of a highly stabilized septal pore model (Figure 3b). Mohamed *et al.* proposed that SpoIIIE is anchored at the edge of the septal pore, and apart from its function in DNA translocation, it also maintains the size and integrity of the septal pore by interacting with PbpG, a forespore peptidoglycan synthase, and SpoIIIM, a mother cell peptidoglycan-binding protein. In the absence of these proteins, SpoIIIE can still translocate the chromosome into the forespore, but the chromosome fails to remain there, leading to chromosome efflux. This suggests that PbpG and SpoIIIM are critical for maintaining pore integrity after chromosome translocation and therefore prevent miscompartmentalization (Figure 3c). The stabilized septal pore model also includes the action of mother cell SpoIIIAH and the forespore SpoIIQ proteins, suggesting that maintenance of the septal pore depends on protein–protein interactions across the septum, as well as peptidoglycan synthesis [36]. SpoIIIAH-SpoIIQ form a molecular bridge connecting mother cell and forespore membranes and act as a zipper (or ratchet) facilitating forespore engulfment [18] (Figure 3b). Interestingly, when both stabilization mechanisms are missing, the asymmetric septum retracts, abolishing compartmentalization and spore development (Figure 3c) [32]. This is in concert with peptidoglycan dissolution performed by the SpoIIDMP complex, which is essential for peptidoglycan remodeling and successful forespore engulfment [36]. SpoIID and SpoIIP are peptidoglycan hydrolyzing enzymes and SpoIIM localizes the complex to the septum — initially to the middle of the polar septum and then to the leading edges of the engulfing mother cell membrane (Figure 3b). The SpoIIDMP complex, while degrading peptidoglycan and making room for the engulfing membrane, was also proposed to tether the membrane to the peptidoglycan and enable the formation of finger-like membrane projections, which advance forespore engulfment [37].

The collective action of membrane proteins, peptidoglycan remodeling enzymes, and septal pore stabilizing factors thus creates and maintains the distinct compartments essential for the proper execution of the sporulation developmental program.

Creating specific microenvironments — translational and metabolic compartmentalization

In bacteria, translation and transcription have been considered coupled for years, and thus, mRNA localization has been used extensively to describe translation localization [5]. However, for over two decades now, increasing evidence has been pointing to translation being uncoupled to transcription in several bacterial species, especially *B. subtilis*, and this conclusion has been emerging from studies employing different methodologies, including fluorescence microscopy [41], genetic and kinetics studies [42], or microscopic and biochemical methods used to investigate RNA localization [43]. In the sporulating bacterium *B. subtilis*, transcription and translation are in fact uncoupled, and the translational machinery presents its own and very distinct spatiotemporal localization, especially during sporulation. The asymmetric septation and the subsequent engulfment play leading roles in this, creating specific microenvironments affecting localization of translation and the translational machinery. It was recently shown

that the ribosomes are mostly excluded from the site of asymmetric division and are only translocated into the forespore once the asymmetric septum is established and the chromosome is translocated, that is, once the spore compartment is created. Such orchestrated ribosomal transport to the developing spore depends on the peptidoglycan remodeling of the asymmetric septum performed by SpoIIDMP, which accompanies engulfment, perhaps through the still open septal pore. The developing spore does not synthesize ribosomes *de novo* but rather, inherits them from the mother cell, and such directional ribosomal transport appears to be in fact required for successful sporulation [38] (Figure3c,d). Moreover, during asymmetric division, the ribosomes are translationally active at the septum highlighting the organizational and regulatory role of the asymmetric septum [38]. Interestingly, by employing RNA-seq and ribosome profiling coupled to fluorescent microscopy assays tracking protein synthesis, entry into the sporulation program was recently shown to coincide with a translational silencing event and major redistribution of the translational machinery within the cell. This further emphasizes both spatial and temporal organization of translation and ribosomes during sporulation in *B. subtilis* [44].

The developing spore constitutes a specific microenvironment not only in terms of translational capacity but, according to the emerging research, also metabolism. SpoIIIAH-SpoIIQ proteins, apart from acting as a membrane zipper during engulfment, assemble the feeding tube channel proteins of the *spoIIIA* operon, SpoIIIAA-SpoIIIAH, which form a gap junction–like channel connecting the mother cell and the forespore and are essential for late sporulation gene expression [45,46]. The structure of the feeding tube channel implied its function during sporulation, and this was demonstrated recently *in vivo*, showing that indeed, the mother cell and the engulfed forespore are metabolically differentiated [39]. Moreover, the developing spore shuts down central and intermediary metabolism including gluconeogenesis, the TCA cycle, and amino acid synthesis by means of proteolysis, and becomes dependent on the mother cell to supply building blocks essential for completion of spore maturation [47,48]. This concept has also been investigated in a latest thought-provoking study using multicell metabolic and gene expression models. Tibocha-Bonilla *et al.* [40] described the metabolic mechanisms for energy production and nucleotide synthesis during sporulation, showing that guanosine-, cytidine-, and uridine triphosphate, respectively synthesizing enzymes are only essential in the mother cell and that these NTPs are transported to the forespore via the feeding tube channel. Moreover, they suggested a sporulation ATP cycle in which high-energy metabolites are supplied to the forespore, which carries out glycolysis to produce ATP and low-energy metabolites, which then shuttle back to the mother cell, thus placing the metabolic burden of energy production on the mother cell (Figure 3d). In summary, the two microenvironments carry out different metabolic processes with the mother cell focused on energy and amino acid production, while the forespore prioritizes the biosynthesis of structural components of the spore coat.

Summary and outlook

The growing body of research has revealed that compartmentalization during sporulation in *B. subtilis* represents a dynamic process involving the coordinated action of multiple molecular machineries. Thanks to technological advancements, we now possess the means to study bacterial compartmentalization in unprecedented detail, overcoming the challenges posed by the small size and curved form of bacterial cells. The field has also progressed due to the integration of multiomics data, encompassing transcriptomics, translatomics, proteomics, and metabolomics, which, together with imaging techniques, facilitated the discovery of novel components involved in sporulation compartmentalization, and revealed new functions of previously identified factors.

Since machine learning is being increasingly applied, we envision that it will also advance our understanding of bacterial compartmentalization, as attempts in the field of sporulation have already been made [49]. Machine learning may be especially interesting in the context of understanding the evolutionary aspects of compartmentalization in terms of both the origins of compartmentalization in sporulation, and bacterial compartmentalization and spore development as precursors of eukaryotic organelles. Moreover, expanding the research beyond the model organism *B. subtilis* to nonmodel bacterial species represents a particularly important frontier that may reveal universal mechanisms of microbial compartmentalization.

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Author Contribution

OI: Writing – original draft. PL: Visualization. AS: Writing – review & editing, Funding acquisition.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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- • of special interest
- •• of outstanding interest

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