Research Article

Microbiology & Infectious Diseases

Spo0A, as the Master Regulator of Multicellularity in *B. subtilis*

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Received: 08 December 2017; **Accepted:** 03 January 2018

Citation: Veronica Donato. Spo0A, as the Master Regulator of Multicellularity in *B. subtilis*. Microbiol Infect Dis. 2018; 2(1): 1-10.

ABSTRACT

B. subtilis is a master of differentiation as it has the ability to display a multitude of distinct cell types. The advantage of a heterogeneous population in the bacterial community can be easily postulated for a better adaptation to unexpected environmental fluctuations. The maintenance of different cell types allows for simultaneous expression of different metabolic pathways with a minimal cost of energy; the division of labor also permits the high optimization of resources. This cooperative community is under the control of the regulatory protein, Spo0A, which governs spore formation, biofilm formation, motility, cannibalism and is also required for competence and lipid synthesis. Here, I revisit all the processes that are under the control of this master regulator.

Keywords

Spo0A, *Bacillus subtilis*, Cell cycle, Chromosome, DNA.

Introduction

Bacterial communities survive in their natural habitats as a result of their ability to perceive environmental changes and respond to them. To do this, bacteria have evolved complex sensing systems to monitor fluctuations in external signals. These signals might be environmental, such as temperature changes or starvation, but they can be derived from neighboring microorganisms in the form of secreted small molecule natural products. These molecules serve as signals to activate distinct pathways and trigger changes in gene expression that allow bacteria to adapt to new scenarios. If the signaling molecules are self-produced, this is known as quorum sensing [1,2]. What is more, *Bacillus subtilis* responds to different environmental signals by differentiating into subpopulations of specialized cell types. The coexistence of numerous cell types implies the presence of a variety of extracellular signals. Furthermore, each subpopulation must have the ability to sense one particular signal and discard the rest. For this purpose, *B. subtilis* possesses at least three different master regulators Spo0A, DegU and ComA that coordinate the activation and regulation of the developmental programs that result in distinct cell types [3]. The phosphorylated form of each master regulator activates the expression of a subset of genes required for the differentiation into a specific cell lineage, while it might also cause inhibition of the other developmental cascades [3].

Extensive research has resulted in a detailed molecular characterization of the physiology of several distinct cell types in dispersed cultures. For example, at the onset of the stationary phase, *B. subtilis* can differentiate into competent cells capable of taking up DNA from the environment [4] or, it can differentiate into dormant spores that are highly resistant to external [5]. Additionally, a subset of cells can produce an extracellular 'killing factor' and toxin that functions to kill cannibalistic cells that have not yet begun sporulation, thereby allowing the cannibalistic cells to delay their own commitment to enter into the sporulation pathway [6]. A fourth cell type is observed during biofilm formation when a portion of the population produces extracellular matrix material that holds cells together [7]. Differentiation has also been observed in cells growing exponentially. Only a fraction of cells express sigD, the sigma factor necessary for flagellar production resulting in heterogeneity in motility [8]. In this review, I have compiled all these mechanisms that are under the control of Spo0A and I have highlighted the most important points of them, describing and presenting a personal interpretation as well as questions remaining for futures studies.

Spo0A as a new player in chromosome replication control

Recently, master transcriptional regulators were demostrated to be involved in controlling chromosome replication as well as other

cellular processes [9].

Bacillus subtilis is widely used as a model organism for studying cell cycle progression and cellular differentiation. Under growth promoting conditions, *B. subtilis* divides symmetrically, giving rise to two identical cell types. When exposed to stress a subpopulation of cells initiates sporulation. Sporulation is a costly and time-consuming process resulting in the development of two different cell types: the larger mother cell and the smaller forespore, both cell types inherit a single copy of the chromosome during sporulation. A strong coordination between DNA replication and sporulation results in correct chromosome copy number, and is a requirement for efficient sporulation [9,10]. So far, two checkpoint factors have been identified that play a role in this: SirA (sporulation inhibitor of replication A) and Sda (suppressor of dnaA1). SirA prevents initiation of DNA replication in cells committed to sporulation. Transcription of sirA is directly activated by Spo0A-P. SirA maintains the diploid state of sporulating cells by directly targeting the DNA replication initiator protein DnaA which consequently becomes displaced from the origin of replication (oriC) [11]. While SirA prevents re-initiation of DNA replication in sporulating cells, the checkpoint function of Sda is to prevent sporulation initiation in replicating cells and during DNA repair [12]. The intrinsically unstable Sda protein binds to the major sporulation kinases (KinA and KinB) and prevents their autophosphorylation [13]. KinA and KinB are proteins of the phosphorelay, which regulates the level of phosphorylated Spo0A (the transcriptionally active, DNA-binding form of Spo0A) [14].

Interestingly, pulses of Sda synthesis occur concomitantly with the initiation of DNA replication via transcriptional activation by DnaA. Following the burst of Sda expression, targeted proteolysis of Sda reduces its concentration to generate a window of opportunity at the end of the replication cycle during which cells can enter sporulation as diploids. Cells that do not enter sporulation during that small time period have to undergo a new round of replication before getting the next opportunity to do so [15]. In order to initiate DNA replication, DnaA binds various perfect and imperfect DnaA-boxes (aA-boxes) present within the oriC region where it assembles into a large nucleoprotein complex. This results in denaturation of an AT-rich region at oriC, which is required for the assembly of a functional replisome [16]. Interestingly, Castilla-Llorente et al. [17] noted that the aA-box sequence (consensus 5′-TGTGNATAA-3′) partially overlaps with the recognition sequence for Spo0A (the 0A-box, consensus 5′-TGTCGAA-3′) [18,19], and indeed the *B. subtilis* origin region contains many Spo0A binding sites [19]. However, it remains unclear whether the binding of Spo0A at the *B. subtilis* origin is physiologically relevant for *B. subtilis*, as studies performed to show a possible direct effect of Spo0A on DNA replication have not led to conclusive results.

Moreover, it was shown that Spo0A can inhibit Dna-dependent DNA duplex unwinding in vitro [19]. On the other hand, Spo0A does not seem to have a significant effect on DNA replication in vivo when sporulation is artificially induced in exponentially growing cells [20]. Spo0A-P also reaches high levels in the mother cell [21] and recently the Piggot lab showed that Spo0A-P inhibits mother cell growth and DNA replication independently of SirA. The molecular mechanism for this Spo0A-dependent repression of replication is yet unknown, although a possible role for Sda in this has been suggested [22]. Spo0A directly controls chromosome copy number by binding to a number of specific Spo0A binding sites present within the oriC region and that this regulation is especially important in the absence of SirA and Sda or when sporulation is induced in actively replicating cells.

I think that an unknown factors also contribute to the coordination of DNA replication with sporulation. For *B. subtilis* it is known that the nutritional status also controls replication. This occurs by the production of the small nucleotides ppGpp and pppGpp upon nutrient starvation. These so-called ‘alarmones’ directly inhibit primase, an essential component of the replication machinery [23]. ppGpp inhibits IMP dehydrogenase and hence the synthesis of GTP [24]. As a result, the precursor inosine monophosphate is available for AMP synthesis and ATP increases in concentration as the amount of GTP decreases. These considerations led to a model in which starvation causes the accumulation of ppGpp, a decrease in the GTP pool and thus the initiation of sporulation [25]. Although the mechanism by which a decrease in GTP triggers spore development is not known, it has been suggested that decreased GTP results in the relief of spo0A repression by CodY, which requires GTP as a co-repressor [26]. This control might indirectly be responsible for correct copy number control during sporulation. Whether this or other mechanisms are at play remains to be investigated.

Phosphorelay and regulatory mechanisms

The Gram-positive bacterium *Bacillus subtilis* has the capacity to decide among a variety of cell fates at the end of the exponential phase of growth. The decision process is under the control of the regulatory protein Spo0A, which governs spore formation, biofilm formation, and cannibalism and is also required for competence [27] and lipid synthesis [28]. The activity of Spo0A is controlled by phosphorylation via a multicomponent phosphorelay, at the head of which are five histidine kinases (KinA to KinE) [29]. Discrimination among alternative cell fates is determined in part by the cellular levels of phosphorylated Spo0A (Spo0A-P), with the regulatory sites for genes with moderate to high affinity for Spo0A-P (e.g., genes involved in cannibalism and biofilm formation) firing at low to intermediate levels of the phosphoprotein and those with low affinity being turned on only at high levels [30]. Phosphorylation induces a conformational change in Spo0A, allowing it to dimerize and bind to target sequences [31].

More than 100 genes are under the direct positive or negative control of Spo0A-P [32]. Genes activated by Spo0A-P include those with promoters recognized by RNA polymerase containing the housekeeping sigma factor σA, as well as genes whose promoters are recognized by RNA polymerase containing the alternative sigma factor σH (including the Ps promoter for spo0A) [32]. The target sites reveal a consensus binding sequence, TTTGTCRAA, which

is known as the 0A box [33]. Spo0A is maintained at relatively high levels (aprox. 2,000 molecules/cell) during exponential phase, rapidly rising to even higher levels (aprox 20,000 molecules/cell) under sporulation-inducing conditions [34]. Losick and Chastanett [35] have attributed the high maintenance level of Spo0A and the rapid increase to yet higher levels to a just-in-time regulatory system that ensures that Spo0A molecules do not become rate limiting at both low and high rates of flux of phosphoryl groups through the phosphorelay. The spo0A gene is transcribed from two promoters, whose start sites are located 204 and 45 bp upstream from the start codon for the open reading frame [36]. The more upstream promoter, Pv, is expressed during the exponential phase of growth under the control of σA -RNA polymerase. This transcription fluctuates strikingly and in a manner that correlates with small changes in the growth rate [37] but then shuts off during the transition to stationary phase [38]. The downstream promoter, Ps, in contrast, is induced after the end of exponential-phase growth under the control of σH-RNA polymerase and Spo0A-P. Thus, transcription of spo0A switches from Pv to Ps as cells exit exponential-phase growth [39]. Transcription from Ps is required in order for Spo0A to reach the high levels needed for the activation of key sporulation genes, such as spoIIA, spoIIE, and spoIIG, but not for the low levels required for efficient entry into competence [40]. Insights into the mechanism of promoter switching has come from the work of Strauch and coworkers, who showed that it is mediated by Spo0A-P and that the protein binds three 0A boxes in the regulatory región [41].

Losick and Chastanet [35] report the discovery of a fourth 0A box that plays a key role in promoter switching and other features of the regulatory region that are conserved among multiple Bacillus spp. They refer to the four 0A boxes as O1, O2, O3, and O4 in the reverse order of their distance from the open reading frame (renaming them to accommodate the newly identified site O2). Their principal findings are that O1 is a negatively acting element that is responsible for repressing Pv at the end of exponential phase, that the newly identified O2 site is a negatively acting element that represses Ps during growth, that O3 is a positively acting element that activates Ps upon entry into stationary phase, and that O4 is dispensable. They also report the discovery of a translational control mechanism that impedes translation of mRNAs originating from Pv but not that originating from Ps. Moreover, they also show that Pv provides a basal level of Spo0A that is required for efficient entry into the state of genetic competence and strong activation of Ps, and they suggest that Pv plays a pump-priming role in the activation of the Spo0A-P-controlled promoter. An intricate regulatory region mediates a just-in-time regulatory system that helps to ensure an adequate supply of Spo0A molecules to meet the needs of the phosphorelay. They propose a pump-priming model for the regulation of spo0A that involves transcriptional, translational, and posttranslational mechanisms. At the heart of the model is promoter switching from the vegetative promoter Pv to the σH -controlled, Spo0A-P-dependent promoter Ps during the transition to stationary phase and the 0A boxes O1, O2, and O3. They propose that early during the exponential phase of growth, synthesis of Spo0A is principally if not exclusively driven by Pv. This synthesis is maintained at a relatively low level by a translational control mechanism that sequesters the start codon and ribosome-binding site for spo0A in the secondary structure of transcripts originating from the upstream promoter. Meanwhile, the downstream promoter, Ps, is silent due to the absence of both Spo0A-P and σH. Then, at the midexponential phase of growth, the gene (sigH) for σH is derepressed [42] and Spo0A-P begins to accumulate due to activation of kinases at the head of the phosphorelay other than KinA [43].

Nonetheless, Spo0A synthesis continues to be maintained at a basal (although significant) level due to repression of Ps via O2. Finally, during the transition to stationary phase, increasing levels of KinA lead to a surge in Spo0A-P levels [44], overpowering O2-mediated repression of Ps and activating Ps via the binding of Spo0A-P to O3. Meanwhile, the binding of Spo0A-P to O1 represses Pv, effecting the switch from the vegetative to the sporulation promoter. The switch results in yet higher rates of Spo0A synthesis as a consequence of unimpeded translation from mRNAs originating from the downstream promoter. Thus, Pv primes the pump by maintaining a pool of Spo0A molecules in growing cells, which upon phosphorylation activates Ps, leading to yet higher levels of Spo0A and downregulation of Pv. In summary, they propose that Pv and O2 maintain Spo0A at a high, basal level (aprox. 2,000 molecules/cell) during growth. As a result, the cells are poised to respond rapidly to signals triggering activation of the phosphorelay. Phosphorylation of Spo0A as a result of flux through the relay sets up a selfreinforcing cycle that rapidly amplifies Spo0A production to extremely high levels (aprox. 20,000 molecules/cell), preventing Spo0A from becoming limiting for the accumulation of Spo0A-P. Whereas the basal level of Spo0A may be relatively constant from cell to cell (as we propose), Spo0A-P levels vary considerably from cell to cell at the start of sporulation [34]. This heterogeneity likely originates from noise in the phosphorelay and is the basis for the diversification of cell types during the transition to stationary phase, resulting in cannibals, biofilm formers, and spore formers [45].

Spo0A and cell differentiation

The developmental pathways that lead to the differentiation of the numerous coexisting subpopulations in *B. subtilis* are triggered by the phosphorylation of three master regulators: DegU, ComA and Spo0A [1]. Phosphorylation of Spo0A, Spo0A-P, activates the cascades towards matrix production and cannibalism when there are low levels of phosphorylated protein in the cell, and when there are high levels of Spo0A-P, the sporulation genes are induced [46]. Activation of these master regulators by phosphorylation is mediated by the action of distinct sensor kinases. Spo0A is phosphorylated by the action of five different kinases, KinA, B, C, D and E [47]. KinA and B are necessary for sporulation while KinC, D and E are important for matrix production and biofilm formation [48].

The action of the master regulators is also controlled by dephosphorylation, which counteracts the action of the kinases. Dephosphorylation is driven by 11 inhibitory proteins termed Rap

(response-regulator aspartyl-phosphatases), which, directly or indirectly, accelerate the dephosphorylation of the phosphorylated version of the respective master regulator. Some of these phosphatases also block the activation of the master regulator by binding to the master regulator and interfering with DNA binding (RapC, F and G) [49]. One of these Rap proteins, RapA, has been shown to be essential for maintaining the bistable expression of Spo0A [50,51].

Spo0A and sporulation

While spores and matrix producers are regulated by Spo0A, they are quite distinct cell types. What dictates the cell fate of a matrix producer or a spore? Spo0A regulates different genes when it is present at different levels. A high threshold level of Spo0A is needed to trigger sporulation and a lower threshold of the regulator is needed to induce sinI expression and derepress matrix genes [52].

Spores are metabolically inactive cells that compartmentalize DNA and essential proteins to allow germination once conditions have improved [53]. *Bacillus subtilis* sporulates in response to environmentally harsh conditions such as nutrient or oxygen deprivation. It is believed that *B. subtilis* senses starvation by measuring the intracellular concentration of key metabolites, such as ATP, GTP or charged tRNAs needed to build proteins. When the cytoplasmic concentration of these effectors decreases abruptly, it stimulates the activation of the master regulator Spo0A-P. High levels of Spo0A-P activate the expression of genes required for sporulation [52]. The cytoplasmic sensor kinase KinA, the most important kinase in *B. subtilis*, responds to starvation by phosphorylating the master regulator Spo0A-P and inducing the process of sporulation. KinA possesses three distinct PAS–PAC sensor domains (PAS-A, PAS-B and PAS-C).

PAS domains have been proposed to monitor changes in light, redox potential, oxygen, small ligands and intracellular energy level [54]. Hence, KinA might use these PAS sensor domains to sense fluctuations in the cytoplasmic concentration of the key metabolites to induce sporulation. This hypothesis remains to be tested, as the signals sensed by the PAS domains of KinA are unknown. While it is known that ATP binds to the sensor domain PAS-A, it might not serve as a signal but rather as a phosphate source for the phosphorylation of the kinase [55]. In addition, it was recently demonstrated that this PAS-A sensor domain is not required to induce the kinase activity of KinA in vivo [1,56], supporting that the binding of ATP to the domain might not constitute a signal. A second line of response to starvation is the DNAbinding repressor CodY, which monitors the intracelular levels of GTP. In excess of nutrients, GTP is produced at high levels. GTP binds and activates the repressor CodY, which inhibits the expression of the spo0A gene. Consequently, depletion of GTP during starvation causes the inactivation of CodY, thus licensing cells to initiate sporulation by allowing spo0A transcription [1,57].

The decrease in intracellular energy levels caused by the scarcity of nutrients also affects the synthesis of amino acids required to build the proteins. Under these conditions uncharged tRNAs bind the ribosome and translation is reduced. Consequently, the energy (GTP) intended for protein synthesis is redirected to the formation of the secondary messenger guanosine tetraphosphate (ppGpp) also termed an alarmone. The synthesis of the alarmone is catalyzed by a small ribosome-associated protein, RelA [58]. The alarmone is a stress indicator that triggers a broad response to minimize damage to cells. Mutants unable to produce the alarmone (DrelA mutants) are defective in sporulation [59,60]. It is proposed that the sporulation defect in a DrelA mutant is not due to the absence of ppGpp itself, but due to the increase of energy levels (GTP) that accumulate when ppGpp cannot be produced [59,60]. Consistent with this hypothesis, sporulation in the DrelA mutant can be recovered using GMP synthase inhibitors that block GTP production [59]. Whether the inhibition of sporulation in the relA mutant due to an increase of GTP levels occurs in a CodY-dependent manner remains to be clarified. While the expression of some CodY-regulated genes has been shown to be compromised in the DrelA-deficient strain [61], other indications suggest that an alternative CodY-independent pathway might possibly regulate the expression of those CodY regulated genes [62]. Because the production of ppGpp triggers the stress response pathway in many different bacteria [63,64], it makes sense that it also serves as a general factor to initiate sporulation in other bacteria in addition to *B. subtilis*. However, *B. subtilis* can also sporulate in response to other extracellular cues that are not related to nutrient deprivation. For instance, the membrane sensor kinase KinB induces sporulation when bound to the membrane associated lipoprotein KapB (kinase-associated protein B); yet, the precise signal that triggers the formation of the complex KinB–KapB remains to be elucidated [65].

Sporulation is also controlled in a quorum-sensing-dependent manner. *Bacillus subtilis* secretes a pentapeptide, PhrA. When the extracellular concentration of PhrA increases, the peptide is imported into the cell and binds to the phosphatase RapA. The interaction between PhrA and RapA inhibits the activity of the phosphatase, which indirectly targets the master regulator Spo0A-P [66]. Hence, inhibition of RapA by the formation of the PhrA–RapA complex stabilizes the active form of Spo0A-P and favors sporulation [66]. This PhrA–RapA quorum-sensing system works redundantly with other similar systems such as PhrC–RapB and PhrE–RapE. All function to stabilize the phosphorylated form of the master regulator Spo0A. Thus, each of these extracelular peptides favors sporulation [66].

In 2013, Ben-Yehuda et al. [67] discovered that under nutrient deprivation, *Bacillus subtilis* initiates the developmental process of sporulation by integrating environmental and extracellular signals. These signals are channeled into a phosphorelay ultimately activating the key transcriptional regulator of sporulation, Spo0A. Subsequently, phosphorylated Spo0A regulates the expression of genes required for sporulation to initiate. They identified a group of genes whose transcription levels are controlled by Spo0A during exponential growth. Among them, three upregulated genes, termed sivA, sivB (bslA), and sivC, encode factors found

to inhibit Spo0A activation. Furthermore they show that the Siv factors operate by reducing the activity of histidine kinases located at the top of the sporulation phosphorelay, thereby decreasing Spo0A phosphorylation. Thus, they demonstrate the existence of modulators, positively controlled by Spo0A, which inhibit inappropriate entry into the costly process of sporulation, when conditions are favorable for exponential growth.

Finally, sporulation in *B. subtilis* can also be triggered in response to natural products derived from other soil microorganisms. For instance, the soil genus Streptomyces produces a plethora of antimicrobials, some of which act against Bacillus species. As both these bacteria live in the soil, it is plausible that *B. subtilis* might have developed a 'tune sensing' mechanism to recognize the presence of Streptomyces, by detecting its secondary metabolites. Among these are decoyinine or angustmycins, both GMP synthase inhibitors that decrease the intracellular energy levels in *B. subtilis* by inhibition of GTP synthesis. Thus, in the presence of decoyinine, *B. subtilis* senses a decrease in energy availability and triggers sporulation [66]. Sporulation preserves *B. subtilis* in a dormant state until the other possible antimicrobials produced by Streptomyces have dissipated and *B. subtilis* can grow again.

Spo0A and biofilm formation

Under certain conditions, *B. subtilis* forms multicelular aggregates known as biofilms [68]. The hallmark of biofilm formation is the production of an extracellular matrix that holds cells together [46,68,69]. Matrix-producing cells specialize to express the protein machinery to secrete the two main components of the matrix: the extracellular polysaccharide (EPS) and the structural matrix-associated protein TasA. Expression of both EPS and TasA is simultaneously triggered by low levels of Spo0A-P [46,69]. Low levels of Spo0A-P in the cell are reached by the action of two membrane-bound sensor histidine kinases: KinC and D [46,69]. KinD is a canonical membrane kinase with two transmembrane segments connected by a 211 amino acid extracellular sensing domain that is presumably involved in signal recognition and binding to a specific extracelular signal. Given the molecular structure of the kinase, it seems feasible that this kinase might serve to sense a specific extracellular small molecule, such as a peptide or a carbohydrate, via direct binding to the large extracellular sensing domain. Unfortunately, the nature of the signal and the mechanism of kinase activation remain to be elucidated. In contrast to KinD, the membrane kinase KinC harbors two transmembrane segments with no extracellular sensor domain. Instead, KinC has a PAS–PAC sensor domain in in the cytoplasmic region of the kinase.

We recently described that the PAS–PAC sensor domain of KinC somehow senses the leakage of cytoplasmic potassium cations. Diverse small molecules that are able to form pores in the membrane of the bacterium can induce this potassium leakage. This triggers the phosphorylation of Spo0A-P, which leads to matrix production [70]. Because of the nature of the stimulus, the various small molecules identified that induce matrix production via KinC differ vastly in their molecular structure. The only property they share is their ability to cause potassium leakage by making pores in the membrane of *B. subtilis*. Among these molecules are the macrolide polyenes nystatin and amphotericin as well as the peptide antibiotics gram gramidicin and valinomycin, which are all produced by soildwelling bacteria. However, perhaps the most important small molecule described to trigger matrix production via KinC is the self-generated lipopeptide, surfactin [70]. Once produced, surfactin causes the leakage of potassium with the formation of pores in the membrane [71], and that is sensed as an autoinducer signal to trigger the subpopulation of matrix producers to differentiate. Recognizing the mode of action of a signaling molecule rather than its structure is a remarkable strategy to allow promiscuous sensing of a large number of signals. This mechanism allows *B. subtilis* to respond not only to self-produced molecules but also to natural products secreted by other soil-dwelling organisms. The production of the quorum-sensing molecule surfactin is tightly regulated by another quorum-sensing pathway that ultimately controls ComA phosphorylation [72]. This sequential quorum-sensing system might serve as a timing mechanism to regulate the activation of diverse metabolic pathways sequentially during the course of development. The initiation of the sequence starts with the production of the peptide pheromone ComX ComX is sensed by the membrane kinase ComP that phosphorylates the response regulator ComA. ComA-P activates the expression of the operon responsible for surfactin production [73]. Only after ComX is sensed and surfactin is produced can surfactin go on to trigger matrix production via activation of KinC [74-76].

Most likely, the activation of ComA is subject to some sort of bimodal regulation because only a subpopulation of cells sense ComX and becomes surfactin producers [74-76]. Furthermore, the subpopulation of surfactin producers is different from the subpopulation of cells that respond to surfactin (matrix producers). Therefore, surfactin acts as a unidirectional signal in which one population produces the molecule and another population responds to it by producing extracellular matrix [74-76]. This mechanism adds more sophistication to the previous concept of 'quorum sensing or 'autocrine', where all cells are physiologically similar and thus able to produce the signal and respond to it [77]. In the case of surfactin, the signaling can be referred to as paracrine signaling because there is a producing cell that is distinct from the nearby cell that can sense the signal [74-76]. Paracrine signaling is a new concept in bacterial cell–cell communication. This led some authors to speculate about additional functions of other extracellular signaling mechanisms in bacteria and their possible roles in sensing more than simply population density. For instance, one direct consequence of having multiple extracellular signaling molecules, where the synthesis of one depends on the prior synthesis of the other (like the mechanism of paracrine signaling of *B. subtilis*), is to generate a sequential timing device, in which a cascade of developmental changes are triggered in response to the presence and concentration of different signaling molecules.

Spo0A and competence

The ability of *B. subtilis* to develop natural competence is very well known and it has been the subject of study for many years. Competence can be defined as a physiological state in which cells

are able to uptake and assimilate exogenous DNA in a process controlled by the cell itself. Only a small fraction of cells shows natural competence (around 10% of the total population). The subpopulation of competent cells within the community arises due to the bistable regulation of the master regulator ComK, which controls the initiation of the cascade of the genes involved in competence [78,79]. The comK gene can be stimulated by its own product ComK [80]; thus, comK expression increases nonlinearly due to a positive feedback loop. This results in a heterogeneous population of cells in which some cells express ComK and others do not [81]. Importantly, promoting the stabilization of ComK protein can also induce the bistable response, due to the induction of the comK expression that ComK protein exerts [82]. The activation of the expression of comK is driven by the induction of the quorum-sensing pathway ComX-ComP-ComA [83]. Briefly, the pheromone ComX activates the histidine kinase ComP to phosphorylate the master regulator ComA. Once ComA is in its active form, ComA-P, it triggers the expression of the regulator ComK to initiate the pathway to competence. Remarkably, ComA-P can also regulate the expression of other features that are associated with sporulation. For instance, the phosphorylated form of ComA can induce the expression of the proteins RapA, RapB, and RapE [50]. These proteins belong to the peptidic system of quorum sensing of *B. subtilis* and they specifically block the phosphorelay route to phosphorylate the master regulator Spo0A. Thus, cells differentiated to be competent have inhibited the process of Spo0A phosphorylation and they will not sporulate. Moreover, activation of the expression of the competence regulator ComK also acts in a second role of inhibiting the expression of the gene spo0A gene [84]. In this case, the activation of the competence leads to the inhibition of Spo0A expression. Because of this regulation, cells committed to be competent are maintained as a distinct population and can only initiate sporulation once the levels of ComK have decreased within the cell.

Spo0A and cannibalism

In natural environments, *B. subtilis* communities are surrounded by other microorganisms, many of them nonsporulating, and *B. subtilis* cells committed to sporulate or spores could be at disadvantage relative to them. Cannibalism helps to sustain a mixed population during the stationary phase with a small percentage of spores and a high percentage of growing cells for a longer period of time, which might be beneficial to the community.

However, not all these genes respond equally to Spo0A; some of them are activated at low concentrations of Spo0A, and others only at high concentrations [29,85]. More recently, transcriptional-profiling experiments using DNA microarrays were applied to identify the Spo0Adependent genes distributed in each category [29,86]. It was observed that genes requiring a high dose of Spo0A to be activated have a low binding affinity for this regulator. Genes directly involved in sporulation, such as spoIIA, spoIIE and spoIIG, are included in this category. On the other hand, genes activated by a low dose of Spo0A either have a high-affinity binding site or are indirectly regulated by Spo0A, which relieves the repression by the AbrB regulator. The operons involved in cannibalism, skf and sdpABC, fall into this category. The promoter of the skf operon has a high-affinity binding site for Spo0A, and the sdpABC operon is repressed by AbrB, and therefore, is indirectly activated at a low concentration of Spo0A through repression of abrB [29]. Moreover, AbrB directly represses transcription of the skf operon; thus, skf is activated by two routes: directly by Spo0A and indirectly by relieving AbrB-mediated repression. What is the biological significance of having differential responses to high and low doses of Spo0A? At an early stage of sporulation, activated Spo0A is produced at a low level in the cells, and this turns on genes involved in auxiliary roles in development, like for instance cannibalism and formation of multicellular aerial structures [29], in which sporulation will take place. The building of these multicellular structures can be seen as a prelude to spore formation. Then, if conditions still promote sporulation, there is a progressive increase in the intracellular concentration of activated Spo0A, and the genes that play a direct role in spore formation are turned on.

Spo0A and lipid synthesis

Roberto Grau's laboratory (I was part of it) demostrated the existence of active and robust de novo fatty acid (FA) and membrane lipid synthesis during sporulation. We also demonstrate that the reactivation of the de novo lipid synthesis during development is due to the Spo0A-dependent reactivation of the synthesis of malonyl-CoA, the central metabolite of FA synthesis and lipid homeostasis in the cell. Among the many genes whose products participate in the de novo lipid synthesis machinery, only one genetic unit, the accDA operon, appears to be controlled by Spo0A. Why does Spo0A only regulate the activity of the operon involved in malonyl-CoA synthesis? And, how does Spo0A control the global synthesis of FAs and membrane lipids by controlling only accDA expression? In addition to its essential role as a precursor of FA synthesis, malonyl-CoA is a direct and specific negative modulator of FapR, which is a conserved transcriptional repressor of several genes involved in FA and membrane lipid synthesis (the fap regulon) in Gram-positive bacteria [87]. The binding of malonyl-CoA to FapR prevents the binding of FapR to (and/or promotes the release of FapR from) its target DNA sequences, an event that derepress de novo lipid synthesis [28]. Therefore, Spo0A functions as the key regulator of de novo lipid synthesis during development by providing appropriate levels of malonyl-CoA for new lipid formation and linking the levels of this central metabolite to the downregulation of the activity of the lipid synthesis repressor FapR [28]. Reinforcing the view of the central role of Spo0A in lipid synthesis homeostasis during development is the fact that the only genes involved in FA synthesis that are not controlled by FapR are those encoding the ACC enzyme responsable for malonyl-CoA formation [87], and these acc genes are the only genes involved in lipid synthesis that are controlled by Spo0A [88].

Spo0A and motility

Multicellular communities of *B. subtilis*, termed biofilms, comprise numerous cell types, including motile cells and matrix-producing cells. These two processes are highly regulated at both

the transcriptional and the post-translational levels. Transcription of genes important for motility and matrix production is regulated by Spo0A. High levels of Spo0A-P repress the fla/che motility operon, whereas Spo0A-P is required for extracellular matrix gene expression via the activation of the regulatory protein SinI [89]. As would be predicted by the inverse regulation, the subpopulation of cells differentiated to produce extracellular matrix does not exhibit induction of the genes related to motility.

Motility requires the induction of a large fla-che operon, which contains 31 genes encoding for proteins that make up the basal body of the flagella, the chemotaxis system, and the sigma factor SigD. SigD is encoded at the end of the fla-che operon and is required for the expression of the hag locus, which encodes flagellin, the protein comprising the actual flagellar filament, as well as for the motA and motB genes, which encode for the motor proteins necessary for flagelar rotation [90]. SigD also controls the expression of autolysins (lytA, lytD, and lytF) that function in cell separation, thus insuring that motile cells are nonfilamentous.

The transition from a motile to a sessile state is mediated by a biased bistable switch from the SigD sigma factor. The activation of SigD is driven by the master regulator DegU, which activates the expression of the operon in its unphosphorylated form. The unphosphorylated form of DegU directly binds the regulatory region of the fla/che operon promoter [91]. The bias in SigD activity relies on the SwrA protein, which enhances expression of the fla-che operon and thus sigD [8]. It has recently been proposed that SwrA is required for DegU binding and thus for the expression of sigD. This finding reveals that swrA expression is controlled by a positive feedback loop, because the expression of swrAA is induced by SigD itself [92].

Flagellar motility requires the expression of numerous genes and the activation of many proteins. This process requires energy and may be costly for the cell. However, it has been observed that some cells inhibit motility on behalf of other processes, such as matrix formation. Extracellular matrix production requires the activation of two operons: the 15-gene epsA-O operon, responsible for the production of the exopolysaccharide component, and the yqxM-sipWtasA operon, responsible for the production and secretion of the major protein component of the matrix, TasA [8,93]. A recent report from Blair et al. [94] exquisitely explains a mechanism that inhibits motility post-translationally once matrix genes are expressed.

During exponential growth, the eps and yqxM operons are repressed in all cells by an inhibitor, SinR. SinI, an antagonist of SinR, is expressed only in a subpopulation of cells in which Spo0A has been activated [95]. SinI antagonizes SinR by binding directly to SinR in a 1:1 stoichiometry. Once SinI has been bound to SinR, the repressor cannot bind DNA and it is inactivated [96]. In this way, as levels of Spo0A-P increase in the cell, SinR-mediated repression is relieved, allowing the expression of the eps operon, and proteins needed for exopolysaccharide are produced. One of these proteins, EpsE, has a putative function as a family II glycosyltransferase, but also acts as an inhibitor of motility. When EPS interacts with a specific flagellum protein, FliG, it inhibits flagellar rotation, similar to a clutch [94]. The clutch provided by EpsE might act as a fail-safe mechanism to prevent any wasted energy; it guarantees the stop of flagella rotation when cells are producing extracelular matrix, which would presumably interfere with rotation. Moreover, if conditions switch to favor motility over matrix production, flagella could be reactivated, allowing cells to reuse the investment of energy in flagella synthesis.

Concluding remarks

The bacterium *B. subtilis* has a remarkably complex network to regulate its gene expression and to ensure that the genetically identical population can display a multitude of behaviors. Many of these behaviors are mutually exclusive and the regulatory system must take into account the alternative paths a cell might take and devise mechanisms to prevent coexpression of the wrong genes.

Recent advances in cell biology have increased our understanding of how replication is regulated at the different steps of the cell cycle. Initiation of chromosomal replication is an essential checkpoint that seems to be common to all domains of life. In bacteria undergoing the transition to a dormant state, master transcription factors (e.g., Spo0A, CtrA, and AdpA) regulate the expression levels of dozens of genes involved in morphological differentiation and inhibit replication initiation by binding to the origin of replication. In the future, an improved understanding of replication regulation could facilitate the experimental control of bacterial replication, potentially allowing us to inhibit DNA replication in pathogens, optimize the production of valuable secondary metabolites, and/or generate synchronized cultures for various physiological and genetic studies.

Differentiation of distinct cells types in *B. subtilis* is necessary for the proper development of the bacterial community. This differentiation is regulated, at least partially, by sensing several extracellular signals. Most of these signals are produced by *B. subtilis* itself. Secretion and sensing of these extracellular signals might regulate the timing of development in concordance with the surroundings. In this manner, the production of extracellular signals and the consequent differentiation of cells can be classified into three sequential steps in development. The first step would be a stage of exponential growth, in which a large portion of the community would differentiate into motile cells. At this point, the concentrations of the quorum-sensing signals ComX and surfactin are insufficient to trigger the differentiation of other cell types. In the next step, cells are entering the stationary phase, and ComX triggers the differentiation of surfactin producers and competent cells. Once surfactin is produced, it is sensed by other cells, which respond by differentiating into matrix producers/ cannibals. These subpopulations are physiologically distinct from the initial population of motile cells. Thus, as arise, the proportion of motile cells decreases. Finally, in the last step of the development, nutrients are exhausted and the production of secondary messengers such as ppGpp, along with other starvation-related signals, would trigger sporulation in some cells as well as a subpopulation of exoprotease

producing miners. To ensure that the timing of the development is appropriate for the particular environmental conditions, this cell differentiation program is also capable of sensing other small molecules produced by diverse soil organisms; different natural products secreted from a large variety of organisms can induce the activation of the three master regulators, for our review, Spo0A, that control differentiation of the distinct cell types. This is an efficient mechanism for *B. subtilis* to adapt its development to a particular environmental niche in which other microorganisms coexist.

The organization of different cell types in the biofilm in *B. subtilis* closely resembles the cellular differentiation and spatial organization observed in structures formed by multicellular eukaryotes, for example fungi. Similarities in development processes between multicelular eukaryotes and bacteria are strongly encouraging to advance investigation in this field. There are numerous questions that remain. Is cell–cell communication involved in the developmental process to coordinate the growth and the spatial distribution of the different cell types within the biofilm? How do other different microorganisms present in the same ecological niche, the soil, affect cellular differentiation? Does population heterogeneity provide an advantage in natural settings? Spo0A could regulate other pathways? The Spo0A and the phosporelay system have another function?. *Bacillus subtilis* is an ideal system in which to ask these types of questions because there is a plethora of information regarding the regulation of differentiation, and the ease of working with a microorganism with a very short doubling time that is amenable to genetic modifications should facilitate future studies.

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