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Spatiotemporally regulated proteolysis to dissect the role of vegetative proteins during *Bacillus subtilis* sporulation: cell-specific requirement of σ^H and σ^A

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Summary

Sporulation in *Bacillus subtilis* is a paradigm of bacterial development, which involves the interaction between a larger mother cell and a smaller forespore. The mother cell and the forespore activate different genetic programs, leading to the production of sporulation-specific proteins. A critical gap in our understanding of sporulation is how vegetative proteins, made before sporulation initiation, contribute to spore formation. Here we present a system, spatiotemporally regulated proteolysis (STRP), that enables the rapid, developmentally-regulated degradation of target proteins, providing a suitable method to dissect the cell- and developmental stage-specific role of vegetative proteins. We have used STRP to dissect the role of two major vegetative sigma factors, σ^H and σ^A, during sporulation. Our results suggest that σ^H is only required in predivisional cells, where it is essential for sporulation initiation, but that it is dispensable during subsequent steps of spore formation. However, we provide evidence that σ^A plays different roles in the mother cell, where it replenishes housekeeping functions, and in the forespore, where it plays an unexpected role in promoting spore germination and outgrowth. Altogether, our results demonstrate that STRP has the potential to provide a comprehensive molecular dissection of every stage of sporulation, germination and outgrowth.

Graphical Abstract

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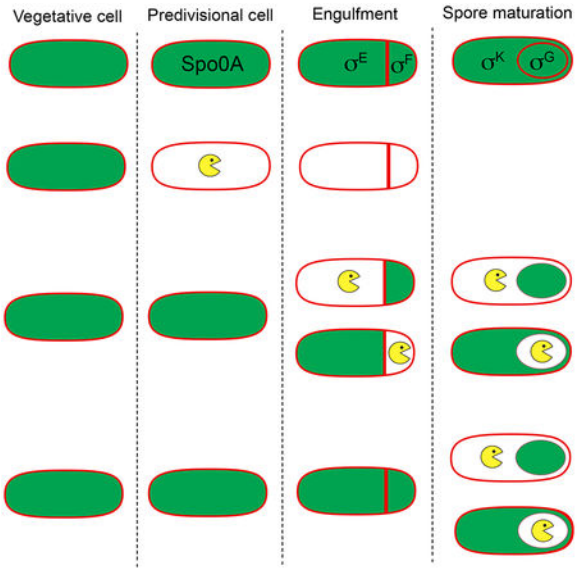
Author contributions

Conception and design of the study: EPR, KP and JLG; acquisition, analysis or interpretation of the data: all authors; writing of the manuscript: EPR, KP and JLG.



Spore formation in *Bacillus subtilis* represents a simple developmental process that involves the interaction between only two cells. Here we present a method, called spatiotemporally regulated proteolysis (STRP), to rapidly deplete target proteins in a cell- and developmental stage-specific manner during sporulation. We show that STRP has the potential to provide a comprehensive molecular dissection of every stage of sporulation, germination and outgrowth.

Keywords

Bacillus subtilis; sporulation; inducible protein degradation; ssrA; sigma factors

Introduction

Cellular differentiation is a pivotal step in every developmental process, from human ontogeny to spore formation in certain bacteria. Sporulation in the bacterium *Bacillus subtilis* has become a paradigm for cell differentiation and development in bacteria (Errington, 2003; Hilbert and Piggot, 2004; Higgins and Dworkin, 2012; Tan and Ramamurthi, 2014; Narula *et al.*, 2016). Triggered by nutrient starvation, spore formation involves the close interaction between two cells that differ in both size and developmental fate: the smaller forespore, which becomes the dormant and highly resilient spore following maturation, and the larger mother cell, which lyses after contributing to the formation of the mature spore.

The sporulation process is depicted in Fig. 1. The forespore and the mother cell are the result of an asymmetrically positioned cell division event in which the septum is formed close to a single cell pole, in a process called polar septation. After polar septation, the membrane of the mother cell engulfs the forespore in a phagocytosis-like process that culminates with the forespore surrounded by two membranes and enclosed within the mother cell cytoplasm. The forespore subsequently matures inside the mother cell, where it is protected from the external medium. Spore maturation involves the formation of a peptidoglycan layer called

the cortex between the inner and outer forespore membranes (Popham and Bernhards, 2015), the assembly of a proteinaceous coat around the outer membrane (McKenney *et al.*, 2013), and the dehydration of the forespore cytoplasm, which endows the spore with a bright appearance under phase-contrast microscopy. Once maturation is complete, the mother cell lyses and the spore is released into the environment, where it can remain dormant for years. Mature spores are resistant to a variety of environmental challenges (Setlow, 2006), including high temperature, desiccation, and a variety of chemicals such as antibiotics, to which spores are impermeable. Spore germination is triggered in response to specific nutrients and other stimuli (Setlow, 2014). During germination the spore core is rehydrated, leading to the resumption of metabolic activities and vegetative growth.

The regulatory program that controls sporulation is relatively well understood (Errington, 2003; Hilbert and Piggot, 2004). Several transcriptional regulators are sequentially activated, which orchestrate distinct programs of gene expression in the mother cell and forespore at different developmental stages (Fig. 1). The entry into sporulation is controlled by the stationary phase-specific σ factor, σ^H, and the phosphorelay response regulator, Spo0A, which together govern gene expression in the predivisional cell. After polar septation, a cascade of cell-specific σ factors become active in the forespore and the mother cell. Immediately after polar septation, σ^F is activated in the forespore, followed by the activation of σ^E in the mother cell. Upon engulfment completion, σ^G becomes active in the forespore and σ^K in the mother cell. Together, the cell-specific σ factors control the transcription of ~560 genes (Eichenberger *et al.*, 2004; Wang *et al.*, 2006; Eichenberger, 2012), most of which are sporulation-specific and not transcribed in vegetative cells.

The study of sporulation has traditionally focused on deciphering the function of genes transcribed by sporulation-specific σ factors. Although there are still many questions to be answered, thanks to the concerted efforts of thousands of researchers over the past fifty years, we have achieved a relatively comprehensive understanding of the function of many sporulation-specific proteins. However, most *B. subtilis* proteins are produced during vegetative growth, before polar septation, and the manner in which they contribute to sporulation remains largely unknown. This critical gap in our knowledge of sporulation is mainly due to the lack of suitable genetic tools to inhibit the function of specific proteins in a precise, cell- and developmental stage-specific manner during spore formation. The precisely regulated inactivation of target proteins is critical because many such proteins are important for growth, so null mutations may be nonviable or unable to enter sporulation. Furthermore, because sporulating cells do not grow or divide following polar septation, methods based on inhibition of transcription or translation to deplete specific proteins have limited utility. Indeed, the average half life of bacterial proteins is ~8–20 h in growing and stationary phase cells (Koch and Levy, 1955; Borek *et al.*, 1958; Mandelstam, 1958; Kock *et al.*, 2004), which, due to the absence of growth during sporulation, would produce a negligible reduction in the amount of protein in the ~3 h between polar septation and forespore maturation (at 37°C). Thus, there is a need for new systems to rapidly deplete proteins, preferably in a cell- and developmental stage-specific manner.

In 2008, Griffith and Grossman developed an inducible, targeted protein degradation system in *B. subtilis* (Griffith and Grossman, 2008), which provides an opportunity to circumvent

these limitations. The system is based on the addition of a modified ssrA tag from *E. coli* (hereafter ssrA*) to the C-terminus of the target protein, and the expression of the *E. coli* SspB ($SspB^{Ec}$) from inducible promoters. When $SspB^{Ec}$ is produced, it binds to the ssrA* tag and delivers the target protein to the endogenous *B. subtilis* protease, ClpXP, for degradation. This system supports the degradation of target proteins within minutes after the induction of $sspB^{Ec}$ expression (Griffith and Grossman, 2008; Eswaramoorthy *et al.*, 2014; Yen Shin *et al.*, 2015; Lamsa *et al.*, 2016; Lopez-Garrido *et al.*, 2018), allowing for the rapid depletion of proteins in non-dividing cells.

We have previously shown that the expression of $sspB^{Ec}$ from sporulation-specific promoters dependent on σ^F and σ^E supports the efficient degradation of ssrA*-tagged proteins in a cell-specific manner during sporulation (Yen Shin *et al.*, 2015; Lopez-Garrido *et al.*, 2018). Here, we expand on our previous study to build a comprehensive framework, called spatiotemporally regulated proteolysis (STRP), to rapidly deplete target proteins in a cell- and developmental stage-specific manner. We demonstrate that our framework is well suited to dissect the cell- and stage-specific requirement of target proteins during sporulation. First, we provide evidence that degradation of abundant target proteins does not affect the turnover of native ClpXP substrates. Second, we show that degradation of control proteins with well-documented roles in sporulation produce phenotypes equivalent to those of the null mutants. Third, we demonstrate that proteins produced before polar septation are rapidly degraded in a spatially- and temporally-controlled manner.

We have applied STRP to dissect the spatiotemporal requirement of the stationary-phase σ factor σ^H, and the essential vegetative σ factor σ^A, during sporulation. Our results indicate that σ^H is only required in predivisional cells, and does not play a major role once polar septation occurs. However, we present evidence that σ^A plays different roles in mother cell and forespore: in the mother cell, it may replenish housekeeping functions needed to complete sporulation; in the forespore, it is required early in sporulation to produce spores that germinate efficiently and it is essential for spore outgrowth.

Results

Degradation of abundant ssrA*-tagged proteins does not saturate ClpXP protease in B. subtilis

Targeted protein degradation systems based on modified ssrA tags have emerged as a powerful genetic tool in different bacteria, including *B. subtilis* (McGinness *et al.*, 2006; Griffith and Grossman, 2008; Wei *et al.*, 2011). Because those systems rely upon endogenous proteases (ClpXP in *B. subtilis*) to degrade target proteins, a potential caveat is that inducing the degradation of an abundant target protein could saturate the protease, preventing processing of its natural substrates (Cookson *et al.*, 2011). To test this possibility, we sought to develop an *in vivo* reporter for ClpXP saturation in *B. subtilis*. Towards this end, we constructed an N-terminal fusion of the green fluorescent protein (GFP) to the stress-related transcriptional regulator, Spx. A strain producing GFP-Spx as the only source of Spx was resistant to diamide (data not shown), suggesting that the fusion protein is functional (Nakano *et al.*, 2003). Under standard laboratory conditions, Spx is maintained at low levels due to proteolysis by ClpXP (Nakano *et al.*, 2002). Accordingly, no GFP-Spx is

observed by fluorescence microscopy (Fig. 2A, +ClpXP). However, when the amount of ClpXP becomes limiting, GFP-Spx accumulates, yielding a clear fluorescent signal that co-localizes with the nucleoid (Fig 2A, -ClpXP). We reasoned that if inducing the degradation of ssrA*-tagged proteins saturated ClpXP, we would observe an accumulation of GFP-Spx signal that co-localizes with the nucleoid. To test this idea, we induced the degradation of an ssrA*-tagged derivative of the red fluorescent protein (Tomato-ssrA*) produced at high levels from a strong constitutive promoter, and looked for the appearance of GFP-Spx (Fig 2B). As a positive control, this strain was also treated with hydrogen peroxide, which has previously been shown to evoke the Spx response (Leelakriangsak *et al.*, 2007). Degradation of Tomato-ssrA* did not result in the accumulation of GFP-Spx, whereas hydrogen peroxide treatment did (Fig 2C). These data suggest that targeting abundant proteins for degradation by ClpXP has little or no effect on ClpXP housekeeping activity. Thus, the phenotypes observed upon degradation of target proteins are likely the result of target protein inactivation rather than a consequence of ClpXP saturation.

Development of a cell-specific protein degradation system during B. subtilis sporulation

To adapt targeted protein degradation to the study of sporulation in *B. subtilis*, we explored the possibility of using developmentally-regulated promoters to drive *sspB*^{Ec} expression. We have previously employed σ^F- and σ^E-dependent promoters to produce SspB^{Ec} and degrade the SpoIIIE DNA translocase after polar septation (Yen Shin *et al.*, 2015; Lopez-Garrido *et al.*, 2018). σ^F and σ^E are rapidly inactivated once engulfment is completed (Li and Piggot, 2001), which limits the ability of this approach to study proteins that are required throughout sporulation. Thus, we decided to expand the set of developmentally-regulated promoters to increase the versatility of the protein degradation system.

We designed a genetic strategy to screen for cell-specific promoters that triggered the efficient degradation of target proteins when driving the expression of *sspB*^{Ec} (Fig. 3A). Specifically, we constructed strains producing ssrA*-tagged versions of the essential sporulation proteins, σ^G and σ^K, which orchestrate cell-specific transcription after engulfment in the forespore and mother cell, respectively (Fig. 1). *B. subtilis* mutants lacking σ^G or σ^K are unable to form spores (Table S1). However, the addition of the ssrA* tags did not produce any observable defect in spore morphogenesis or titer (Fig. 3B–D; Table S1), suggesting that both tagged proteins are fully functional. Expression of *sspB*^{Ec} from a xylose-inducible promoter, however, yielded a dramatic reduction in spore titer for both strains (Fig. 3B; Table S1), indicating that σ^G-ssrA* and σ^K-ssrA* were efficiently degraded. Note that the addition of 1% of xylose alone to sporulating cultures did not reduce spore titers (Fig. 3B) nor affect the progression of sporulation (Fig. S1). We then chose sporulation cell-specific promoters to drive *sspB*^{Ec} expression and tested if they triggered the efficient degradation of σ^G-ssrA* and σ^K-ssrA*. We selected promoters belonging to three different temporal classes:

i. Early promoters, active immediately after polar septation in the forespore (σ^F-dependent) or in the mother cell (σ^E-dependent), but inactive after engulfment. We selected the σ^F-dependent promoters P$_{spoIIR}$ (Karow *et al.*, 1995) and P$_{spoIIQ}$ (Londoño-Vallejo *et al.*, 1997), and the σ^E-dependent promoters P$_{spoIID}$ (Clarke *et al.*, 1986; Rong *et al.*, 1986) and P$_{spoIVA}$ (Roels *et al.*, 1992).

ii. Late promoters, active in the forespore (σ^G-dependent) or the mother cell (σ^K-dependent) after engulfment. Here we used the σ^G-dependent promoters P_{sspA} and P_{sspB} (Nicholson *et al.*, 1989) and the σ^K-dependent promoters P_{gerE} (Cutting *et al.*, 1989) and $P2_{cotE}$ (Zheng and Losick, 1990) for late forespore and mother cell expression, respectively. Note that P_{sspB} drives the expression of the *Bacillus sspB* gene, which encodes the major β-type small acid-soluble protein and is unrelated to the degradation adaptor protein $SspB^{Ec}$.

iii. Sustained promoters, continuously active in the forespore (σ^F- and σ^G-dependent) or the mother cell (σ^E- and σ^K-dependent) during and after engulfment. The synthetic $P_{sspE(2G)}$ (Sun *et al.*, 1991), which is recognized by both σ^F and σ^G, was used to continuously transcribe $sspB^{Ec}$ in the forespore. To continuously transcribe $sspB^{Ec}$ in the mother cell, we used the two *cotE* promoters, the first of which is activated by σ^E ($P1_{cotE}$), and the second by σ^K ($P2_{cotE}$) (Zheng and Losick, 1990).

As shown in Fig. 3B, forespore expression of $sspB^{Ec}$ from P_{spoIIQ}, $P_{sspE(2G)}$, P_{sspA} and P_{sspB} resulted in significant spore titer defects in strains expressing σ^G tagged with ssrA*. Similarly, mother cell expression of $sspB^{Ec}$ from P_{spoIVA}, P_{cotE}, P_{gerE} and $P2_{cotE}$ significantly reduced the spore titer of the σ^K-ssrA* strain. Importantly, no spore titer defects were observed in the σ^G-ssrA* and σ^K-ssrA* strains when $sspB^{Ec}$ was expressed in the cell in which the transcription factor is not active. Only the early promoters P_{spoIIR} and P_{spoIID} failed to trigger efficient degradation of σ^G-ssrA* and σ^K-ssrA*, respectively. Both promoters are only transiently active during engulfment (Clarke *et al.*, 1986; Karow *et al.*, 1995; Wu and Errington, 2000; Eichenberger *et al.*, 2004). In addition, P_{spoIIR} is particularly weak (Sharp and Pogliano, 2002; Ojkic *et al.*, 2016). Thus, it is possible these promoters do not yield enough $SspB^{Ec}$ to mediate the complete and/or sustained degradation of σ^G-ssrA* and σ^K-ssrA* once engulfment is complete. To avoid complications due to inactivation of promoters after engulfment, we focused on sustained and late promoters for further characterization. We specifically selected the sustained promoters $P_{sspE(2G)}$ and P_{cotE} and the late promoters P_{sspA} and P_{gerE}. Although $P2_{cotE}$ produced a stronger spore titer defect than P_{gerE} when σ^K was tagged with ssrA* (Fig. 3B), we observed that its pattern of expression was variable, leading to premature degradation of target proteins in a fraction of the sporangia (not shown). We therefore decided to exclude $P2_{cotE}$ from further studies. The sustained and late promoters that we selected for use in our system are indicated by a blue box in Figure 3B.

Characterization of cell-specific degradation from sustained and late promoters

We next performed experiments to confirm that the selected promoters conferred the expected temporal and spatial regulation on $sspB^{Ec}$ expression, and that they yielded enough $SspB^{Ec}$ to completely degrade target proteins. First, we made transcriptional fusions to *gfp* to confirm the expression pattern from the different promoters using fluorescence microscopy (Fig. S2). As expected $P_{sspE(2G)}$ and P_{cotE} conveyed sustained forespore- and mother cell-specific *gfp* expression that could be visualized shortly after polar septation, whereas P_{sspA} and P_{gerE} conveyed late expression in these cells. Expression of $sspB^{Ec}$ from these promoters had no detectable effect on sporulation (Fig. 3B; Table S1). In addition, we

compared the expression levels from the selected sustained or late promoters with the expression level of a xylose-inducible promoter (P_{xylA}) induced with a xylose concentration (0.01 %) that we determined was enough to completely degrade abundant ssrA* tagged proteins during vegetative growth (Fig. S3). As shown in Fig. S3E, expression from sustained and late promoters yielded intracellular GFP concentrations that were at least 20 fold higher than the concentration achieved when GFP was expressed from P_{xylA} induced with 0.01 % of xylose, suggesting that, when produced from the selected sustained and late promoters, $SspB^{Ec}$ is not limiting for degradation. Finally, we performed microscopy to determine if the cytological profiles obtained upon cell-specific degradation of σ^G-ssrA* and σ^K-ssrA* matched those of the σ^{G-} and σ^{K-} mutants. We used a combination of fluorescence and phase contrast microscopy to assess the completion of two developmental milestones: engulfment membrane fission, which depends on early cell-specific gene expression, and forespore dehydration, which depends on late cell-specific gene expression. To assess the completion of engulfment, we simultaneously stained sporangia with two membrane dyes: FM 4–64, which fluoresces red and is membrane-impermeable, and Mitotracker Green (MTG), which fluoresces green and is membrane-permeable (Sharp and Pogliano, 1999). During engulfment, the membrane of the forespore is exposed to the culture medium, and is accessible to both FM 4–64 and MTG. After engulfment culminates with membrane fission, the forespore is completely enclosed within the mother cell cytoplasm and the forespore membranes can only be stained by the membrane-permeable dye MTG, but not FM 4–64. This allows sporangia that have completed engulfment membrane fission to be easily distinguished from those that have not (Sharp and Pogliano, 1999). To assay forespore dehydration, we used phase-contrast microscopy and observed the appearance of phase-bright spores.

Degradation of σ^G-ssrA* from the sustained promoter $P_{sspE(2G)}$ or the late promoter P_{sspA} produced cytological profiles that phenocopied that of the σ^{G-} null mutant, with completion of engulfment membrane fission, but no forespore dehydration (Fig. 3C). Production of $SspB^{Ec}$ from the mother cell-specific promoters P_{cotE} or P_{gerE} in strains producing σ^G-ssrA*, however, resulted in wild type profiles (Fig. 3C). Similarly, degradation of σ^K-ssrA* from P_{cotE} or P_{gerE} allowed completion of engulfment membrane fission, but yielded less dehydrated, phase-gray forespores, similar to the σ^{K-} null mutant (Fig. 3D). As expected, production of $SspB^{Ec}$ from forespore-specific promoters did not trigger σ^K-ssrA* degradation and resulted in wild-type cytological profiles (Fig. 3D). To confirm that σ^G and σ^K were efficiently degraded, we tagged each protein with GFP and ssrA*. A clear GFP fluorescence signal was observed in the forespore and the mother cell in strains producing σ^G-GFP-ssrA* and σ^K-GFP-ssrA*, respectively (Fig. S4). The signal completely disappeared when $sspB^{Ec}$ was expressed in the appropriate compartment from either sustained or late promoters (Fig. S4), indicating that both proteins were efficiently degraded. The cytological profiles and spore titers obtained after degradation of σ^G-GFP-ssrA* and σ^K-GFP-ssrA* were equivalent to those obtained after of σ^G-ssrA* and σ^K-ssrA* degradation (Fig. S4; Table S1).

It is interesting to note that degradation of σ^G and σ^K from late promoters produced cytological profiles equivalent to those of the null mutants (Fig. 3C, D; Fig. S4). Because transcription from late promoters depends on σ^G and σ^K, those σ factors must be initially

activated, allowing a burst of expression of the genes under their control, including *sspB*^{Ec}. Once enough SspB^{Ec} has accumulated, it will trigger the degradation of σ^G-ssrA* and σ^K-ssrA*. Thus, these results suggest that σ^G and σ^K activity is required continuously during forespore maturation, and not just transiently after engulfment has been completed.

Overall, the above results demonstrate that production of SspB^{Ec} from developmentally-regulated promoters can be used to trigger the cell-specific degradation of sporulation-specific target proteins, yielding cytological profiles equivalent to the null mutants.

Cell- and stage-specific degradation of vegetative proteins

To assess whether expression of *sspB*^{Ec} from the selected promoters led to an efficient, spatiotemporally-regulated degradation of target proteins made before polar septation, we studied the degradation of a YtsJ-GFP-ssrA* fusion protein (Lamsa *et al.*, 2016). YtsJ is an abundant, non-essential cytoplasmic protein that is not required for sporulation. We visually evaluated degradation based on the disappearance of GFP fluorescence (Fig. 4A). The results obtained can be summarized as follows: (i) When *sspB*^{Ec} was not expressed, a clear fluorescent signal was observed filling the cytoplasm of the mother cell and the forespore, as well as the cytoplasm of predivisional cells. (ii) Production of SspB^{Ec} from sustained promoters triggered the continuous degradation of YtsJ-GFP-ssrA* in the forespore ($P_{sspE(2G)}$) or the mother cell (P_{cotE}), starting shortly after polar septation. We also triggered degradation in both cells simultaneously by producing SspB^{Ec} from $P_{sspE(2G)}$ and P_{cotE} at the same time; in this case, fluorescence was only observed in cells before polar septation. (iii) Production of SspB^{Ec} from the late promoters triggered degradation in the forespore (P_{sspA}), in the mother cell (P_{gerE}) or in both (P_{sspA} P_{gerE}) only after engulfment completion. In every case, no fluorescence was detected in the cell types in which YtsJ-GFP-ssrA had been degraded, indicating that SspB^{Ec} production led to the rapid and efficient degradation of the target protein.

Next, we performed timelapse microscopy to determine how quickly YtsJ-GFP-ssrA* was degraded upon induction of *sspB*^{Ec} expression. We specifically used sustained promoters, which are active immediately after polar septation, to drive the expression of *sspB*^{Ec}, since polar septation provides a clear marker for the onset of degradation. We stained sporangia with the membrane dye FM 4–64 and monitored the disappearance of YtsJ-GFP-ssrA* fluorescence signal upon induction of *sspB*^{Ec} expression in the mother cell or the forespore from P_{cotE} and $P_{sspE(2G)}$, respectively (Fig. 4B). To correct for GFP photobleaching due to continuous imaging, we calculated the ratio between the GFP intensity in the compartment in which degradation was induced and the other compartment. Once degradation started, the decrease in GFP fluorescence followed an exponential decay. Degradation was slightly faster in the forespore (protein half-life 4.6±1.3 min) than in the mother cell (protein half-life 6.7±2.3 min) (Fig. 4C). It has been reported that ClpXP is more active in the mother cell than in the forespore (Kain *et al.*, 2008). It seems therefore unlikely that the faster degradation kinetics in the forespore is due to differences in ClpXP activity between both cells, in which case we would expect degradation to happen faster in the mother cell. Instead, the difference might reflect a slower accumulation of SspB^{Ec} in the mother cell due to its larger volume or differences in promoter strength. The kinetic data from cell-specific

degradation are in good agreement with degradation speeds estimated at the population level in vegetative cells (Griffith and Grossman, 2008; Eswaramoorthy *et al.*, 2014; Lamsa *et al.*, 2016). Importantly, according to the estimated degradation rates, cell-specific expression of *sspB*^{Ec} would allow complete depletion of target proteins within ~20–30 min, which is significantly less time than required for engulfment under these conditions (~90 min, Ojkic et al. 2016).

These results show that expression of *sspB*^{Ec} from sustained and late promoters allow rapid degradation of proteins made before polar septation in a spatially and temporally controlled manner. Sustained promoters remain active in the forespore or mother cell throughout sporulation, while late promoters are active only after engulfment. Therefore, the use of both temporal classes of promoters to trigger degradation of target proteins, in combination with various assays to assess the completion of developmental milestones (Harwood *et al.*, 1990), allows for the dissection of the temporal and cell-specific requirement of target proteins during sporulation (Fig. 4D). We have named this experimental approach spatiotemporally regulated proteolysis (STRP). In the next sections, we have used STRP to dissect the cell-specific role that two key vegetative sigma factors, σ^H and σ^A, play during sporulation.

The stationary phase sigma factor σ^H is only required in predivisional cells

The initiation of sporulation requires the activation of the stationary-phase sigma factor, σ^H, which is required for polar septation. Accordingly, σ^{H-} mutants are unable to undergo polar septation and do not initiate sporulation (Fig. 5C, Δ*sigH*). While its importance in sporulation initiation is well established, it is unclear if σ^H activity is required in the mother cell or forespore after polar septation. To evaluate this possibility, we constructed a σ^H-ssrA* fusion protein, which supports sporulation at levels comparable to the wild type (Table S1). First, we validated that degradation of σ^H-ssrA* prior to polar septation suppressed the formation of the polar septum. For this purpose we used two different strategies. First, we induced degradation of σ^H-ssrA* by expressing *sspB*^{Ec} from a xylose-inducible promoter (Fig. S5), which triggers σ^H-ssrA* degradation continuously during the growth of the culture. Second, we expressed *sspB*^{Ec} from the Spo0A-dependent promoter P_{spoIIE}, which becomes active in predivisional cells, shortly before polar septation (Fig. 5A). This second strategy restricts degradation to predivisional cells that are about to commit to sporulation, a point that we confirmed by monitoring the disappearance of YtsJ-GFP-ssrA* (Fig. 5B). In both cases, degradation of σ^H-ssrA* prior to polar septation resulted in the inhibition of polar septum formation (Fig. 5C; Fig. S5), confirming the requirement of σ^H-dependent transcription in predivisional cells to start sporulation. We next determined the cytological profiles obtained upon cell-specific degradation of σ^H-ssrA* from forespore- and mother cell-specific sustained promoters. As shown in Fig. 5C, degradation of σ^H-ssrA* in the forespore or mother cell yielded cytological profiles equivalent to the control strain. These results suggest that σ^H does not play a critical role in either the forespore or the mother cell. Altogether, our results indicate that σ^H-dependent transcription is required in predivisional cells until the polar septum is formed, but that it is dispensable after polar septation.

Effect of cell-specific degradation of σ^A on spore formation

σ^A is the only essential sigma factor in *B. subtilis*, and it controls the transcription of housekeeping genes in vegetative cells (Haldenwang, 1995). During sporulation, σ^A is present in both the mother cell and the forespore (Yen Shin *et al.*, 2015), and it is bound to a fraction of the core RNA polymerase (Ju *et al.*, 1999; Fujita, 2000). In addition, it has been shown that σ^A is active and that it mediates gene transcription in both cells (Li and Piggot, 2001). However, the actual relevance of σ^A-mediated transcription during sporulation is not known.

To address this question, we used a σ^A-ssrA* version that supported a vegetative growth rate close to that of wild type in the absence of degradation, but did not support growth when $sspB^{Ec}$ expression was induced from a xylose-dependent promoter (Lamsa *et al.*, 2016). First, we studied the effect of cell-specific degradation of σ^A-ssrA* from sustained and late promoters on spore titers, measured as the ability of heat-treated spores to form colonies in the presence of nutrients. In the absence of degradation, a strain containing σ^A-ssrA* showed a mild spore titer reduction of ~2–3 fold compared to the wild type (Fig. 6A), suggesting that the functionality of the tagged protein was somewhat impaired, but still enough to support spore formation. When degradation was induced in the mother cell from the sustained promoter P_{cotE}, there was a modest additional 3-fold reduction in spore titer. No additional reduction was observed when $SspB^{Ec}$ was produced from the late mother cell-specific promoter P_{gerE} (Fig. 6A). These results suggest that σ^A-dependent transcription does not play a major role in the mother cell during sporulation, although it might contribute to replenish the pool of housekeeping proteins early during development. When degradation was induced in the forespore from sustained ($P_{sspE(2G)}$) or late (P_{sspA}) promoters, however, there was a dramatic reduction in the spore titer of ~five orders of magnitude (Fig. 6A). A similar reduction was observed when σ^A-ssrA* was degraded simultaneously in the mother cell and in the forespore (Table S1).

To determine the specific sporulation steps affected by cell-specific degradation of σ^A-ssrA*, we performed microscopy and observed the cytological profiles upon degradation of σ^A-ssrA* from sustained promoters. As shown in Fig. 6B, tagging σ^A with ssrA* had no detectable effect on engulfment or on the formation of phase bright spores, compared to the wild type. When degradation was induced in the mother cell, we observed occasional cell lysis, which might explain the mild spore titer defect obtained upon σ^A-ssrA* degradation in the mother cell (Fig. 6A; Table S1). Nevertheless, a significant fraction of the forespores were phase bright by *t*6. Surprisingly, when σ^A-ssrA* was degraded in the forespore there was no detectable defect in spore formation, indicating that σ^A is not required in the forespore for the production of phase-bright spores. Altogether, these results indicate that σ^A-dependent transcription is dispensable in both the forespore and the mother cell for the production of apparently mature spores.

Forespore σ^A is required for spore germination and outgrowth

The above results suggest that the dramatic spore titer reduction observed when σ^A-ssrA* is degraded in the forespore is not due to defects in forespore development. We noted that when σ^A-ssrA* degradation was induced in the forespore from either sustained or late

promoters, the few colonies formed by the germinating spores after overnight incubation at 30°C were remarkably small (not shown). We therefore reasoned that the spore titer defects observed after degradation of σ^A-ssrA* in the forespore might be the result of deficient germination or outgrowth of spores devoid of σ^A, rather than the consequence of compromised spore development. In agreement with this idea, we observed the appearance of an increasing number of colonies after longer incubation of the plates at 30°C (Fig. S6).

To further explore this possibility, we monitored germination and outgrowth of individual spores over time using phase-contrast timelapse microscopy. During germination, the spore core is rehydrated, which causes the spores to transition from phase-bright to phase-dark. Simultaneously, the spore cortex is degraded and the coat is detached, allowing the germinated spore to resume vegetative growth during a process called outgrowth. We purified spores from the wild type strain, σ^A-ssrA* (no degradation) strain, and from strains in which σ^A-ssrA* degradation was induced in the forespore from either sustained or late promoters. We induced germination by placing the spores on an LB agarose pad supplemented with 10 mM of L-alanine, and used timelapse phase-contrast microscopy to monitor germination and outgrowth over time (Fig. 7A). We also estimated germination efficiency at the population level by monitoring the loss in optical density of spore suspensions upon addition of 10 mM of L-alanine (Fig. S7). σ^A-ssrA* spores exhibited germination kinetics similar to that of wild-type spores (Movie S1 and S2; Fig. S7), with more than 90% phase-dark spores 80 minutes after imaging onset (Fig. 7B). Outgrowth was slightly delayed, however, compared to wild type (Fig. 7A), consistent with the idea that the σ^A-ssrA* fusion protein is not completely functional. Spores from a strain in which σ^A-ssrA* was degraded in the forespore from the late forespore promoter P_{sspA} (Fig. 7A–B; Movie S4; Fig. S7) germinated normally, but showed no outgrowth, demonstrating that σ^A is required for the resumption of vegetative growth after germination, as expected. Surprisingly, spores from a strain in which σ^A-ssrA* was degraded from the sustained promoter $P_{sspE(2G)}$ showed a dramatic germination defect, with only ~50% of the spores transitioning to phase-dark four hours after adding the germinant L-alanine (Fig. 7A–B; Movie S3; Fig. S7). Those spores that germinated showed no outgrowth. This suggests that σ^A activity is required early in sporulation to produce spores that germinate efficiently, and that σ^A activity in the spore is necessary for outgrowth. Potential models to explain these results are discussed below.

Discussion

Here we present a method, called spatiotemporally regulated proteolysis (STRP), to rapidly deplete target proteins in a cell- and developmental stage-specific manner during sporulation in *B. subtilis*. STRP allows the first systematic evaluation of the cell- and developmental-stage specific requirement of proteins made before polar septation, and has the potential to complete the list of the cellular machineries required to assemble a spore. It also provides a versatile new tool to study proteins that play critical roles at several stages of sporulation, such as the peptidoglycan biosynthetic machinery, which is required for polar septation, engulfment, and cortex biosynthesis.

A key feature of STRP is that it is based on the direct degradation of target proteins, rather than on the inhibition of transcription or translation. Our data indicate that target proteins are depleted within minutes after polar septation in the mother cell and in the forespore. This is critical to study the role of specific proteins during sporulation, when the lack of growth prevents the dilution of proteins and mRNAs that is required by other methods such as CRISPR interference.

An essential requirement to achieve the spatiotemporally controlled degradation of target proteins is the use of tightly regulated promoters to drive the expression of *sspB*^{Ec}. The sporulation regulatory program provides an excellent genetic framework for this purpose, since it includes a plethora of promoters that become active in specific cell types and developmental stages. Because these promoters show no ectopic expression, target protein degradation is only triggered in the cell and developmental stage in which the promoters are active. We have explored cell-specific promoters that are continuously active in the mother cell or the forespore after polar septation, which support the sustained degradation of target proteins throughout sporulation, and promoters that become active in either cell only when engulfment is completed, which trigger the late degradation of target proteins. This design provides a systematic method to determine the requirement of target proteins in the mother cell or the forespore at different developmental stages. Proteins required only during engulfment would cause sporulation defects only when subjected to sustained degradation, but not when subjected to late degradation. However, proteins required after engulfment would also produce sporulation defects upon late degradation. Altogether, our results show that STRP is well suited to dissect the spatiotemporal role of proteins made before polar septation, including essential proteins, during development. This opens new avenues in the study of sporulation, since it provides the possibility to investigate how vegetative cellular processes are compartmentalized and coordinated between different cell-types.

We here focus our efforts on the transcriptional machinery of sporulation, which provides an ideal system to test our methodology. In addition, there are key outstanding questions concerning the role played by vegetative transcription factors late in sporulation. We have used STRP to dissect the spatiotemporal requirement of two vegetative σ factors, σ^H and σ^A, during sporulation. σ^H controls gene expression upon entry into stationary phase, and some of the genes in the σ^H regulon are essential for sporulation initiation. Degradation of σ^H-ssrA* in predivisional cells by expressing *sspB*^{Ec} from a high-threshold Spo0A~P dependent promoter that is activated shortly before polar septation (P_{spoIIE}; Fujita *et al.*, 2005) blocks polar septation (Fig. 5C). This suggests that the initial σ^H activity after entry into stationary phase is not sufficient for initiation of sporulation. However, degradation of σ^H-ssrA* after polar septation in the mother cell, forespore or both does not produce any sporulation defects (Fig, 5C). Altogether, these results indicate that σ^H activity is required in predivisional cells until the polar septum is formed, but that after polar septation, σ^H activity is dispensable.

σ^A is required for the transcription of housekeeping genes in vegetative cells, and is the only essential σ factor in *B. subtilis*. During sporulation, σ^A is found in both mother cell and forespore (Yen Shin *et al.*, 2015), and at least a fraction is bound to the core RNA polymerase (Ju *et al.*, 1999; Fujita, 2000). Accordingly, σ^A-dependent transcription has been demonstrated in both cells (Li and Piggot, 2001). However, our results show that σ^A activity

is asymmetrically required in mother cell and forespore. The sustained degradation of σ^A in the mother cell produces only a mild spore titer defect, and no spore titer defect is observed after late degradation (Fig. 6A). These results suggest that σ^A plays a minor role in the mother cell, perhaps to replenish housekeeping functions in sporangia in which they become limiting. It is therefore possible that the σ^A-dependent functions provided before polar septation are enough to support sporulation in most sporangia, or that transcription of key genes under σ^A-control can be mediated by either σ^E or σ^K during sporulation, relieving the need for σ^A-dependent transcription in the mother cell. In support of this latter hypothesis, it has been shown that the transcription of genes involved in the synthesis of peptidoglycan precursors becomes dependent on mother cell-specific σ factors during sporulation (Eichenberger *et al.*, 2003; Eichenberger *et al.*, 2004), which leads to the accumulation of peptidoglycan precursors for cortex synthesis after engulfment (Vasudevan *et al.*, 2007).

Degradation of σ^A in the forespore produces a dramatic spore titer reduction (Fig. 6A) that our data suggest is the result of germination and outgrowth defects (Fig. 6; Fig. 7). Germination of dormant, dehydrated spores is triggered by specific germinants that are recognized by receptors in the inner spore membrane. This triggers the release of dipicolinic acid, probably through channels made of SpoVA proteins, which leads to a partial rehydration of the spore core. Subsequently, cell wall lytic enzymes that degrade the cortex are activated, allowing the full expansion and rehydration of the spore core, in preparation for outgrowth (Setlow, 2014). During outgrowth, the germinated spore is transformed into a rod-shaped, growing, vegetative cell. Outgrowth is completely abolished when σ^A is degraded from either sustained or late promoters (Fig. 7; Movies S3 and S4). This is expected, since σ^A-mediated gene expression is likely required to resume growth after exiting dormancy. It seems reasonable to think that degradation of σ^A-ssrA* in the forespore results in mature spores devoid of σ^A. Therefore, when transcription restarts after germination, σ^A-dependent housekeeping transcripts will not be produced, preventing the growth of the reviving spore. Even if new σ^A-ssrA* molecules are produced during outgrowth, they will be quickly degraded due to the presence of residual $SspB^{Ec}$ in the spores, which will target σ^A-ssrA* for degradation by ClpXP.

The germination defect is only observed when σ^A is continuously degraded in the forespore, starting shortly after polar septation. We conceive two potential models to explain this observation: First, it is possible that σ^A-dependent transcription is required in germinating spores to mediate the transition from phase-bright to phase-dark spores, which is consistent with recent results suggesting that translation happens during germination and that it is required to complete the germination process (Sinai *et al.*, 2015). However, we consider this possibility unlikely, since late degradation of σ^A in the forespore completely blocks outgrowth, but does not produce a significant germination defect (Fig. 7). Of course, it is possible that some intact σ^A remains in the spores after late degradation, and that this supports germination. However, the outgrowth defect after late σ^A-ssrA* degradation argues against this possibility.

The second model to explain the germination defect observed when σ^A is continuously degraded in the forespore is that σ^A-dependent transcription is required in the developing forespore, early during spore formation, to produce germination-proficient spores. In this

context, it is possible that specific factors produced in the forespore under σ^A-control are required for the germination of mature spores. Candidates for such factors are translation proteins that are required for efficient germination, which must be present in mature spores or quickly synthesized at the onset of germination (Sinai *et al.*, 2015). It is also possible that σ^A-dependent transcription plays a more general role in developing forespores, by providing housekeeping functions that confer to the forespore an optimal metabolic or structural state that promotes the formation of a functional germination machinery. Nevertheless, further experiments are required to clarify the requirement of σ^A in the forespore for germination.

Altogether, these results outline a potential application of STRP to identify cellular pathways involved in spore germination and outgrowth. This is particularly appealing, since it is unclear whether specific biosynthetic activities are required in the dehydrated, dormant spore to mediate germination (Sinai *et al.*, 2015; Boone and Driks, 2016; Korza *et al.*, 2016; Sinai and Ben-Yehuda, 2016).

As discussed above, the use of targeted degradation to deplete proteins has key advantages compared to other methods. However, it also has limitations that are important to consider. Perhaps the most apparent limitation is that not all proteins are suitable for degradation. The ssrA*/SspB^{Ec} degradation system requires that target proteins have an accessible cytoplasmic C-terminus for degradation, which excludes all extracellular proteins and membrane proteins with extracytoplasmic C-termini as potential targets, as well as cytoplasmic proteins in which the tag is sterically inaccessible due to the folding of the protein. The use of degradation systems based on N-terminal tags might provide an alternative to degrade some of the membrane proteins with extracytoplasmic C-termini (Sekar *et al.*, 2016). Second, the inducible protein degradation system entails the modification of the target protein by adding the fifteen amino acid ssrA* tag, which might significantly affect protein functionality. However, ~95% of the ~200 proteins we have tagged so far (see below) tolerate the addition of the tag, suggesting that the ssrA* tag does not tend to significantly interfere with protein folding nor function.

Altogether, our results show that STRP holds great potential for providing a comprehensive molecular dissection of not only spore development, but also germination and outgrowth. We have undertaken a genome-wide approach to tag with ssrA* every essential protein with cytoplasmic C-termini in *B. subtilis*. The systematic exploration of our collection will provide a comprehensive overview of the spatiotemporal role of essential vegetative cellular pathways during the different developmental stages in *B. subtilis*.

Experimental procedures

Strain construction

All the strains used in this study are derivatives of *B. subtilis* PY79, and are listed in Table S2. A list of plasmids and primers as long with detailed descriptions of plasmid construction, is provided in supplementary information (Tables S3 and S4). In every case, ssrA* was added to the appropriate gene in its native locus. The amino acid sequence of the ssrA* used in our constructs was AANDENYSENYALGG, and was previously described by Griffith and Grossman (2008). *sspB^{Ec}* was inserted in different ectopic loci in *B. subtilis*

chromosome, depending on the cell in which expression was taking place. For forespore-specific expression, we selected the *amyE* locus, which is trapped in the forespore after polar septation (Wu and Errington, 1998), allowing immediate expression before the forespore chromosome is fully translocated. For mother cell-specific expression, we inserted $sspB^{Ec}$ in the terminus-proximal *pelB* locus, which encodes a pectate lyase involved in polygalacturonic acid degradation. Because *pelB* (174°) is located in the chromosome close to the *dif* site (172°) (Sciochetti *et al.*, 2001), it is one of the last loci to be translocated to the forespore (Ptacin *et al.*, 2008), which can transiently boost $sspB^{Ec}$ expression in the mother cell after polar septation. We previously inserted $sspB^{Ec}$ in the ectopic *thrC* locus for mother cell-specific expression. This creates a threonine auxotrophy, and therefore, the sporulation medium has to be supplemented with threonine. Under those conditions sporulation progress normally through engulfment, but we have observed that forespore maturation is delayed compared to threonine prototroph strains (not shown). For expression in predivisional cells, $sspB^{Ec}$ was inserted in the *lacA* locus. Xylose inducible expression was achieved by inserting $sspB^{Ec}$ in the *xylA* locus (Lamsa *et al.*, 2016), which simultaneously creates a mutant unable to use xylose as a carbon source, preventing the depletion of the inducer.

Culture conditions

For microscopy experiments, sporulation was induced by resuspension (Sterlini and Mandelstam, 1969), except that the bacteria were grown in 25% LB prior to resuspension, rather than CH medium. Cultures were grown at 37°C for batch culture microscopy experiments, and at 30°C for timelapse fluorescence microscopy experiments.

For determination of heat-resistant spore titers and spore purification, cultures were grown and sporulated in DSM broth (Schaeffer *et al.*, 1965).

To study YtsJ-GFP-ssrA* degradation and P_{xylA} expression in vegetative cells (Fig. S3), cultures of the appropriate strains were grown in LB at 37°C until $O.D._{600}$ 0.2. The cultures were than split into 2 ml aliquots and incubated for 45 minutes in LB or LB supplemented with different concentrations of xylose, at 37°C. Then 12 µl aliquots were used for microscopy, and 1 ml aliquots were used for protein extraction for Western blot.

Spore titer assay

Strains were cultured in 2 ml of DSM for 24 h at 37°C. Cultures were then heated at 80°C for 20 min, serially diluted, and plated on LB. Spore titers were calculated based on colony counts.

Spore purification

Sporulation was induced by exhaustion in 250 ml of DSM. Strains were grown for 72 h and then collected and washed with 4°C sterile water. The spores were incubated in sterile water at 4°C to lyse any remaining vegetative cells. Spores were further purified over a phosphate-polyethylene glycol aqueous biphasic gradient (Harrold *et al.*, 2011). The top organic phase containing the spores was removed and extensively washed in at least 50 vol of 4°C sterile water. The spores were pelleted, resuspended in sterile water, and stored at 4°C. Sample

purity was checked using phase contrast microscopy. Spore samples of >95% purity (phase-bright spores) were used in the studies.

Batch culture microscopy

Samples from sporulation cultures were taken at the indicated time points and transferred to 1.2% agarose pads for imaging. When appropriate, membranes were stained with 0.5 μg ml^{-1} FM 4–64 (Life Technologies) and 1 μg ml^{-1} Mitotracker green (Life Technologies). FM 4–64 was added to the agarose pad, whereas Mitotracker green was mixed with the cells before transferring them to the pad. Cells were visualized on an Applied Precision DV Elite optical sectioning microscope equipped with a Photometrics CoolSNAP-HQ^2 camera. Pictures were deconvolved using SoftWoRx v5.5.1 (Applied Precision). The median focal plane is shown.

Timelapse fluorescent microscopy to visualize YtsJ-GFP-ssrA* degradation

Sporulation was induced at 30°C. 1.5 h after sporulation induction, 0.5 μg ml^{-1} FM 4–64 was added to the culture and incubation continued for another 1.5 h. Seven-μl samples were taken 3 h after resuspension and transferred to agarose pads prepared as follows: 2/3 volume of supernatant from the sporulation culture; 1/3 volume 3.6% agarose in fresh A+B sporulation medium; 0.17 μg ml^{-1} FM 4–64. Pads were partially dried, covered with a glass slide, and sealed with petroleum jelly to avoid dehydration during timelapse imaging. Pictures were taken in an environmental chamber at 30°C every 5 min for 5 h. To visualize FM 4–64 stained membranes, excitation/emission filters were TRITC/CY5, excitation light transmission was set to 5%, and exposure time was 0.1 s. To visualize GFP signal, excitation/emission filters were FITC/FITC, excitation light transmission was set to 50%, and exposure time was 0.5 s.

Quantification of YtsJ-GFP-ssrA* degradation kinetics

To calculate YtsJ-GFP-ssrA* degradation kinetics, we measured the mean cytoplasmic GFP fluorescence in the mother cell and the forespore over time by drawing polygons encompassing either cell. After subtracting the mean background fluorescence, we calculated the mean fluorescence ratio between the cell in which degradation was triggered and the other cell of the same sporangium. This allowed us to correct for GFP photobleaching. We defined time zero as the time frame immediately before GFP degradation was first observed, and relativized the fluorescence ratios of subsequent time points with respect to time zero, that was arbitrarily given the value of one. The decrease in the fluorescence ratio over time followed an exponential decay curve. We fitted the data for each sporangia to an exponential decay function and determined the protein half-life upon degradation induction for individual sporangia. The reported half-lives are the averages ± standard deviations of 17 and 14 sporangia for forespore and mother cell degradation, respectively. The graph in Fig. 4C shows the average fluorescence ratio of the different sporangia over time.

Timelapse phase contrast microscopy to visualize spore germination and outgrowth

Purified spores were diluted in water to an $O.D._{600}$ of 0.3, and 10 μl of the spore suspension were placed on 1.2% agarose pads prepared in LB and supplemented with the germinant L-alanine (10 mM). Pads were partially dried, covered with a glass slide, and sealed with petroleum jelly to avoid dehydration during timelapse imaging. Phase contrast pictures were taken at 30°C every 3 minutes during 10 hours. Light transmission was set to 32% and exposure time was 0.1 s. Note that, after placing the spores in the agarose pads, there was a time lag of ~15 minutes until we started taking pictures. To minimize the number of spores that germinated during this time, spores were not heat-activated before timelapse microscopy. We quantified the number of spores that transitioned from phase-bright to phase-dark during the first four hours of imaging, focusing only on spores that were phase bright at the onset of imaging.

Quantification of spore germination by loss of optical density

Germination assays by loss of $O.D._{580}$ were performed as described in Harwood et al. 1990. Briefly, spores were heat activated at 70°C for 20 min in water, and resuspended in 10 mM Tris-HCl, pH 8.4, to obtain an $O.D._{580}$ of 0.3. The resuspended spores were incubated at 37°C for 20 min, and then germination was induced by the addition of L-alanine 10 mM. $O.D._{580}$ was measured every 5 min for an hour.

Protein extracts and Western blot analysis

Bacterial cultures were grown as described in “Culture conditions”. Bacterial cells present in 1 ml aliquots of the cultures were collected by centrifugation (5,000 × g, 2 min) and suspended in 60 μl of Tris-sucrose buffer [33 mM Tris-HCl, pH8.0; 40% sucrose; 1 mM EDTA] containing 1 mg ml^{-1} lysozyme, 160 μg ml^{-1} PMSF, and 1:100 diluted Sigma P2714 protease inhibitor cocktail, and incubated at 37°C for 10 min. Then, membranes were dissolved by adding 2 μl of Triton X-100, samples were mixed with 60 μl of Laemmli loading buffer [1.3% SDS; 10% (v/v) glycerol; 50 mM Tris-HCl; 1.8% β-mercaptoethanol; 0.02% bromophenol blue, pH 6.8] and boiled for 10 min. Proteins present in 5-μl aliquots of each sample were resolved by Tris-Tricine-PAGE, using 12% gels. Conditions for protein transfer have been described elsewhere. Optimal dilutions of anti-$SspB^{Ec}$ and anti-σ^{A} primary antibodies were 1:5,000. Goat anti-rabbit horseradish peroxidase-conjugated antibody (1:20,000) was used as secondary antibody. Proteins recognized by the antibodies were visualized by chemoluminescence using the luciferin-luminol reagents, in a BioRad Gel Doc XR+ System. For quantification, the intensity of the bands was determined using ImageJ. σ^{A} was used as loading control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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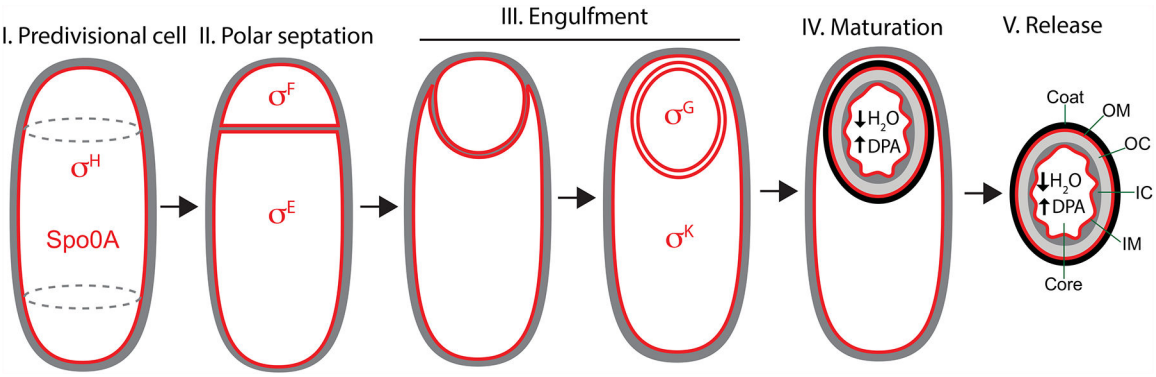

Figure 1. The sporulation pathway in *B. subtilis*.

Membranes are in red, and peptidoglycan in gray. Transcription factors active in different cells and stages are shown red. I. In predivisional cells, the division sites (dotted ovals) are shifted to polar positions. II. Polar septation produces a small forespore and a large mother cell. III. After polar septation, the mother cell engulfs the forespore in a phagocytosis-like process. After engulfment, the forespore is enclosed within the mother cell cytoplasm and delimited by two membranes. IV. The forespore matures through the formation of a peptidoglycan cortex (gray) between the inner and outer forespore membranes, the assembly of a proteinaceous coat around the outer membrane (black), and the dehydration of the forespore core, which accumulates dipicolinic acid (DPA). V. Once maturation is completed, the mother cell lyses and the spore is released into the environment, where it remains dormant until conditions are appropriate for germination. OM, outer membrane; OC, outer cortex; IC, inner cortex; IM, inner membrane.

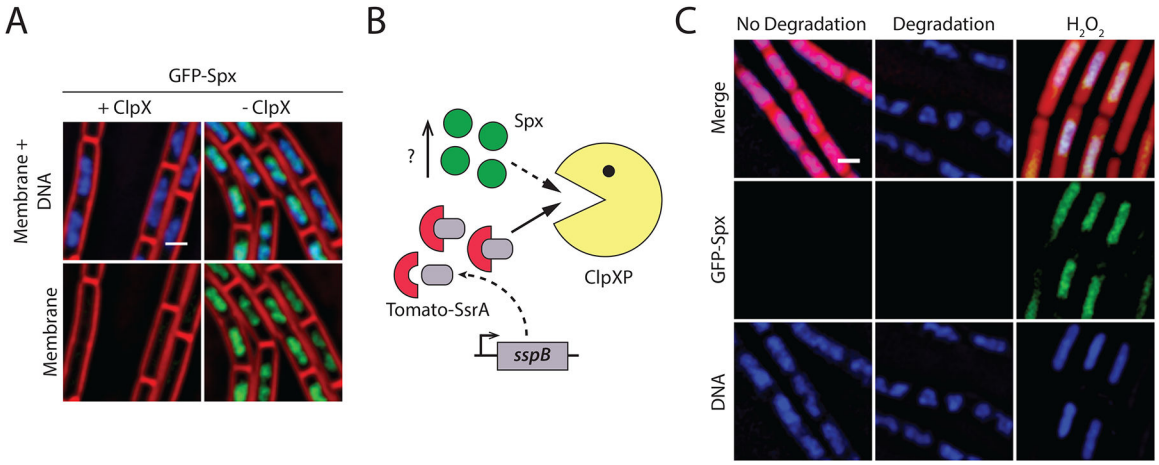

Figure 2. Degradation of an abundant protein does not affect turnover of a native ClpXP substrate.

A. Fluorescence microscopy of a strain carrying a GFP-Spx fusion that expresses ClpXP under the control of an IPTG-inducible promoter (BER0552), grown in the presence (left, +ClpXP) or absence (right, -ClpXP) of 1mM IPTG. When ClpX is limiting, GFP-Spx accumulates. Membranes are stained with FM4–64 (red) and DNA with DAPI (blue). Scale bar, 1μm.

B. Inducing degradation of abundant ssrA*-tagged proteins should cause accumulation of GFP-Spx if ClpXP is saturated.

C. Fluorescence microscopy of a strain carrying a GFP-Spx fusion, producing the red fluorescent protein, tomato, from a strong promoter and $SspB^{Ec}$ from a xylose-inducible promoter (BER0577), grown in the absence (left, no degradation) or in the presence (middle, degradation) of 1% xylose, or with 2mM H_2O_2 (right) as a positive control. Imaging was performed 2 hours after treatment. Inducing degradation does not yield an accumulation of GFP-Spx, but treatment with hydrogen peroxide, which is known to evoke the Spx response, does. Membranes are stained with FM4–64 (red) and DNA with DAPI (blue). Scale bar, 1 μm.

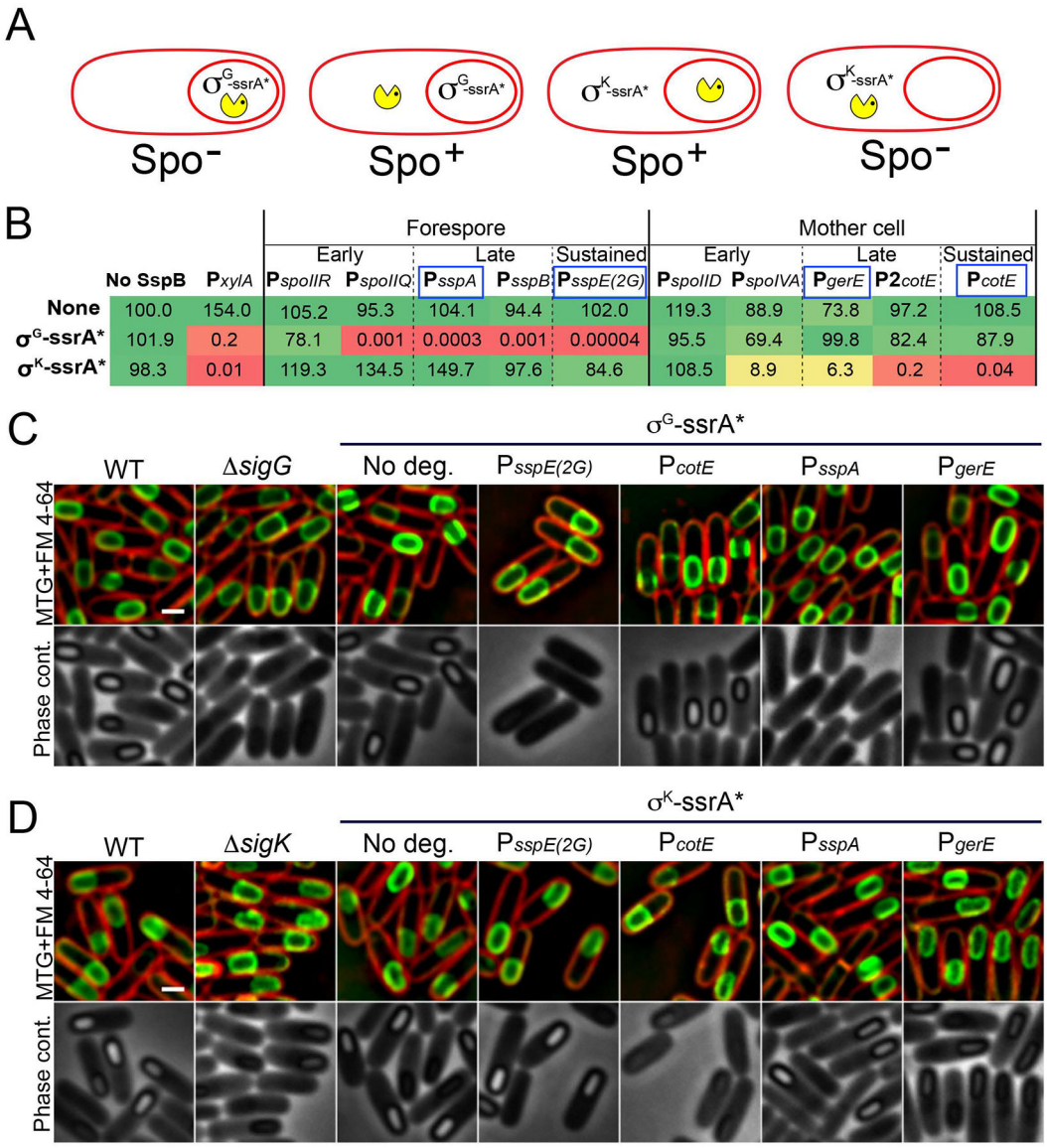

Figure 3. Optimization of cell- and stage-specific protein degradation system.

A. Diagram representing the genetic strategy used to screen for cell-specific promoters that trigger the efficient degradation of target proteins when driving the expression of *sspB*^{Ec}. We constructed ssrA*-tagged versions of the σ^{G} and σ^{K}, which are essential for sporulation and required in the forespore and mother cell, respectively. Degradation of either protein in the cell in which they are required should yield a strong sporulation defect (Spo^{-}), while expression of *sspB*^{Ec} in the opposite cell should have no effect (Spo^{+}).

B. Relative spore titers of strains expressing *sspB*^{Ec} from different cell-specific promoters (columns) in the absence of ssrA*-tagged protein (none), when σ^{G} is tagged with ssrA* (σ^{G}-ssrA*) or when σ^{K} is tagged with ssrA* (σ^{K}-ssrA*). To facilitate data comparison, the spore titer of the wild type strain (no ssrA*, no SspB^{Ec}) was relativized to 100. The actual values are shown in Table S1. The color indicates the severity of the spore titer defect (green, no defect; yellow, moderate defect; red, severe defect). Expression from P$_{xylA}$ was induced by

the addition of 1 % xylose. Sporulation-specific promoters promoters were classified in three temporal classes according to their expression pattern: early promoters are activated shortly after polar septation, and inactivated after engulfment; late promoters activated after engulfment; sustained promoters are continuously active throughout engulfment and forespore maturation. The promoters selected for further characterization are indicated by a blue box.

C. Micrographs of wild type sporangia (WT), σ^G mutant sporangia (Δ*sigG*) and sporangia in which σ^G is tagged with ssrA* (σ^G-ssrA*) in the absence of $SspB^{Ec}$ (No deg.) or when $SspB^{Ec}$ is produced from the sustained promoters $P_{sspE(2G)}$ and P_{cotE} or the late promoters P_{sspA} and P_{gerE}. The top row shows membranes stained with FM 4–64 (red) and Mitotracker green (green). The bottom row shows phase contrast pictures. Pictures were taken six hours after sporulation induction (*t6*). Scale bar, 1 μm.

D. Micrographs of wild type sporangia (WT), σ^K mutant sporangia (Δ*sigK*) and sporangia in which σ^K is tagged with ssrA* (σ^K-ssrA*) in the absence of $SspB^{Ec}$ (No deg.) or when $SspB^{Ec}$ is produced from the sustained promoters $P_{sspE(2G)}$ and P_{cotE} or the late promoters P_{sspA} and P_{gerE}. The top row shows membranes stained with FM 4–64 (red) and Mitotracker green (green). The bottom row shows phase contrast pictures. Pictures were taken six hours after sporulation induction (*t6*). Scale bar, 1 μm.

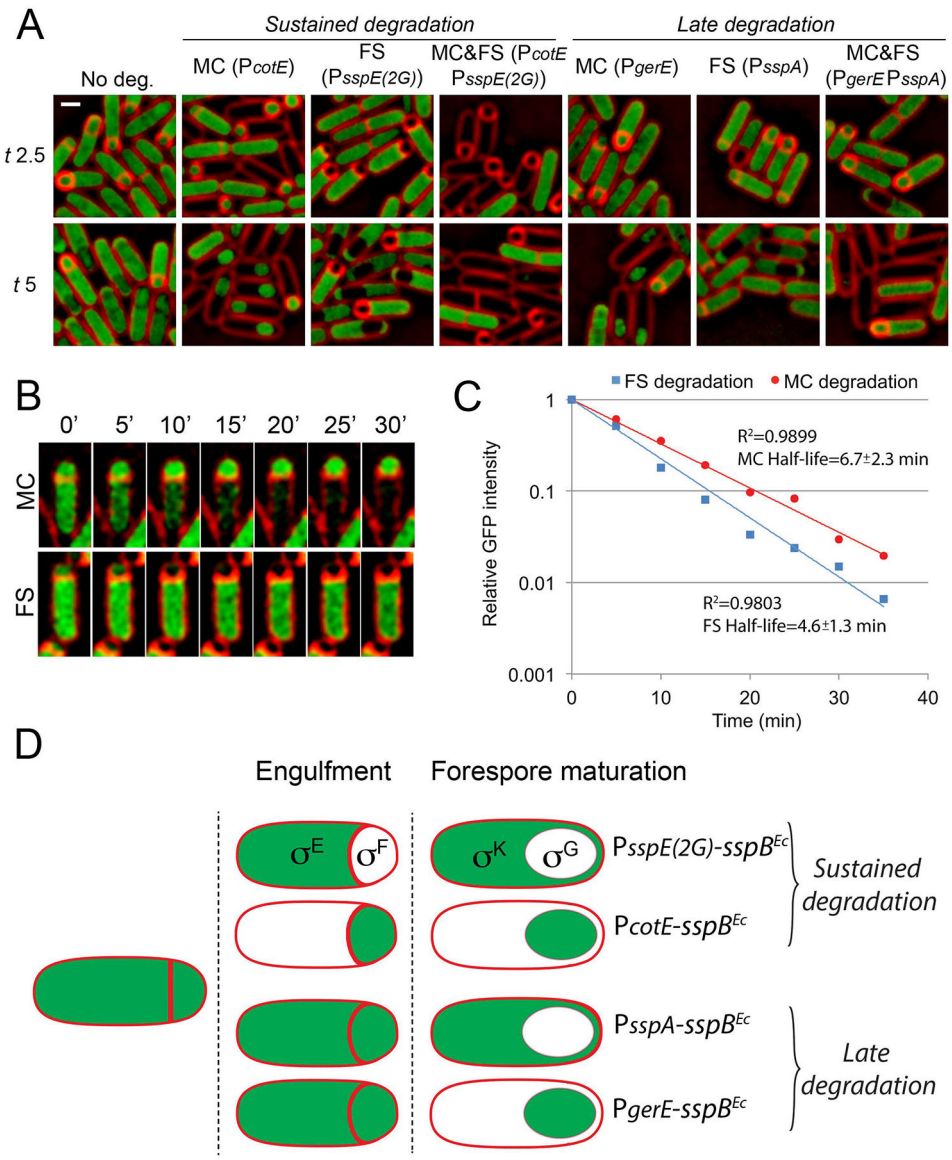

Figure 4. Cell- and stage-specific degradation of vegetative proteins.

A. Fluorescence microscopy showing the cell-specific degradation of YtsJ-GFP-ssrA*. SspB^{Ec} was produced from sustained promoters (indicated in parentheses) in the mother cell (MC), forespore (FS), or both (MC&FS) to induce the sustained degradation of YtsJ-GFP-ssrA*. To induce degradation only after engulfment (late degradation), *sspB^{Ec}* was expressed from late promoters. Membranes were stained with FM 4–64. Scale bar, 1 μm.

B. Timelapse microscopy showing the disappearance of YtsJ-GFP-ssrA* fluorescence signal when degradation is induced in the mother cell (MC, *sspB^{Ec}* expressed from P_{cotE}) or in the forespore (FS, *sspB^{Ec}* expressed from $P_{sspE(2G)}$). Images taken every 5 minutes are shown. Membranes were stained with FM 4–64.

C. YtsJ-GFP-ssrA* degradation kinetics when SspB^{Ec} is produced in the forespore (blue squares and line) or in the mother cell (red circles and line). In both cases, the disappearance of the GFP signal follows an exponential decay. The coefficient of determination of the

regression curves (R^2), and the half-lives of YtsJ-GFP-ssrA* when degradation is induced in the mother cell or the forespore are indicated in the graph. Data represent the average of 17 and 14 sporangia for forespore and mother cell degradation, respectively.

D. STRP setup. The use of sustained promoters ($P_{sspE(2G)}$ and P_{cotE}) allows the sustained degradation of target proteins in the mother cell or the forespore. Expression of $sspB^{Ec}$ from late promoters (P_{sspA} and P_{gerE}), however, triggers degradation of target proteins only after engulfment is completed. The combination of both classes of cell-specific promoters allows the evaluation of the spatial and temporal requirement of proteins made before polar septation.

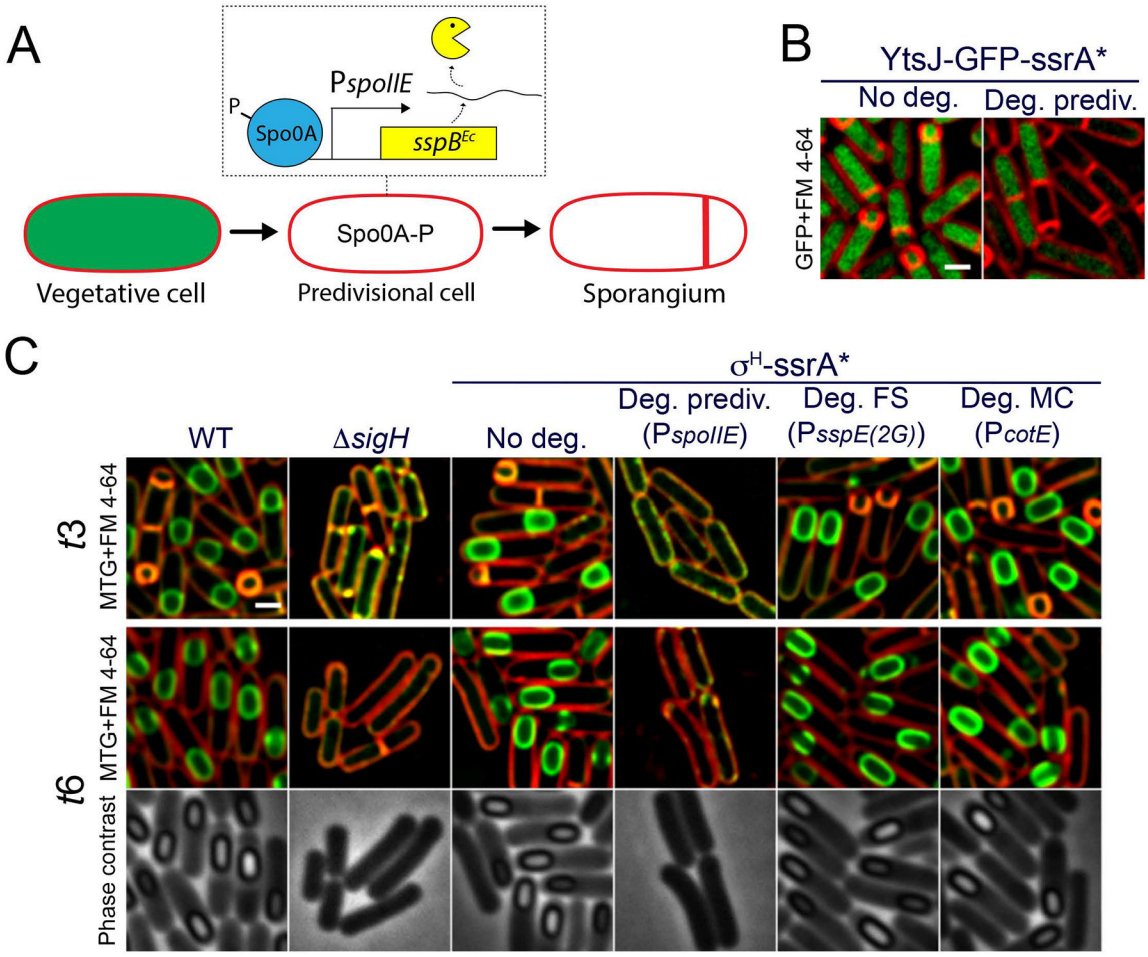

Figure 5. σ^H activity is only required in predivisional cells.

A. Diagram representing the strategy used to induce the degradation of target proteins in predivisional cells about to undergo polar septation. Before polar septation, Spo0A becomes phosphorylated and controls the expression of several genes required for entry into sporulation. Expression of *sspB*^{Ec} from promoters activated by Spo0A would trigger degradation in predivisional cells, before polar septation occurs. We specifically used the promoter of *spoIIE* to drive *sspB*^{Ec} expression.

B. Degradation of YtsJ-GFP-ssrA* in predivisional cells. Membranes were stained with FM 4–64. Scale bar, 1 μm.

C. Micrographs of wild type sporangia (WT), σ^H mutant sporangia (Δ*sigH*) and sporangia in which σ^H is tagged with ssrA* (σ^H-ssrA*) in the absence of SspB^{Ec} (No deg.) or when SspB^{Ec} is produced in predivisional cells from P_{spoIIE} (Deg. prediv.), in the forespore from $P_{sspE(2G)}$ (Deg. FS) or in the mother cell from P_{cotE} (Deg. MC). Pictures were taken 3 (*t3*) and 6 (*t6*) hours after sporulation induction. Membranes stained with FM 4–64 (red) and Mitotracker green (green) are shown for *t3* and *t6*. Phase contrast pictures are only shown for *t6*. Scale bar, 1 μm.

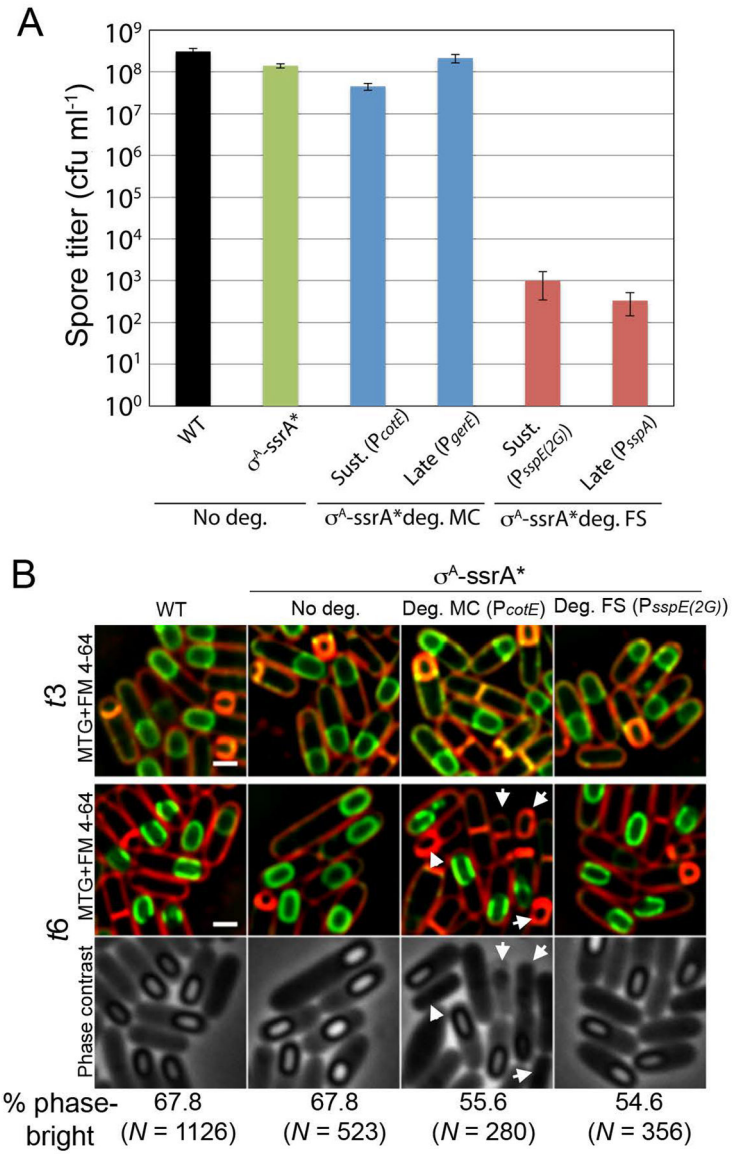

Figure 6. Cell-specific degradation of σ^A.

A. Spore titers, measured as the number of colony forming units per ml (cfu ml^{-1}) in heat-killed sporulating cultures, of wild type (WT) and σ^A-ssrA* strains, and strains in which σ^A-ssrA* degradation is triggered in the mother cell (σ^A-ssrA* deg. MC) or in the forespore (σ^A-ssrA* deg. FS) from sustained (Sust.) or late promoters. The promoters used to drive the expression of *sspB*^{Ec} are indicated in parentheses. Data represent the average and standard deviation of at least three independent experiments.

B. Micrographs of wild type sporangia (WT) and σ^A-ssrA* sporangia in the absence of degradation (No deg.) or when degradation is induced in the forespore (Deg. FS), mother cell (Deg. MC) or both (Deg. both) from sustained promoters (in parentheses). Pictures were taken 3 (*t3*) and 6 (*t6*) hours after sporulation induction. Lysed cells upon mother cell degradation at *t6* are indicated by arrows. The percentage of sporangia containing phase-bright spores at *t6* is shown at the bottom, including the umber of sporangia (*N*) analyzed for

each strain. Lysed sporangia were excluded from the quantification. Membranes stained with FM 6–64 (red) and Mitotracker green (green) for each time point. Phase contrast pictures are shown in the bottom row for *t6*. Scale bar, 1 μm.

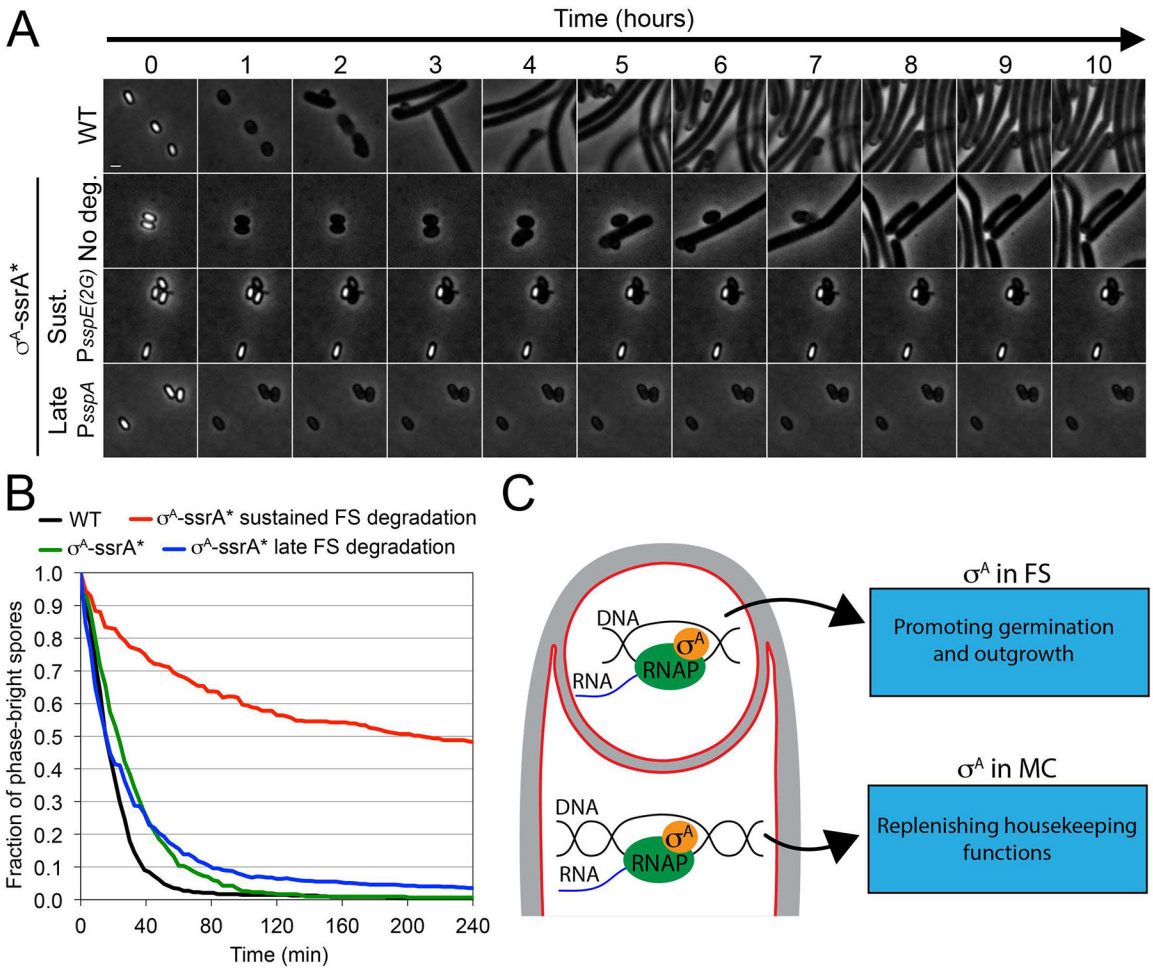

Figure 7. Forespore σ^A contributes to spore germination and outgrowth

A. Phase-contrast timelapse microscopy of germinating spores from wild type (WT), σ^A-ssrA* (No deg.), σ^A-ssrA* sustained forespore degradation (Sust.) and σ^A-ssrA* late forespore degradation (Late) strains. Images taken every hour for 10 hours are shown. Complete time series of images taken every 3 minutes for 10 hours are provided in movies S1–S4.

B. Fraction of phase-bright spores over time after germination induction, calculated from timelapse microscopy shown in B. Black line, wild type spores (N = 547); green line, σ^A-ssrA* spores (N = 318); red line, σ^A-ssrA* sustained forespore degradation spores (N = 434); blue line, σ^A-ssrA* late forespore degradation spores (N = 668). The fraction of phase bright spores at the onset of imaging was relativized to 1 for every strain. More than 84% of the spores were phase-bright initially.

C. σ^A-dependent transcription in the mother cell might contribute to replenish housekeeping functions during sporulation. In the forespore, σ^A-dependent transcription makes critical contributions to the germination and outgrowth of mature spores.