Phosphatases modulate the bistable sporulation gene expression pattern in *Bacillus subtilis*

Jan-Willem Veening, Leendert W. Hamoen, and Oscar P. Kuipers*

Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, the Netherlands.

Summary

Spore formation in the Gram-positive bacterium *Bacillus subtilis* is a last resort adaptive response to starvation. To initiate sporulation, the key regulator in this process, Spo0A, needs to be activated by the so-called phosphorelay. Within a sporulating culture of *B. subtilis*, some cells initiate this developmental program, while other cells do not. Therefore, initiation of sporulation appears to be a regulatory process with a bistable outcome. Using a single cell analytical approach, we show that the autostimulatory loop of *spo0A* is responsible for generating a bistable response resulting in phenotypic variation within the sporulating culture. It is demonstrated that the main function of RapA, a phosphorelay phosphatase, is to maintain the bistable sporulation gene expression. As *rapA* expression is quorum regulated, it follows that quorum sensing influences sporulation bistability. Deletion of *spo0E*, a phosphatase directly acting on Spo0A-P, resulted in abolishment of the bistable expression pattern. Artificial induction of a heterologous Rap phosphatase restored heterogeneity in a *rapA* or *spo0E* mutant. These results demonstrate that with external phosphatases, *B. subtilis* can use the phosphorelay as a tuner to modulate the bistable outcome of the sporulating culture. This shows that *B. subtilis* employs multiple pathways to maintain the bistable nature of a sporulating culture, stressing the physiological importance of this phenomenon.

Introduction

Spore formation in *Bacillus subtilis* is a complex adaptive response to starvation (Sonenshein, 2000). The process of sporulation is governed by a multicomponent phosphorelay which consists of five histidine kinases (KinA, KinB, KinC, KinD and KinE) and two phosphorelay proteins (Spo0F and Spo0B) (Perego and Hoch, 2002) (see Fig. 1). Multiple environmental and physiological signals are fed into this system, and under appropriate conditions this leads to phosphorylation of Spo0A, the key sporulation transcription factor (for reviews see Grossman, 1995; Sonenshein, 2000). Within an isogenic culture of sporulating *B. subtilis*, some cells initiate the developmental program of sporulation, whereas others do not (Chung et al., 1994). Therefore, initiation of sporulation appears to be a regulatory process with a bistable outcome. Phenotypic variation could benefit the fitness of the species, because the heterogeneous population is able to quickly react to changing environments (Balaban et al., 2004). As sporulation is an energy intensive process, and irreversible after its earliest stage (Parker et al., 1996), cells that are delayed to commit to sporulate could have an advantage over sporulating cells if food resources were to become plentiful again. Theoretical modelling and experiments in both prokaryotic and eukaryotic model systems have demonstrated that positive feedback of a transcriptional regulator, together with a non-linear response to an activator, can lead to a bimodal probability distribution in expression (Hasty et al., 2000; Becskei et al., 2001; Isaacs et al., 2003). It is believed that stochastic fluctuations cause some cells to reach the threshold level to activate the feedforward loop and these cells will end up in the 'high expressing' population, whereas others do not reach this threshold and remain in the 'low expressing' state (Ferrell, 2002; Rao et al., 2002). In the present study, we examined whether the positive feedback architecture of *spo0A* regulation is responsible for bistability in sporulation gene expression and studied the influence of various phosphatases on the maintenance of bistability.

Activity of Spo0A is subject to several auto-stimulatory loops (Strauch et al., 1992; Strauch et al., 1993; Fujita and Sadaie, 1998). These loops involve transcription of *spo0A* and phosphorylation of Spo0A. Transcription of *spo0A* is directly activated by Spo0A-P and indirectly activated by induced expression of *sigH*. The *sigH* gene encodes an RNA polymerase sigma factor (σ^H^) that recognizes an alternative promoter located upstream of *spo0A* (Predich et al., 1992) and activates transcription of genes involved in the phosphorylation of Spo0A such as kinA and spo0F.
exponential growth, AbrB represses expression of kinA

KinA is the primary kinase in the phosphorelay and is necessary for the phosphorylation of Spo0A (Burbuly
s, 1991). A major role of phosphorylated Spo0A is to repress the expression of abrB, a gene encoding a
transcriptional regulator that represses various stationary phase processes (Robertson et al., 1989). During exponen
tial growth, AbrB represses expression of sigH, kinA and abrB itself (Strauch, 1995). Thus, alleviation of AbrB repression by Spo0A–P at the beginning of the stationary growth phase, stimulates sigH and kinA expression and therefore spo0A transcription and indirectly phosphorylation of Spo0A (see Fig. 1). In conclusion, the complex autostimulation of spo0A could be the basis of the bistable sporulation gene expression.

KinA is the primary kinase in the phosphorelay and is necessary for the phosphorylation of Spo0A (Burbuly
er al., 1991). It has been demonstrated that the fraction of cells that initiate sporulation is decreased in a kinA mutant background (Chung et al., 1994). This result suggests that a certain threshold concentration of Spo0A–P is necessary to initiate sporulation and that the activity of the phosphorelay determines the threshold level for autostimulation of Spo0A. This implies that influences on the phosphorelay by external phosphatases could alter the heterogeneous sporulation gene expression. An interesting candidate is RapA. RapA is an aspartyl-phosphatase that dephosphorylates one of the components of the phosphorelay, Spo0F–P, and consequently reduces accumulation of Spo0A–P (Perego and Hoch, 2002) (see Fig. 1). In an earlier study it was shown that rapA, by that time known as gsiAA (glucose starvation induced protein A), was activated under glucose limiting conditions and high cell densities, signals which also trigger initiation of sporulation (Mueller et al., 1992). Apparently, RapA increases in concentration coincidentally with the activation of Spo0A–P. This seeming contradiction suggests that RapA might play a role in the heterogeneous induction of sporulation. This possibility is especially interesting because RapA activity is subject to a complex quorum sensing regulation. First of all, expression of rapA is acti
vated by the ComA-ComP two-component signal transduction system, which is activated by cell density signals via the quorum sensor molecule, ComX (Mueller et al., 1992; Lazazzera et al., 1999). This regulatory system is essential for another stationary growth phase process; the development of genetic competence. Secondly, the phosphatase activity of RapA is negatively modulated by a small protein, PhrA, encoded by an adjacent gene on the same transcript as rapA. PhrA is exported by the SecA-dependent system and subsequently processed to the carboxyl-terminal five-amino acid active inhibitor (ARNTQ). The pentapeptide presumably functions as a quorum sensor signal. PhrA is re-imported by the oligopeptide permease transport system (Opp) and inhibits the dephosphorylating action of RapA (Perego and Hoch, 2002). Deletion of the phrA gene causes uncontrolled RapA phosphatase activity leading to poor sporulation (Perego and Hoch, 1996).

To study the role of the RapA-PhrA system and the autostimulation of Spo0A in sporulation heterogeneity, a single cell approach was used. By flow cytometric analyses and fluorescence microscopy, we demonstrate that the autostimulatory activation of spo0A is responsible for the bistable expression pattern in sporulating cultures. In addition, we show that external phosphatases, such as RapA, Spo0E and Rap60, can modulate sporulation bistability.

Fig. 1. Simplified schematic representation of the regulatory network that governs initiation of sporulation. Perpendiculars and arrows represent the negative and positive actions respectively.

**Results**

**Initiation of sporulation is heterogeneous**

When *B. subtilis* cells reach the end of exponential growth, and various environmental signals promote the activation of the sporulation response regulator Spo0A, this protein in turn activates more than 40 genes directly, including the sigma-H dependent spoIIA operon comprising the early sporulation genes spoIIAA, spoIIAB and sigF (Molle et al., 2003). To determine how the initiation of sporulation is distributed among cells within an isogenic population, we constructed a strain in which the gfp gene (encoding Green Fluorescent Protein) is under control of the spoIIA promoter (integrated at the spoIIA promoter region, leaving the original operon intact). The resulting strain, IIAgfp (PspoIIA-gfp), was grown in sporulation medium (SM), and...
cells were collected at different time points for analysis by flow cytometry (Fig. 2). As a control for GFP specific fluorescence, parental B. subtilis 168 strain was analysed as well (indicated with the asterisk). Arrows indicate cells in a low and high spoIIA expression state.

Activation of the spoIIA promoter is not subject to transcriptional noise

Previous studies have shown that substantial stochastic fluctuations in promoter activity may account for non-genetic variability within isogenic populations (McAdams and Arkin, 1997; Elowitz et al., 2002; Ozbudak et al., 2002; Blake et al., 2003; Raser and O’Shea, 2004). Theoretically, such stochastic effects could contribute to the large cell-to-cell variation in spoIIA expression. Intrinsic noise of a given gene can be defined as the extent to which the activities of two identical copies of that gene, in the same intracellular environment, fail to correlate. To verify that the observed heterogeneity is not a consequence of intrinsic transcriptional noise of the spoIIA promoter itself, a double-labelled strain that enables the detection of stochasticity in gene expression was constructed. Therefore, the spoIIA promoter was fused to improved variants of the cyan- and yellow fluorescent proteins (ICFP and IYFP, respectively, Veening et al., 2004). In the resulting strain (IIA/double), the P_\text{spoIIA-icfp} construct was integrated at the amyE locus and the P_\text{spoIIA-iyfp} construct was integrated at the spoIIA promoter region (a map showing the chromosomal locations of the constructs is indicated in Fig. S2 of the Supplementary material). The experimental set-up and fluorescence microscopy measurements were performed in a similar fashion as described by Elowitz et al. (2002). They showed that in the absence of intrinsic noise, the CFP and YFP signals fluctuate in a correlated fashion over time in a single cell. However, the amount of fluorescent proteins produced will differ between cells because of extrinsic noise. The correlated YFP and CFP signals in the individual cells in Fig. 3, suggest that the levels of intrinsic noise are negligible for the spoIIA promoter (cells that exhibit ICFP production, expressed from the spoIIA promoter at the amyE locus, show a similar level of IYFP expressed from the spoIIA promoter at the spoIIA promoter region). The results of a more quantitative analysis are presented in Table 1. To quantify noise, a statistical analysis was performed in a similar fashion as described previously (Elowitz et al., 2002). Strain IIA/double was grown in SM and cells were collected for fluorescent microscopy two hours after entry into stationary growth phase. Images were analysed and noise data were calculated as described in the Experimental procedures. Table 1 shows the calculated noise levels for strain IIA/double. The value for intrinsic noise (\(\eta_{\text{intr}}\)) in strain IIA/double was approximately 0.09, indicating that the two colours, on average, differ by about 0.09% within individual cells. In the work of Elowitz et al. (2002), similar intrinsic noise values were obtained with *Escherichia coli* strains that displayed low levels of intrinsic noise (using strong constitutive promoters). The extrinsic noise levels were somewhat higher compared with the levels of intrinsic noise (\(\eta_{\text{extr}}\sim 0.13\)) but still quite low. This indicates that once a cell is activated, the expression level reaches similar heights among cells. Because the measured extrinsic noise is significantly lower than the mean expression level, it is assumed that cells that are unable to activate Spo0A expression state.

![Image](image-url)

**Fig. 2.** Heterogeneous expression of P_\text{spoIIA-gfp}. Cells were grown in sporulation medium and collected for flow cytometric analysis. Time is given in hours relative to the transition point between the exponential and stationary growth phase (T0). As a control for GFP specific fluorescence, the parental B. subtilis 168 strain was analysed as well (indicated with the asterisk). Arrows indicate cells in a low and high spoIIA expression state.

**Table 1.** Noise of the spoIIA promoter.

<table>
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<tr>
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<th>Intrinsic noise, (\eta_{\text{intr}}) ((\times 10^{-5}))</th>
<th>Extrinsic noise, (\eta_{\text{extr}}) ((\times 10^{-5}))</th>
<th>Total noise, (\eta_{\text{tot}}) ((\times 10^{-5}))</th>
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<tr>
<td>IIA/double*</td>
<td>9.0 ± 0.8</td>
<td>13.1 ± 2.6</td>
<td>16.1 ± 2.4</td>
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* Measurements are from four independent experiments in which at least 100 cells per experiment were counted; ± indicates the standard error in these data.
higher than the intrinsic noise, it is the major source for the total observed variation ($\eta_{\text{tot}}$) because the three values are related as $\eta_{\text{int}}^2 + \eta_{\text{ext}}^2 = \eta_{\text{tot}}^2$ (Elowitz et al., 2002). Overall, these results indicate that intrinsic noise does not play a role in the observed heterogeneity of spoIIA gene expression, but is a consequence of specific regulation.

**Spo0A autoactivated cells initiate sporulation**

To confirm that the spoIIA promoter activity is a good indicator for cells that activate Spo0A, we constructed a strain that enables the visualization of activation of both spoIIA and spo0A transcriptional units. The double-labelled strain spo0A/IIA ($P_{\text{spo0A-iyfp}}$, $amyE::P_{\text{spoIIA-icfp}}$) was grown in SM and collected at hourly intervals for fluorescence microscopic analyses. After entry into the stationary growth phase, production of IYFP from the spo0A promoter could be observed in most cells (Fig. 4). However, some cells clearly show stronger spo0A expression. These cells also show high activation of the spoIIA promoter, indicating that activation of the spoIIA promoter can be used to monitor the Spo0A activation state.

**The Spo0A autostimulatory loop is essential for sporulation bistability**

As described in the introduction, positive feedback can be the basis for bistable gene expression. To examine whether Spo0A autoactivation is the mechanism responsible for bistable induction of sporulation, we bypassed the transcriptional autostimulation by replacing the wild-type spo0A promoter with an IPTG inducible promoter. In addition, a constitutively active form of Spo0A (Spo0A-Sad67) was used in order to bypass the autostimulatory effects on the phosphorelay (Ireton et al., 1993). All strains contained the $P_{\text{spoIIA-gfp}}$ reporter. Strains IIA/spo0A + ($P_{\text{spoIIA-}}$

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**Fig. 3.** Transcriptional noise of the spoIIA promoter. Strain IIA/double was grown in sporulation medium and cells were collected for fluorescence microscopy two hours after entry into the stationary growth phase. IYFP and ICFP images were combined in the red and green channels respectively. The left panel shows IYFP produced from activation of the spoIIA promoter construct integrated at the spoIIA locus (red cells) and the middle panel indicates ICFP produced from the $P_{\text{spoIIA-icfp}}$ construct integrated at the amyE locus (green cells). An overlay of both channels is depicted in the right panel. Yellow cells express both transcriptional units.

**Fig. 4.** Activation of spo0A and spoIIA within a sporulating culture. Strain spo0A/IIA was grown in sporulation medium and collected for fluorescence microscopy one hour after entry into the stationary growth phase. IYFP and ICFP images were combined in the red and green channels respectively. The left panel shows IYFP produced from activation of the spo0A promoter (red cells) and the middle panel indicates ICFP produced from the spoIIA promoter (green cells). An overlay of both channels is depicted in the right panel. Yellow cells express both transcriptional units.
gfp, P_{spac-spo0A}, IIA/sad67 (P_{spoIIA-gfp}, P_{spac-spo0A-sad67}) and IIA/spo0A/sad67 (P_{spoIIA-gfp}, Δspo0A, P_{spac-spo0A-sad67}) were grown at varying IPTG concentrations, and cells were collected for flow cytometric measurements and immunoblot analyses (Fig. 5). To reduce possible effects of the phosphorelay, cells were grown in rich medium and samples were taken at the logarithmic growth phase. As depicted in panel A, the spoIIA promoter could not be activated by induction of wild-type Spo0A. In accordance with this, the intracellular Spo0A levels in strain IIA/spo0A + accumulate with increasing IPTG concentrations (Fig. 5D, upper panel). This demonstrates that unphosphorylated Spo0A (Spo0A is not activated under these growth conditions) is not able to activate the spo0A autostimulatory loop. This is in agreement with previous results (Ireton et al., 1993). Figure 5B shows the expression patterns of spoIIA expression upon the induction of the constitutively active form of Spo0A (Sad67) in a wild-type background in which the autostimulatory loop is present. At low IPTG levels, all cells are in the low expression state, whereas at high induction levels all cells are in the high expression state. At intermediate induction levels (with a threshold concentration of approximately 50 μM of IPTG), a clear bistable expression pattern can be observed. In agreement with this, immunoblot analysis of strain IIA/sad67 (Fig. 5D, middle panel) shows that the spo0A autostimulatory loop is activated when the constitutively active Spo0A-Sad67 is induced at levels higher than 50 μM of IPTG, indicated by an increase of wild-type Spo0A. It should be noted that the Sad67 protein migrates faster than wild-type Spo0A because of the N-terminal deletion. This demonstrates that Spo0A-Sad67 is not only able to activate transcription of spo0A, but also results in increased Spo0A phosphorylation. This is most likely caused by increased expression of phosphorelay genes like kinA and spo0F (Hoch, 1991). In contrast to this, when wild-type spo0A is deleted and only the IPTG inducible spo0A-sad67 is present, a graded response in spoIIA gene expression can be observed (Fig. 5C). At low IPTG concentrations, little activation of the spoIIA promoter could be observed. Upon increasing IPTG levels, the population fluorescence is shifted to higher intensities in a Normal-Distribution fashion without the occurrence of a dual population. Also, at maximal induction, the average

Fig. 5. Bistability requires a positive feedback loop. Strains containing the P_{spoIIA-gfp} reporter fusion were grown for 4 h in TY medium containing varying concentrations of IPTG and samples were analysed on a flow cytometer. Fluorescence distributions of strain IIA/spo0A + (P_{spoIIA-gfp}, P_{spac-spo0A}) (A), strain IIA/sad67 (P_{spoIIA-gfp}, P_{spac-sad67}) (B) and (P_{spoIIA-gfp}, P_{spac-sad67}, Δspo0A) (C) grown in the presence of the indicated IPTG concentration. D shows an immunoblot analysis using polyclonal anti-Spo0A antibodies and polyclonal anti-GroEL antibodies on whole-cell extracts which were subjected to SDS-PAGE (12%) and immunoblotting. The anti-GroEL antibody served as a control for loading.

fluorescence intensity of strain IIA/spo0A/sad67 does not reach the level of strain IIA/sad67, in which the autoloop is present. This trend is also seen in the corresponding immunoblot analysis (Fig. 5D, lower panel) in which upon increasing IPTG levels a linear increase in Sad67 protein levels is shown, and wild-type Spo0A is not present because of the spo0A mutation. In summary, these results demonstrate that the spo0A autostimulatory loops are responsible for the bistable expression of spoIIA.

**RapA modulates sporulation bistability**

RapA lowers the phosphorelay activity and could influence the bistable sporulation gene expression as described in the introduction. In one of the first studies on rapA it was shown that the expression of this gene was strongly repressed by Spo0A–P (Mueller and Sonenshein, 1992). Because expression of rapA is activated by factors outside the Spo0A–P regulatory network (ComA/P/X) but is repressed by Spo0A–P, it is conceivable that cells which do not reach the threshold level of Spo0A–P in time, will accumulate high levels of RapA, thus delaying sporulation initiation. To examine whether there is a correlation between activation of rapA and initiation of sporulation, the rapA promoter was fused to the iyfp gene, and this construct was combined with a strain carrying the spoIIA promoter fused to the icfp gene. The resulting strain, rapA/IIA (P_{rapA-iyfp}, amyE::P_{spoIIA-icfp}), was grown in SM and cells were collected for fluorescence microscopy. As shown in Fig. 6, at the end of logarithmic growth cells begin to express rapA, albeit at low levels. At this time-point (T = -1), activation of the spoIIA operon cannot be observed. Later in growth (T = 2), some cells have activated the spo0A autostimulatory loop and highly express spoIIA. Cells that do not show clear activation of spoIIA, show a strong induction of rapA expression. In the spoIIA active cells, this rapA induction is clearly absent. Thus, expression of the two genes is reciprocally distributed within the culture (Fig. 6C–E). To further substantiate these observations, strain rapA-iyfp (P_{rapA-iyfp}) was subjected to flow cytometric analyses (Fig. 6F). Again, it is shown that at the end of logarithmic growth, cells express

![Reciprocal expression of rapA and spoIIA](image-url)

**Fig. 6.** Reciprocal expression of rapA and spoIIA. Strain rapA/IIA was grown in sporulation medium and cells were collected one hour before (T = -1) and two hours after (T = 2) entry into the stationary growth phase for analysis by fluorescence microscopy. IYFP and ICFP images were combined in the red and green channels respectively. Red cells produce IYFP of which expression is driven by activity of the rapA promoter; green cells represent production of ICFP of which expression is driven by activity of the spoIIA promoter.

A. Light phase contrast picture of cells at T = -1.
B. IYFP image of cells from panel A.
C–D. Fluorescent microscopic graphs of cells from T = 2.
E. An overlay of panels C and D.
F. Strain rapA-iyfp was grown in sporulation medium and cells were collected one hour before (T = -1) and two hours after (T = 2) entry into the stationary growth phase for analysis by flow cytometry. Because YFP has different spectral properties compared with GFP, settings of the flow cytometer were set more sensitive. Therefore, the parental 168 strain was also included in the fluorescence histogram (T = 2).
YFP from the rapA promoter at low levels but in a mono-modal distribution. In the stationary phase, a fraction of cells express rapA at high levels and the other fraction at a low level.

Because induction of rapA seems to prevent activation of spoIIA, a mutation in rapA could have an effect on sporulation bistability. To test this hypothesis, we introduced a rapA mutation in our reporter strain. The resulting strain, IIA-gfp/ΔrapA (PspoIIA-gfp, ΔrapA), was grown in SM and cells analysed by flow cytometry. As shown in Fig. 7A, the final bistable expression of spoIIA almost completely disappeared in a rapA mutant background, and the vast majority of cells express high levels of spoIIA. Figure 7B shows a quantitative analysis of the effect of a rapA mutation. When we consider cells with signals higher than fluorescent channel 670 to be in a high expressing state, approximately 70% of the wild-type population reaches the spoIIA high expression state, compared with more than 90% in the rapA mutant. Initially, induction of sporulation is still heterogeneous and a similar fraction of cells highly express spoIIA in the rapA mutant when compared with the wild type (Fig. 7B; T0). This indicates that sporulation bistability is not caused by RapA, but stabilized by RapA.

**Phosphorelay phosphatases modulate the bistable outcome**

The dephosphorylating action of RapA reduces the activity of the phosphorelay and consequently will raise the threshold level of Spo0A~P to fully initiate autostimulation. This suggests that other phosphatases that act on the sporulation phosphorelay, such as Spo0E, have the potential to alter sporulation bistability. The Spo0E phosphatase can dephosphorylate Spo0A~P (Ohlsen et al., 1994). Overproduction of Spo0E was shown to inhibit sporulation, whereas deletion of this locus resulted in premature sporulation (Perego and Hoch, 1991). A spo0E mutation was introduced in our reporter strain. The resulting strain, IIA-gfp/Δspo0E (PspoIIA-gfp, Δspo0E), was grown in SM and cells analysed by flow cytometry. As shown in Fig. 8, the final bistable expression of spoIIA is completely abolished in a spo0E mutant background, and the vast majority of cells express high levels of spoIIA. Although activation of the spoIIA operon is premature in the spo0E mutant, the initial expression distribution is still bistable (data not shown). This result suggests that by modulating phosphorelay activity through external phosphatases, the kinetics of the autostimulatory Spo0A acti-
The sporulating culture can be used as a tuner to modulate the bistable outcome of sporulation. To substantiate this supposition, we tested the effect of an artificial induction of a heterologous RapA homologue, Rap60. The Rap60 phosphatase is encoded on pHt1060, a rolling-circle plasmid of Bacillus amyloliquefaciens (Meijer et al., 1998). Previously, it was shown that Rap60, which is highly homologous to RapA, could dephosphorylate a component of the phosphorelay of B. subtilis (Koetje et al., 2003). The rap60 gene was placed under a xylose-inducible promoter and introduced into the amyE locus of a strain in which either rapA or spo0E was deleted. Strains IIA/0E/Xrap (P_spoIIA-gfp, Δspo0E, amyE::P_xyl-rap60) and IIA/rapA/Xrap (P_spoIIA-gfp, ΔrapA, amyE::P_xyl-rap60) were grown in SM and xylose was added 1 h before entry into the stationary growth phase. Cultures were analysed using flow cytometry two hours after entry into the stationary growth phase (Fig. 9). As shown in Fig. 9, xylose induction of Rap60 complements the abolished bistable expression pattern of a rapA and spo0E mutant. These results show that the phosphorelay can be used as a tuner to modulate the bistable outcome of the sporulating culture.

**Discussion**

The spoIIA operon, encoding the sporulation sigma factor σF, is one of the earliest targets that is activated by Spo0A–P at the onset of sporulation (Sonenshein, 2000). Using GFP as a reporter, activation of this operon was shown to be heterogeneously distributed within the sporulating culture (Fig. 2). By following an approach pioneered by Elowitz and coworkers, we showed that the heterogeneity in activation of the spoIIA promoter is not a consequence of transcriptional noise of this promoter (Fig. 3). Elowitz et al. have shown that low intrinsic noise of target promoters can be observed when intracellular concentrations of activator proteins are high (Elowitz et al., 2002). This suggests that the spoIIA promoter requires relatively high amounts of regulator protein(s), such as Spo0A, to activate its transcription. In support of this, we demonstrate that cells that have a high spo0A expression state are the ones that also activate expression of the spoIIA operon (Fig. 4). These results agree with previous findings (Bird et al., 1992; Chung et al., 1994). The spoIIA promoter is therefore a useful reporter to study the heterogeneous activation of Spo0A in a sporulating culture.

Modelling of autostimulatory gene regulation networks has shown that stochastic fluctuations of regulatory components in these systems can lead to a bistable response (Hasty et al., 2000). This was biologically supported using relative simple well-defined, positive feedback circuits as models, such as that derived from bacteriophage lambda in E. coli (Hasty et al., 2000; Isaacs et al., 2003), and a tetracycline-responsive transactivator system in yeast (Becskei et al., 2001). Although sporulation is initiated by the autostimulatory expression of spo0A, we cannot speak of a simple positive feedback circuit. As indicated in Fig. 1, and described in the introduction, there are several positive feedback loops working on the spo0A promoter. The activation of Spo0A by the phosphorelay is also subject to autostimulatory influences (Strauch et al., 1992; Strauch et al., 1993; Fujita and Sadaie, 1998). By using an inducible and constitutively active variant of Spo0A, we demonstrated that sporulation bistability is abolished when the Spo0A-autostimulation is replaced by a graded induction (Fig. 5). This corresponds with previous described regulatory systems in which autostimula-

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**Fig. 9.** A RapA homologue can modulate sporulation bistability. Strains were grown in sporulation medium and cultures were split in equal volumes and xylose was added to the indicated concentration 1 h before entry into the stationary growth phase. Cultures were analysed by flow cytometry two hours after entry into the stationary growth phase. As a control for a possible xylose effect on sporulation heterogeneity, strains IIA/gfp and IIA-gfp/Δspo0E were also analysed and showed no decrease in expression of spoIIA, rather a slight increase (data not shown). Fluorescence distributions of strain IIA/rapA/Xrap (P_spoIIA-gfp, ΔrapA, amyE::P_xyl-rap60) (A) and IIA/0E/Xrap (P_spoIIA-gfp, Δspo0E, amyE::P_xyl-rap60) (B) grown in the presence of the indicated xylose concentration.
tion of a regulator can convert a graded response to a bistable response (Gardner et al., 2000; Hofer et al., 2002). In conclusion, these results indicate that the complex autostimulatory Spo0A regulation cascade can be considered as a classical autostimulatory loop with a bistable output. The mechanism that decides which cell reaches the Spo0A threshold first, is likely based on a random process (Dawes and Thornley, 1970; Hasty et al., 2000; Maughan and Nicholson, 2004).

Notably, during the revision of this manuscript, Fujita and coworkers have also shown, using different methodologies and independent of our work, that initiation of sporulation requires a threshold level of Spo0A and can be regarded as a bistable switch, results consistent with the work presented here (Fujita et al., 2005).

Heterogeneity in gene expression in B. subtilis cultures is not only limited to the process of sporulation but also takes place during competence development (Cahn and Fox, 1968; Hadden and Nester, 1968). Because this process is also influenced by Spo0A–P (Hahn et al., 1995), it raises the question whether heterogeneity observed in this process is the consequence of spo0A autoactivation. However, in our group we have recently obtained evidence that the autostimulatory transcription loop of comK (encoding the Competence Transcription Factor), is responsible for bistability in competence development (Smits et al., 2005).

lacZ reporter studies has shown that rapA expression coincides with the initiation of sporulation, which is peculiar considering the negative effect of RapA on this process. We showed that this discrepancy is resolved when looking at expression levels in individual cells. As a result of the bistable nature of sporulation initiation, some cells initiate sporulation and repress rapA gene expression while the non-sporulating cells continue to accumulate RapA (Fig. 6). When rapA was deleted, the final bistable distribution of sporulation gene expression was almost abolished (Fig. 7). Based on these findings, we propose that the main function of RapA is to maintain the bistable gene expression that originates from the autostimulatory spo0A activation.

The fascinating aspect of rapA regulation is that the induction is subjected to multiple quorum sensing pathways. The initial rapA expression is not heterogeneous, which suggests that induction of all ComA-regulated genes is homogeneous (Fig. 6). This corresponds with the observation that srfA induction, directly activated by the ComA/P/X system, also showed homogeneous expression (Hahn et al., 1994). One could imagine that within shaken liquid cultures, quorum sensors, such as ComX, are evenly distributed. Quorum sensing might be a mechanism to reduce noise in signal transduction pathways: although cells might differ in the expression of regulatory systems, they will sense the same level of pheromones. Of course this situation will be different in dense biofilm populations.

The phosphatase activity of RapA is modulated by PhrA, a small-secreted peptide encoded by an adjacent gene on the same transcript as rapA. Previously, it was shown that sporulation is poor in a phrA mutant strain (Perego and Hoch, 1996). Eventually, the accumulation of PhrA seems to antagonize the dephosphorylating action of RapA, and thus allows delayed cells to initiate sporulation. Perego suggested that the RapA-PhrA system is not a cell-density control mechanism but that processed PhrA primarily accumulates in the periplasmic space (Perego, 1999). This was based on the observation that: (i) free PhrA peptide could only be detected in medium of a opp mutant strain (owing to a lack of re-import) and (ii) the peptide is susceptible to the high amounts of extracellular proteases produced by B. subtilis. Because of the temporal sequence of export, processing and import, the PhrA pheromone might provide a proscribed and finite time frame for information processing and decision-making meaning that the RapA-PhrA system functions as a sporulation timing device (Perego, 1999). In this work, we show that only a limited number of cells express high levels of rapA and are delayed in sporulation. As a result of cotranscription, these cells are also likely to express high levels of phrA. It is envisioned that in time, RapA inhibition can be counteracted by the continuous accumulation of PhrA. Whether this would occur in cis (PhrA in periplasm) or in trans (PhrA in medium) remains to be established. However, Perego’s idea that the RapA-PhrA functions as a sporulation-timing device corresponds with our postulated role as stabilizer of bistable gene expression in sporulating cultures.

Surprisingly, deletion of comA, the activator of rapA, did not promote sporulation under the conditions tested (J.-W. Veening, L. W. Hamoen and O. P. Kuipers, unpubl. results), whereas overexpression of ComA was previously shown to inhibit sporulation (Weinrauch et al., 1989). This suggests that the ComA-ComP system, besides activating expression of negative regulators like rapA, also regulates genes that promote sporulation initiation. A possible candidate is the srfA operon, which is activated by ComA–P (Nakano et al., 1991a). The induction of srfA is also important for the development of genetic competence. In the 3’ end of this operon a small gene, comS, is embedded which is essential for the activation of ComK. Interestingly, the 3’ part of this operon was found to be required for sporulation (Nakano et al., 1991b). Whether there is another (small) gene present in this operon that is required for sporulation remains to be investigated.

Another mechanism that could act as a sporulation delay system was previously described by Gonzalez-Pastor et al. (2003). They showed that, within a sporulating culture, sporulating cells are able to kill their siblings that
have not yet initiated this process. This phenomenon is responsible for the reduction in cell density of a sporulating culture. The operon responsible for producing the sporulation killing factor (skf) is activated by Spo0A-P. The Spo0A-inactive subpopulation is sensitive to the sporo-
lation-killing factor secreted by the Spo0A-active cells, and will subsequently lyse and release nutrients available for the remainder of the population. Early in the stationary growth phase, the low Spo0A-active population (Fig. 2 left peak) seems to be resistant to the killing factor. This is most likely caused by the high efficiency of the coexpressed export pump proteins SkfE and SkfF, which confer resistance against the killing factor (J.E. Gonzalez-Pastor, pers. comm.). The Skf system could provide the sporulating culture with an alternative mechanism to maintain sporulation bistability by impeding cell growth via the Skf antibiotic. However, a disruption of the skf operon did not change the bistable expression of spoIIA (data not shown). Using a dye that specifically stains cells with damaged membranes (propidium iodide), no dead cells were detected (data not shown). Thus, the RapA-active subpopulation is not targeted for lysis, but is able to commit to other differentiation pathways than sporulation.

In conclusion, the RapA results show that phosphorelay phosphatases have a modulating action on the bistable expression pattern of a sporulating culture by altering the threshold-level of Spo0A-P. By monitoring spoIIA expression in a spo0E mutant, and in strains containing artificially inducible Rap60, we substantiated that the phosphorelay can be used as a tuner to modulate the bistable outcome of the sporulating culture (Figs 8 and 9). The exact function of Spo0E in sporulation regulation is unclear. AbrB represses spo0E gene expression (Strauch et al., 1989; Perego and Hoch, 1991). Apparently, cells that initiate spo0A autoactivation also induce Spo0E levels. This coexpression suggests that Spo0E serves as some kind of a ‘safety lid’ to prevent over-stimulation of spo0A autoactivation. Rap phosphatases are common among Bacillus species and many of them are encoded on plasmids isolated from industrially relevant strains. These strains are used for high production of enzymes, and generally show a reduction in sporulation (Meijer et al., 1998). Apparently, natural selection chose to alter the sporulation outcome by using external phosphatases to modulate the phosphorelay.

**Experimental procedures**

**Plasmids, bacterial strains and media**

Table 2 lists the plasmids and bacterial strains used. TY medium (tryptone/yeast extract) contained Bacto-Tryptone (1%), Bacto-yeast extract (0.5%) and NaCl (1%). SM contained dehydrated nutrient broth (0.8%), NaOH (0.5 mM), MgSO4 (1 mM), KCl (1 g l-1), Ca(NO3)2 1 mM, MnCl2 (0.01 mM) and FeSO4 (0.001 mM). Minimal medium for B. subtilis was prepared as described before (Veening et al., 2004). When required, medium for E. coli was supplemented with ampicillin (Ap; 100 µg ml-1); media for B. subtilis were supplemented with chloramphenicol (Cm; 5 µg ml-1), tetracycline (Tc; 6 µg ml-1), spectinomycin (Sp; 100 µg ml-1) or kanamycin (Km; 10 µg ml-1). When indicated, IPTG (isopropyl-β-D-thiogalactosidase) or xylose was added to the medium.

**Table 2. Bacterial strains and plasmids.**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td>F-, araD139, Δ[ara-leu]7696, Δ[lac]X74, galU, galK, hsdR2, mcrA, mcrB1, rspL</td>
<td>(Wertman et al., 1986)</td>
</tr>
<tr>
<td><strong>B. subtilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>trpC2</td>
<td>(Kunst et al., 1997)</td>
</tr>
<tr>
<td>IIA-gfp</td>
<td>168, P^spac^-gfp, Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>IIA/double</td>
<td>168, P^spac^-gfp, Cm', amyE::P^spac^-icfp, Km'</td>
<td>This study</td>
</tr>
<tr>
<td>spoA/IIA</td>
<td>168, P^spac^-gfp, Cm', amyE::P^spac^-icfp, Km'</td>
<td>This study</td>
</tr>
<tr>
<td>rapA/IIA</td>
<td>168, P^spac^-gfp, Cm', amyE::P^spac^-icfp, Km'</td>
<td>This study</td>
</tr>
<tr>
<td>rapA-lyf</td>
<td>168, P^spac^-lyf, Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>IIA-gfp/rapA</td>
<td>168, P^spac^-gfp, Cm', rapA::Sp, Sp'</td>
<td>This study</td>
</tr>
<tr>
<td>IIA-gfp/spo0E</td>
<td>168, P^spac^-gfp, Cm', spo0E::Sp, Sp'</td>
<td>This study</td>
</tr>
<tr>
<td>IIA/spo0A+</td>
<td>168, P^spac^-gfp, Sp', amyE::P^spac^-spo0A, Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>IIA/sad67</td>
<td>168, P^spac^-gfp, Cm', amyE::P^spac^-sad67, Tc'</td>
<td>This study</td>
</tr>
<tr>
<td>IIA/0E/Xrap</td>
<td>168, P^spac^-gfp, Cm', spo0E::Sp, Sp', amyE::P^spac^-rap60, Km'</td>
<td>This study</td>
</tr>
<tr>
<td>IIA/rapA/Xrap</td>
<td>168, P^spac^-gfp, Cm', rapA::Sp, Sp', amyE::P^spac^-rap60, Km'</td>
<td>This study</td>
</tr>
</tbody>
</table>

| Plasmids | | |
| pGFP-IIA | bla, cat, P^spo0A-gfp | This study |
| plYFP-spo0A | bla, cat, P^spo0A-lyf | This study |
| plYFP-rapA | bla, cat, P^rapA-lyf | This study |

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Recombinant DNA techniques and oligonucleotides

Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis and transformation of E. coli were carried out as described before (Sambrook et al., 1989). Enzymes were obtained from Roche (Mannheim, GER). Oligonucleotides were purchased from Biologeo (Malden, NL). B. subtilis was transformed as described before (Veening et al., 2004).

Construction of plasmids

To construct plasmid pGFP-IIA, carrying the B. subtilis spoIIA promoter region fused with the gfpmut1 gene, a polymerase chain reaction (PCR) with the primers spoIIA-F (5'-CCCAAGCTTAGGCAAGCTTGCACT-3') and spoIIA-R (5'-TCTGGTCATCGAGG-3') was performed, using chromosomal DNA of B. subtilis 168 as a template. The amplified fragment was subsequently cleaved with HindIII and PstI, and ligated into the corresponding sites of pSG1151 (Lewis and Marston, 1999), resulting in plasmid pGFP-IIA.

To construct plasmid pIYFP-spo0A, carrying the B. subtilis spo0A promoter region fused with the ifp gene, a PCR with the primers spo0A-F (5'-CCTGGTCATCGAGG-3') and spo0A-R (5'-CGGAATTCTCTGTTTCTCTCCCCAATG-3') was performed, using chromosomal DNA of B. subtilis 168 as a template. The amplified fragment was subsequently cleaved with HindIII and EcoRI, and ligated into the corresponding sites of pIYFP (Veening et al., 2004), resulting in plasmid pIYFP-spo0A.

To construct plasmid pIYFP-rapA, carrying the B. subtilis rapA promoter region fused with the ifp gene, a PCR with the primers rapA-F (5'-CCAAGCTTAGGCAAGCTTGCACT-3') and rapA-R (5'-CTGGTCATCGAGG-3') was performed, using chromosomal DNA of B. subtilis 168 as a template. The amplified fragment was subsequently cleaved with HindIII and EcoRI, and ligated into the corresponding sites of pIYFP (Veening et al., 2004), resulting in plasmid pIYFP-rapA.

Construction of bacterial strains

Bacillus subtilis strain IIA-gfp was obtained by a Campbell-type integration of plasmid pIYFP-IIA into the chromosomal spoIIA promoter region of B. subtilis 168. B. subtilis strain rapA-gfp was obtained by a Campbell-type integration of plasmid pIYFP-rapA into the chromosomal rapA promoter region of B. subtilis 168. Transformants were selected on TY agar plates containing Cm and Km, after overnight incubation at 37°C. Correct integration was verified by PCR (data not shown).

Bacillus subtilis strain IIA/double was obtained by transformation of strain IIA-gfp (Veening et al., 2004) with chromosomal DNA of strain icp-IIA-amyE (Veening et al., 2004). Transformants were selected on TY agar plates containing Cm and Km, after overnight incubation at 37°C. Correct integration into the amyE gene was tested and confirmed by lack of amylase activity upon growth on plates containing 1% starch.

Bacillus subtilis strains spo0A/IIA and rapA/IIA were obtained by a Campbell-type integration of plasmids pIYFP-spo0A and pIYFP-rapA, respectively, into the chromosome of B. subtilis icp-IIA-amyE (Veening et al., 2004). Transformants were selected on TY agar plates containing Cm and Km, after overnight incubation at 37°C. Correct integration was verified by PCR (data not shown).

Bacillus subtilis strain IIA-gfp/ΔspoOE (PspoOE-gfp, ΔspoOE) was obtained by transformation of strain IIA-gfp with chromosomal DNA of strain oES (Hosoya et al., 2002). Transformants were selected on TY agar plates containing Cm and Sp, after overnight incubation at 37°C. B. subtilis strain ΔrapA-Sp was obtained by transformation of strain ΔrapA-Cm (Tjalsma et al., 2004) with plasmid pCm::Sp (Steinmetz and Richter, 1994). Transformants were selected on TY agar plates containing Sp, after overnight incubation at 37°C. To confirm correct switching of the Cm resistance marker to the Sp resistance marker, transformants were scored on Cm sensitivity and Sp resistance. B. subtilis strain IIA-gfp/ΔrapA (PspoOE-gfp, ΔrapA) was obtained by transformation of strain IIA-gfp with chromosomal DNA of strain ΔrapA-Sp. Transformants were selected on TY agar plates containing Cm and Sp, after overnight incubation at 37°C.

Bacillus subtilis strain IIA-gfp::Sp was obtained by transformation of strain IIA-gfp with plasmid pCm::Sp (Steinmetz and Richter, 1994). Transformants were selected on TY agar plates containing Sp, after overnight incubation at 37°C. To confirm correct switching of the Cm resistance marker to the Sp resistance marker, transformants were scored on Cm sensitivity and Sp resistance. B. subtilis strain IIA-gfp::Sp was obtained by transformation of strain IIA-gfp::Sp with chromosomal DNA of strain Sik243 (Ireton et al., 1993). Transformants were selected on TY agar plates containing Sp and Cm, after overnight incubation at 37°C.

Bacillus subtilis strain Xrap::Neo was obtained by transformation of strain R (Koetje et al., 2003) with plasmid pCm::Nm (Steinmetz and Richter, 1994). Transformants were selected on TY agar plates containing Km, after overnight incubation at 37°C. To confirm correct switching of the Cm resistance marker to the Km resistance marker, transformants were scored on Cm sensitivity and Km resistance. B. subtilis strain IIA-gfp::Xrap was obtained by transformation of strain IIA-gfp with chromosomal DNA of strain Xrap::Neo. Transformants were selected on TY agar plates containing Sp and Cm, after overnight incubation at 37°C.
sensitivity and Tc resistance. To obtain *B. subtilis* strain IIA-gfp/sad67, strain sad67::Tc was transformed with chromosomal DNA of strain IIA-gfp. Transformants were selected on TY agar plates containing Tc and Cm, after overnight incubation at 37°C. Finally, *B. subtilis* strain IIA/spo0A/sad67 was obtained by transforming strain IIA-gfp/sad67 with chromosomal DNA of strain SWV215 (spo0A::Km) (Xu and Strauch, 1996). Transformants were selected on TY agar plates containing Tc, Cm and Km, after overnight incubation at 37°C.

**Microscopy**

Cells were prepared for microscopy and applied to agarose slides as described before (Veening et al., 2004), and images were acquired using an Axioshot microscope equipped with an AxioVision camera (Zeiss, Oberkochen, GER). Fluorescence filter sets used to visualize the green, cyan and yellow fluorescent proteins were obtained from Zeiss. Fluorescent signals of GFP were visualized using set 09 (excitation, 450–490 nm; emission, > 520 nm), fluorescent ICFP signals were visualized using set 47 (excitation, 426–446 nm; emission, 520–550 nm) and fluorescent signals of IYFP using set 46 (excitation, 490–510 nm; emission, 520–550 nm). AxiosVs20 software (Zeiss) was used for image capturing and figures were prepared for publication using Corel Graphics Suite 11 (Corel Corporation).

**Flow cytometry**

Cells were 100× diluted in 0.2 μM filtered minimal medium and directly measured on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Mijdrecht, NL) operating an argon laser (488 nm). For each sample, at least 20 000 cells were analysed. Data containing the green fluorescent signals were collected by a FITC filter and the photomultiplier voltage was set between 700 and 800 V. Data were captured using EXPO32 software (Beckman Coulter) and further analysed using WinMDI 2.8 software (http://facs.scripps.edu/software.html). Figures were prepared for publication using WinMDI 2.8 and Corel Graphics Suite 11. To distinguish background fluorescence from GFP specific fluorescence, parental strain *B. subtilis* 168 was also analysed with each flow cytometric experiment.

**Calculation of noise**

Strain IIA/double was grown in SM and cells were collected for fluorescent microscopy two hours after entry into stationary growth phase. Images were analysed using ImageMaster 2D elite software v3.1 (Amersham Pharmacia biotech) and Microsoft Excel (as described by Smits et al., 2005). Data were calculated essentially as described before (Elowitz et al., 2002). In brief, intrinsic noise (\(\eta_{int}\)), extrinsic noise (\(\eta_{ext}\)) and total variation (\(\eta_{tot}\)) were defined as follows:

\[
\eta_{int}^2 = \frac{\langle c - y^2 \rangle}{2\langle c\rangle\langle y\rangle}, \quad \eta_{ext}^2 = \frac{\langle cy \rangle - \langle c\rangle\langle y\rangle}{\langle c\rangle\langle y\rangle},
\]

\[
\eta_{tot}^2 = \frac{\langle c^2 + y^2 \rangle - 2\langle c\rangle\langle y\rangle}{2\langle c\rangle\langle y\rangle}
\]

Here the \(i\)th element of vectors \(c\) and \(y\) contain the average CFP or YFP intensity, respectively, of the \(i\)th cell in the sample. Angled brackets denote means over the cell population.

**Western blot analysis and immunodetection**

Before harvesting of the cells, the optical density at 600 nm was measured and was used to correct for the total amount of protein loaded on the gel. Cells were separated from the growth medium by centrifugation (14 000 r.p.m., 1 min, room temperature). Pelleted cells were resuspended in protoplast buffer (20 mM potassium phosphate pH 7.5, 15 mM MgCl₂, 20% sucrose and 1 mg ml⁻¹ of lysozyme) and incubated at 37°C for 30 min. The resulting protoplasts were diluted with 2× SDS sample buffer, incubated at 95°C for 5 min and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described (Sambrook et al., 1989). Next, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Roche) as described (Sambrook et al., 1989). Spo0A and Spo0A-Sad67 were detected with polyclonal anti-Spo0A antibodies (kindly provided by M. Fujita), GroEL was detected with polyclonal anti-GroEL antibodies (our laboratory collection) and horseradish peroxidase-antirabbit-IgG conjugate (Amersham Biosciences, Little Chalfont, UK). Anti-Spo0A and anti-GroEL was added in a 1:5000 dilution and anti-rabbit at a 1:7500 dilution. Anti-Spo0A antibodies can be used to detect both wild-type Spo0A and the N-terminal deletion mutant Spo0A-Sad67 (Fujita and Losick, 2003).

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**Supplementary material**

The following supplementary material is available for this article online:

Appendix S1. Specific growth conditions used in this study.

Fig. S1. Typical growth of *B. subtilis* IIA-gfp grown in sporulation medium.

Fig. S2. Chromosome map of strain IIA/double.

**References**


Mueller, J.P., Bukusoglu, G., and Sonenshein, A.L. (1992) Transcriptional regulation of *Bacillus subtilis* glucose starvation-inducible genes: control of gsiA by the ComP-