

Single-cell measurement of the levels and distributions of the phosphorelay components in a population of sporulating *Bacillus subtilis* cells

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Upon nutrient starvation, the Gram-positive bacterium *Bacillus subtilis* switches from growth to sporulation by activating a multicomponent phosphorelay consisting of a major sensor histidine kinase (KinA), two phosphotransferases (Spo0F and Spo0B) and a response regulator (Spo0A). Although the primary sporulation signal(s) produced under starvation conditions is not known, it is believed that the reception of a signal(s) on the sensor kinase results in the activation of autophosphorylation of the enzyme. The phosphorylated kinase transfers the phosphate group to Spo0A via the phosphorelay and thus triggers sporulation. With a combination of quantitative immunoblot analysis, microscopy imaging and computational analysis, here we found that each of the phosphorelay components tested increased gradually over the period of sporulation, and that Spo0F was expressed in a more heterogeneous pattern than KinA and Spo0B in a sporulating cell population. We determined molecule numbers and concentrations of each phosphorelay component under physiological sporulation conditions at the single-cell level. Based on these results, we suggest that successful entry into the sporulation state is manifested by a certain critical level of each phosphorelay component, and thus that only a subpopulation achieves a sufficient intracellular quorum of the phosphorelay components to activate Spo0A and proceed successfully to the entry into sporulation.

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INTRODUCTION

Sporulation in the Gram-positive bacterium *Bacillus subtilis* is induced by nutrient starvation, producing a metabolically dormant cell type known as a spore. Nutrient deprivation stops the vegetative cell growth phase and initiates a developmental process. This process involves a single-round asymmetrical division to form two morphologically distinct cell compartments: a large mother cell and a small forespore (the future spore) (Grossman, 1995; Hoch, 1993; Piggot & Losick, 2002; Sonenshein, 2000). The signal transduction network that initiates sporulation

consists of sequential phosphotransfer reactions, termed a ‘phosphorelay’, involving at least three sensor kinases, KinA, KinB and KinC, two phosphotransferases, Spo0F and Spo0B (Burbulys *et al.*, 1991; Hoch, 1993; Stephenson & Hoch, 2002), and two phosphatases, RapA and Spo0E (Perego & Hoch, 2002). The end result of the phosphorelay is the activation of Spo0A, the master transcriptional regulator of sporulation, by its phosphorylation (Burbulys *et al.*, 1991; Hoch, 1993). It has been suggested that one of the inputs to activate the phosphorelay requires activation of the major sporulation kinase, KinA, through autophosphorylation by some metabolic and/or environmental effectors upon nutrient limitation (Grossman, 1995; Hoch, 1993).

Additional complexity of the phosphorylation initiation network arises at the level of transcriptional regulation, which involves multiple feedback loops. The Spo0A activated via the phosphorelay represses the *abrB* gene, which encodes a negative regulator of *sigH* (also known as *spo0H*), a gene encoding the RNA polymerase σ^H factor (Weir *et al.*, 1991). Subsequently, increased expression of σ^H stimulates synthesis of the phosphorelay components, including KinA, Spo0F and

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Abbreviation: CV, coefficient of variation.

A set of supplementary methods, describing plasmid construction, three supplementary tables, listing strains, plasmids and primers, and two supplementary figures, showing the results of immunoblot analyses, are available with the online version of this paper.

Spo0A, and thereby affects the level of active Spo0A. This feedback regulation results in complex phosphorelay transcriptional patterns during early sporulation. Transcription of *kinA*, known to be under the control of σ^H , reaches a maximum level after the onset of sporulation (Antoniewski *et al.*, 1990; Asai *et al.*, 1995; Predich *et al.*, 1992). The *spo0B* gene is preferentially transcribed during growth under the control of σ^A (a housekeeping sigma factor) (Bouvier *et al.*, 1984; Ferrari *et al.*, 1985). Furthermore, expression of Spo0B protein appears to be regulated at the translational level by its mRNA secondary structure encompassing the ribosome-binding site (Asayama *et al.*, 1998). The transcription of *spo0F*, known to be under the control of both σ^H and Spo0A~P, is increased upon entry into sporulation (Asayama *et al.*, 1995; Bai *et al.*, 1990; Strauch *et al.*, 1993) and then repressed with increased concentrations of phosphorylated Spo0A (Fujita & Sadaie, 1998). Transcription of the *spo0A* gene is known to be differentially controlled by two promoters: during the initiation of sporulation, the relatively weak vegetative promoter P_v (σ^A type) is shut off, whereas the strong sporulation-specific promoter P_s (σ^H type) is turned on as a result of the autoregulation of Spo0A (Chibazakura *et al.*, 1995; Strauch *et al.*, 1992).

Our previous report indicates another important feature of the sporulation initiation network. We have shown that a gradual increase in Spo0A activity is essential for the success of sporulation and ‘just-in-time expression’ of Spo0A-dependent genes (Fujita *et al.*, 2005). We also found that Spo0A activation via the phosphorelay increases gradually and that the activity beyond a threshold level results in ‘digital’ on/off responses of the sporulation gene regulation programme. Furthermore, Veening and co-workers have shown that a sporulating population of genetically identical *B. subtilis* cells is heterogeneous, with a certain subpopulation being sporulated in a non-uniform manner across the population (Veening *et al.*, 2005). This phenotypic variation within a population may help a bacterium to survive in unexpected environmental circumstances (Veening *et al.*, 2008c).

Since phosphorelay components are essential to activate Spo0A for entry into sporulation, all these components should be expressed and present at sufficient levels to ensure efficient phosphate transfer in the early sporulation phase. However, the dynamics of distribution of the phosphorelay components in individual cells during sporulation have not been fully quantified. To quantitatively understand the phosphorelay dynamics, we need to know when and how each component in the phosphorelay is expressed upon starvation, both in individual cells and in a population. As a first step to address some of these questions, we report the determination of the molecule numbers and concentrations of each phosphorelay component under physiological sporulation conditions at the single-cell level, using a combination of quantitative immunoblot analysis, microscopy imaging, and mathematical and computational analysis.

METHODS

Strain and plasmid construction. The parent strain for all experiments was *B. subtilis* strain PY79 (Youngman *et al.*, 1984). Details regarding the full genotypes of strains are provided in Supplementary Table S1. All plasmid constructions were performed in *Escherichia coli* DH5 α using standard methods. Details of plasmid construction are described in the supplementary material. The plasmids used in this study are listed in Supplementary Table S2. Deletion strains of *spo0F* and *spo0B* were generated using the technique of long flanking homology PCR (Wach, 1996). The oligonucleotide primers used are listed in Supplementary Table S3.

Media. For sporulation induction, the resuspension method described by Sterlini & Mandelstam (1969) was employed. In brief, *B. subtilis* cells were grown in casein hydrolysate (CH) medium at 37 °C. At the mid-exponential phase of growth (OD₆₀₀ 0.5) in CH medium, cells were induced to sporulate by resuspension in Sterlini–Mandelstam (SM) medium. An *E. coli* strain harbouring a plasmid was grown in Luria–Bertani (LB) medium supplemented with ampicillin (100 μ g ml⁻¹).

Immunoblot analysis. Immunoblot analysis was done with polyclonal anti-GFP (Rudner & Losick, 2002), anti-Spo0A (Fujita, 2000) and anti- σ^A (Fujita, 2000) antibodies. Bound antibody was detected with secondary antibodies coupled to alkaline phosphatase, using 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium chloride (NBT) as a colour development substrate (Promega). GFP purification and antibody production have been described elsewhere (Rudner & Losick, 2002). The density of the signal generated after immunostaining was quantified with a FluorChem digital imaging system (Alpha Innotech) and autocorrected for background.

Fluorescence microscopy. Fluorescence microscopy was performed as described previously (Eswaramoorthy *et al.*, 2009). Cells expressing GFP-tagged protein under sporulation conditions were examined using a fluorescence microscope (Olympus BX-61).

To randomize sampling, all cells within a field were analysed. Intensities of GFP (arbitrary pixel units) were analysed electronically by the software Slidebook (Intelligent Imaging Innovations).

Measurement of cell length and cell width by digital image analysis. Phase contrast images of cells under sporulation conditions were captured and analysed electronically by the Slidebook software. Cell length (the long axis of the cell) and cell width (the short axis of the cell) were measured directly from images as pixel values and converted to actual sizes (μ m). We confirmed that our data were essentially consistent with published results (Gregory *et al.*, 2008; Kubitschek & Friske, 1986; Sharpe *et al.*, 1998).

Computing population distributions of phosphorelay concentrations. The fluorescence intensity data for Spo0B, Spo0F and KinA were normalized by the background as follows:

$$I_{\text{norm}}^i(t_k) = (I_{\text{original}}^i - \langle I_{\text{background}}^i \rangle) / \langle I_{\text{background}}^i \rangle$$

where I_{original}^i is a vector of the original intensities (arbitrary units) for each of the functional fusions ($i=1, 2$ and 3 for KinA, Spo0B and Spo0F, respectively), and $\langle I_{\text{background}}^i \rangle$ is the mean over 10 intensity measurements of the background. These normalizations were done at four time points, $t_k=1, 1.5, 2$ and 2.5 h. The next step was to run a zero-slope linear regression for the concentration of each of the proteins (Table 2) versus the mean normalized fluorescence intensities (Fig. 4):

$$C_i(t_k) = \alpha_i \langle I_{\text{norm}}^i(t_k) \rangle$$

Different coefficients, α_i , were assumed for different GFP fusions to account for potentially different fractions of fluorescent proteins. A

zero intercept regression implies that a zero concentration would correspond to a background level of fluorescence, i.e. that the normalized intensity is zero. As a possible alternative, we also performed a regression for a constant autofluorescence model in which the same intercept is assumed for all three proteins. The results (not shown) implied that no significant autofluorescence was present. Finally, concentration distributions for each protein were recomputed from normalized intensities as:

$$C_i(t_k) = \alpha_i I_{\text{norm}}^i(t_k)$$

RESULTS AND DISCUSSION

Protein levels of phosphorelay components during the initiation of sporulation

It has been reported that Spo0F protein levels increase upon entry into sporulation (Bai *et al.*, 1990). Furthermore, levels of KinA and Spo0A proteins increase gradually after the onset of sporulation (Fujita & Losick, 2005). To understand the dynamic properties of the sporulation initiation network, it is important to know when and how much of each component is produced upon starvation both in individual cells and in a population. However, relative protein levels of each component have not been characterized comprehensively under identical culture conditions. Thus, to quantify the protein content in a cell at the population level, we employed a quantitative immunoblot analysis of phosphorelay proteins. For this purpose, we created strains that expressed functional GFP fusion proteins with Spo0F and Spo0B. Spo0F and Spo0B tagged with GFP were constructed as a C-terminal fusion and an N-terminal fusion under the control of the native promoter, respectively. Each gene fusion was integrated at the *amyE* locus, which encodes a nonessential α -amylase, and then a knockout mutation for each endogenous gene was introduced at its normal chromosomal position. The synthesis of each gene fusion was under the control of its native promoter. The fusions were apparently functional because the replacement of the endogenous gene with the corresponding gene fusion showed no measurable impairment of growth or sporulation (data not shown). In contrast, each null mutation of a phosphorelay component was severely defective in sporulation (data not shown). We note that the *spo0B* gene exists as an operon with the downstream gene for Obg, an essential GTP-binding protein (Trach & Hoch, 1989). A tetracycline-resistance gene with no transcriptional terminator was inserted into the Spo0B-coding region by using the long flanking homology PCR strategy (Wach, 1996). The strain harbouring the *spo0B* gene knockout showed a severe sporulation defect, but no growth impairment (data not shown). Thus, we concluded that our *spo0B* knockout mutation had no polar effect on the expression of *obg*, as reported previously (Trach & Hoch, 1989). We should note for the record that a carboxyl-terminal fusion of GFP to Spo0B was not functional (data not shown). To construct an in-frame GFP-tagged Spo0B, the DNA fragment containing the

native promoter and ribosome-binding site of *spo0B* was ligated to the start codon (ATG) followed by the *HindIII* linker and the complete GFP sequence fused to Spo0B (strain MF3675). A functional KinA–GFP fusion under the control of the native promoter and ribosome-binding site has been constructed as a C-terminal fusion protein and reported previously (Fujita & Losick, 2005), and was also used in this study. We note that a GFP fusion of Spo0A, at either the N or the C terminus, was not functional (data not shown), so that it could not be used in this study, especially for fluorescence microscopy experiments. Therefore, anti-Spo0A antibodies were used in the immunoblot analysis for Spo0A quantification (Fujita, 2000), whereas anti-GFP antibodies were used to quantify KinA, Spo0A and Spo0B concentrations.

Cells expressing each GFP-tagged protein were induced to sporulate in SM medium, and cell samples were taken periodically. Cells of the wild-type strain were processed in the same way for Spo0A detection. Then, the phosphorelay components were detected by anti-GFP antibodies for GFP-tagged proteins and anti-Spo0A antibodies for Spo0A (Fig. 1a). Constitutively expressed housekeeping σ^A protein, detected using anti- σ^A antibodies, was used as an internal normalization control for each experiment (Fujita, 2000) (Fig. 1a). KinA–GFP was detected at relatively low levels at the early stage of sporulation and then its level gradually increased during sporulation (Fig. 1b), essentially consistent with previous results (Fujita & Losick, 2005). Furthermore, in support of previous results (Bai *et al.*, 1990; Fujita & Losick, 2005), levels of Spo0F–GFP and Spo0A increased gradually upon entry into sporulation (Fig. 1b). During the course of the experiments, we found that the expression of GFP-tagged Spo0B (strain MF3675) was low and extremely difficult to quantify accurately with an immunodetection procedure and a fluorescence microscope. Therefore, we replaced the endogenous ribosome-binding site for *spo0B* with an optimized one (Fujita & Losick, 2002), which was used for the promoter fusion to GFP as described below, and confirmed that this alternative GFP–Spo0B was functional in conjunction with a complementation assay (data not shown). The alternative GFP-tagged version of Spo0B protein was detectable with an immunoblot assay and by fluorescence microscopy (MF1377, shown below). We found that GFP–Spo0B increased gradually upon entry into sporulation. We then confirmed that levels of GFP-tagged Spo0B in two strains (original MF3675 and mutated MF1377) were different: expression levels in the strain harbouring the optimum ribosome-binding site (MF1377) were approximately fivefold higher than those in the strain harbouring the native ribosome-binding site (MF3675) (Supplementary Fig. S1). Although the protein levels were different between these two strains, the expression pattern of a gradual increase appeared to be similar in the two strains (Supplementary Fig. S1). These results indicate that the levels of all the phosphorelay components show a gradual increase from basal to peak levels during the early

sporulation phase, while the control mechanisms of the expression of the phosphorelay components remain unknown. Furthermore, our results suggest the idea that the entry into the sporulation phase is accompanied by an increase in the intracellular concentration of the phosphorelay components. To confirm that the expression level of a gene located at the *amyE* locus is similar to that at the endogenous locus, as a representative example we measured the level of Spo0A expressed at the two different loci. The results shown in Supplementary Fig. S2 indicated that the two levels were indistinguishable, suggesting that the level of a protein ectopically expressed at the *amyE* locus is equivalent to that expressed from the endogenous locus.

Expression profile of genes encoding phosphorelay components during the initiation of sporulation at the single-cell level

Thus far, our methods only allowed us to assess the mean level of phosphorelay components in a cell population. At a cell-population level, the transcriptional profiles of the genes involved in the phosphorelay have also been analysed by the use of *lacZ* transcriptional fusions to each of the individual promoters (Antoniewski *et al.*, 1990; Asai *et al.*, 1995; Asayama *et al.*, 1995; Bai *et al.*, 1990; Bouvier *et al.*, 1984; Chibazakura *et al.*, 1995; Ferrari *et al.*, 1985; Predich *et al.*, 1992; Strauch *et al.*, 1992, 1993). In contrast to these measurements, visualization of gene expression in individual cells by the use of fluorescence microscopy reveals heterogeneous gene expression in a population of genetically identical bacteria. Various studies on population heterogeneity have been reported for genetic competence, sporulation and cannibalism, and swimming and chaining (Chung *et al.*, 1994; Dubnau & Losick, 2006;

Gonzalez-Pastor *et al.*, 2003; Kearns & Losick, 2005; Veening *et al.*, 2008a, b, c). These types of studies of sporulation have revealed that the size of the subpopulation expressing early developmental genes that are directly activated by Spo0A~P correlates well with the fraction of cells that produce spores (Veening *et al.*, 2008c). It has been speculated that both positive and negative regulatory loops are involved in maintaining the two subpopulations and that the threshold of Spo0A~P (phosphorelay components) is a principal barrier that cells in a population overcome to initiate sporulation (Fujita *et al.*, 2005; Veening *et al.*, 2005). Also, one of the above groups has demonstrated that sporulation bistability and the formation of heterogeneity in a culture population are dependent on the phosphorelay, suggesting that the sporulation signal is memorized by the positive-feedback architecture (Veening *et al.*, 2008c). For a further understanding of population heterogeneity, we next attempted to investigate the expression profiles of the phosphorelay components, at both the transcription and the protein level, at the single-cell level. For this, we constructed promoter fusions to the *gfp* gene and introduced each of the promoter-fused reporter genes at the *amyE* locus in the chromosome of the wild-type strain as a single copy. To exclude the ambiguity of the translational control, each of the endogenous promoters was fused to an optimized ribosome-binding site (5'-AAGGAGGAA-3') (Vellanoweth & Rabinowitz, 1992) and then followed by the gene for GFP. Each of the strains was induced to undergo sporulation using the resuspension method in SM medium (Sterlini & Mandelstam, 1969). We confirmed that the sporulation efficiency of each of the reporter strains was indistinguishable from that of the wild-type strain (data not shown). We then captured a series of images during sporulation and quantified the fluorescence signals, which were uniformly

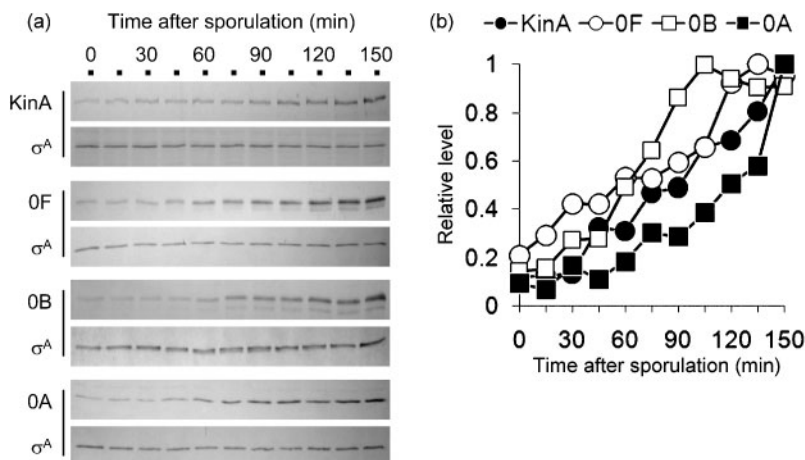


Fig. 1. Immunoblot analysis to measure protein levels of phosphorelay components during the initiation of sporulation. (a) Cell extracts were prepared from strains harbouring the genes for GFP-tagged KinA (MF929), Spo0F (MF1373) and Spo0B (MF1377) at the indicated times after suspension in SM medium. Protein samples were subjected to SDS-PAGE followed by immunoblot analysis with anti-GFP antibodies. In addition, the accumulation of Spo0A prepared from the wild-type strain (PY79) was analysed by immunoblotting with anti-Spo0A antibodies. (b) Quantification of the time-course of protein accumulation from the immunoblot analysis shown in (a), normalized to the constitutively expressed σ^A levels and then to the maximum level of each phosphorelay component. The intensity of each band was quantified with an image analyser, as described in Methods. Representative results from one of three independent experiments are shown.

distributed throughout the cytoplasmic space in individual cells. As shown in Fig. 2, the fluorescence intensities of GFP from each of the promoters increased over the time of the initiation process of sporulation (1–2 h), suggesting that transcription from the promoters continues during the early sporulation phase, as demonstrated by the use of *lacZ* reporter systems in the published literature (Antoniewski *et al.*, 1990; Asai *et al.*, 1995; Asayama *et al.*, 1995; Bai *et al.*, 1990; Bouvier *et al.*, 1984; Chibazakura *et al.*, 1995; Ferrari

et al., 1985). However, the intensities of GFP signals derived from each promoter showed variability among individual cells at each time point. With a closer look at each image, signals from the promoters for *spo0F* and *spo0A* were more heterogeneously distributed throughout the culture population than those from the other two promoters: after hour 2 (t_2), GFP signals from *kinA* and *spo0B* promoters peaked at around 100 arbitrary units (AU), with ranges between 60 and 200 AU, while those from *spo0F* (100–700 AU) and

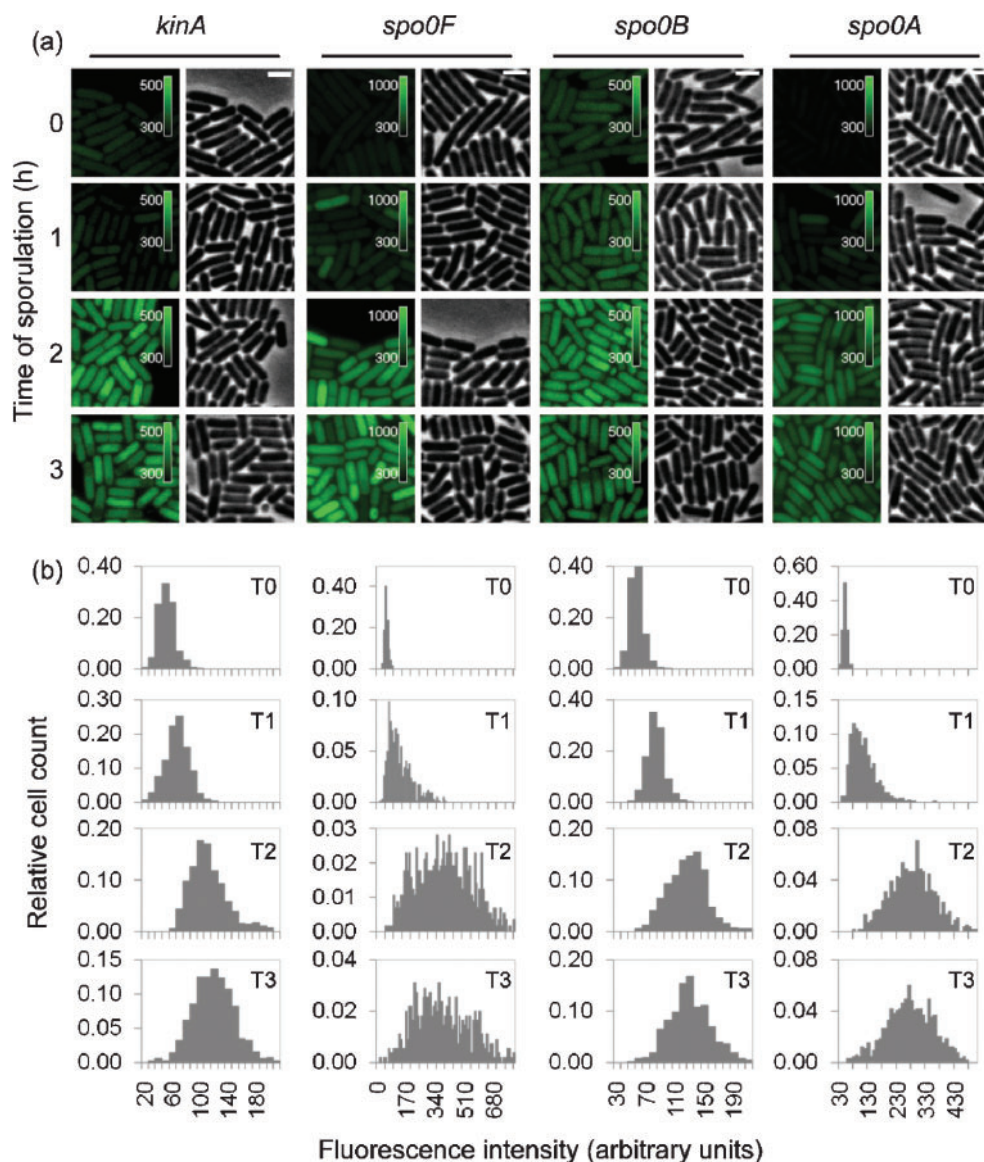


Fig. 2. Single-cell transcriptional profiles of genes for phosphorelay components during the initiation of sporulation. (a) Microscope images for GFP fluorescence (green) and phase contrast (greyscale) were captured in the strains harbouring the *gfp* fusion to the following promoters: *kinA* (MF934), *spo0F* (MF935), *spo0B* (MF943) and *spo0A* (MF280). Cells were collected at the indicated times after suspension in SM medium to induce sporulation. (b) The same data shown in (a) were processed and the pixel intensities are shown as an intensity plot in the histogram for each image. GFP intensities were measured in cells selected randomly (>200 cells) at 0, 1, 2 and 3 h after the onset of sporulation. Relative cell numbers are shown on the y axis. Pixel values (arbitrary units) are shown on the x axis. Bars, 2 μ m.

spo0A (100–400 AU) were widely distributed. These results suggest that transcription from the genes for *kinA* and *spo0B* is at a relatively low level and distributed evenly in individual cells, while that from *spo0F* and *spo0A* genes is at a relatively high level but distributed unequally in individual cells. Generally, it is expected that variability of protein distribution within a population of cells is linked to multiple factors, including the number of protein molecules per cell (Kaern *et al.*, 2005). This factor is expected to be less important for more abundant proteins. In other words, as the protein concentration increases, a smaller variability in the protein distribution pattern would be expected. However, our observations show an opposite trend, possibly indicating that the heterogeneity of protein levels arises from their transcriptional regulation but not from the abundance of the respective proteins. Regulation of transcription for each promoter is different and complicated: (1) *kinA* is under the control of σ^H ; (2) *spo0F* is under the control of both σ^H and Spo0A~P; (3) *spo0B* is under the control of σ^A ; (4) *spo0A* is controlled by two promoters, σ^A -controlled P_v and σ^H - and Spo0A~P-controlled P_s. Nevertheless, we found that the level of transcription from each of the promoters for the phosphorelay component genes was heterogeneous and variable in a population, though to differing extents. During the initial review process of our manuscript, Kuipers and co-workers have reported that the transcription levels of phosphorelay components increases over time and that the activity of transcription from each phosphorelay gene shows a cell-to-cell variability (de Jong *et al.*, 2010). Their results are essentially consistent with our data presented here, although the culture and experimental conditions are different in the two laboratories.

It should be noted that in many of the experiments using reporter systems, including *gfp* and *lacZ*, the stability of the reporter proteins (GFP and β -galactosidase) may hinder direct interpretation of the results (Pessi *et al.*, 2001). The stability of the reporter gene products might affect the accuracy of measurements of transcriptional activity. For example, synthesis, accumulation and degradation of the protein cannot be distinguished in the reporter system. Thus, the reporter assays do not fully report gene expression. Although we used the transcriptional fusion system in this study, these are technical limitations that we currently cannot overcome.

Based on our transcriptional profiles shown in Fig. 2, we hypothesized that not only Spo0A activity, as demonstrated in earlier studies (Gonzalez-Pastor *et al.*, 2003; Veening *et al.*, 2005), but also the expression of the other phosphorelay components, KinA, Spo0F and Spo0B proteins, display heterogeneous patterns in a cell population. Thus, to investigate the population distribution as well as the subcellular localization of the KinA, Spo0F and Spo0B proteins in more detail, and to determine whether the distribution pattern of these components is bimodal, as suggested in the case of *spoIIA* transcription for monitoring the level of phosphorylated Spo0A (Veening *et al.*, 2008b, c), we collected images of cells expressing each functional

GFP fusion protein by fluorescence microscopy. Under sporulation conditions, all these proteins were distributed more or less uniformly throughout the cytoplasmic space in a single cell (Fig. 3a). However, we found that the levels of the phosphorelay components, as measured by GFP intensity, varied among individual cells in a population: some cells expressed phosphorelay components more efficiently than others (Fig. 3). Despite the heterogeneity in the distribution of phosphorelay components, our results did not show a clear bimodal distribution of the components in the population.

A comparison between the distributions shown in Figs 2 and 3 suggests similar trends in the levels of noise in the transcriptional activities and protein concentrations of the respective components. To quantify statistically the distribution of noise levels of a transcriptional GFP reporter and of a GFP fusion protein, we computed coefficients of variation (CVs), defined as the standard deviation divided by the mean. This dimensionless quantity corresponds to the fraction of noise in the distribution, i.e. a CV of 0.2 correspond to 20 % noise. The computed CVs are shown in Table 1. Statistical analysis indicated a strong and significant linear correlation (correlation coefficient, $r=0.8770$, $P=0.0217$) between the CVs of transcriptional reporter and protein distributions measured at the same time points (1 and 2 h). Based on this statistical analysis, we concluded that the distribution patterns of each of the components were essentially consistent with those in the transcriptional profile shown in Fig. 2: (1) Spo0F was more heterogeneously distributed throughout the culture population than KinA or Spo0B; and (2) the level of the Spo0F protein was higher than that of the other two proteins. Furthermore, we found that these distribution patterns were relatively unimodal and broader than those in the published literature (Veening *et al.*, 2008b, c). It is important to note that several different cell types, such as sporulating, motile and competent, can be identified within a single culture due to population heterogeneity (Chung *et al.*, 1994; Dubnau & Losick, 2006; Gonzalez-Pastor *et al.*, 2003; Kearns & Losick, 2005; Maamar & Dubnau, 2005; Veening *et al.*, 2008b, c), so that some cells induce the developmental programme of gene expression and others do not, even under identical sporulation conditions. Our results indicate that the stochasticity of transcription is an important regulatory mechanism for the heterogeneity of the phosphorelay components in a population and thus for cell type determination, although in this study our data do not provide a clear separation between sporulating and non-sporulating cells.

Single-cell measurement of molecule numbers and concentrations of phosphorelay components

In an attempt to further understand the regulation of the phosphorelay, we determined molecule numbers and concentrations of each component in a single cell. For this purpose, a quantitative immunoblot analysis in

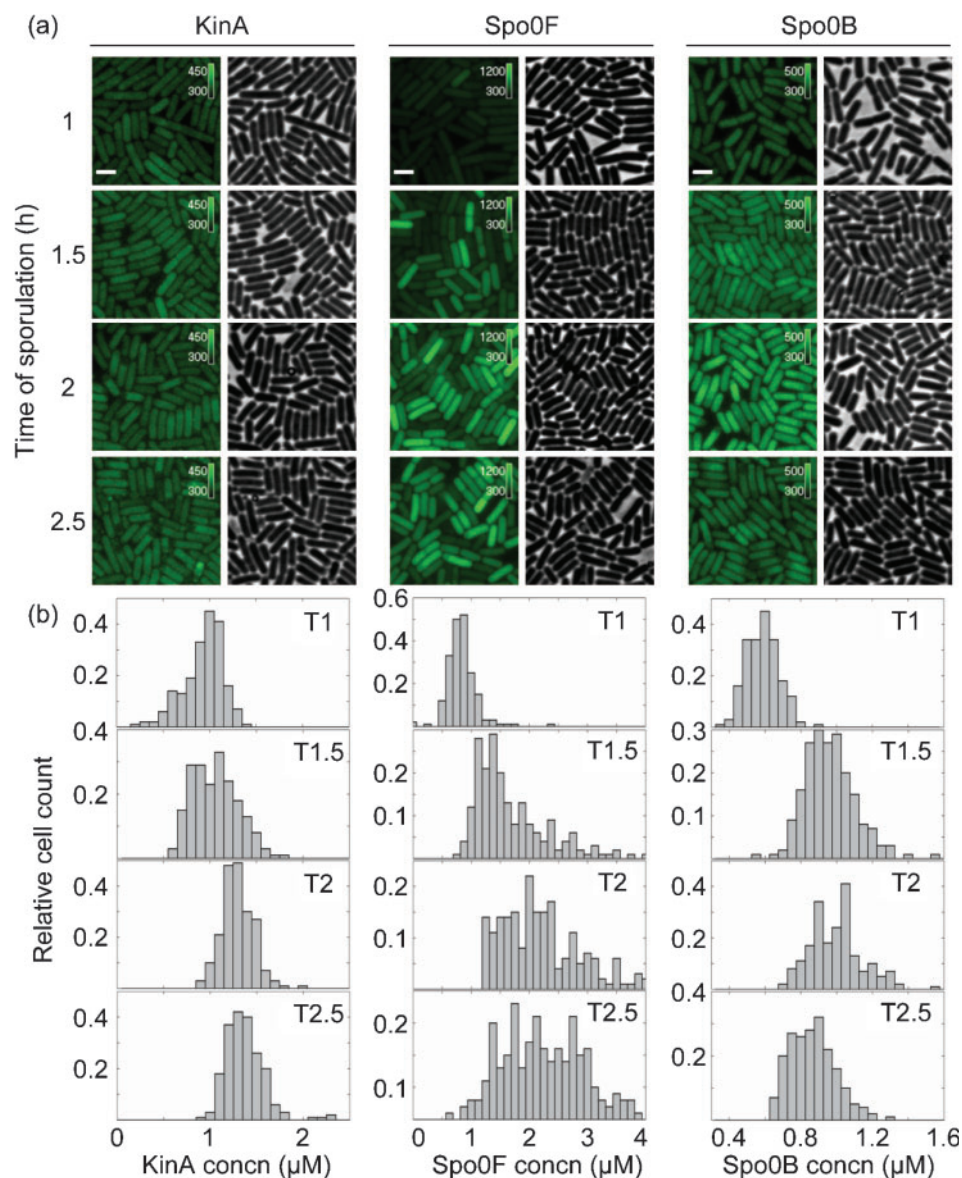


Fig. 3. Distribution of KinA, Spo0F and Spo0B in a cell population during the initiation of sporulation. (a) Sporulating cells of the strains harbouring GFP-tagged KinA (MF929), Spo0F (MF1373) and Spo0B (MF1377) were collected at 1, 1.5, 2 and 2.5 h after the start of sporulation and analysed by fluorescence microscopy. (b) Distributions of GFP intensities for KinA, Spo0F and Spo0B in 200 randomly selected cells were quantified for the images for each time point. The distributions of concentrations (μM) for each component in a cell population (number of cells) were determined from normalized fluorescence intensities using linear regression and plotted in the histograms (see Methods and Fig. 5). The pixel intensities are shown as an intensity plot in the histogram for each image. Bars, 2 μm.

combination with fluorescence microscopy imaging was performed. First, we used the same samples of cell culture prepared for the immunoblotting experiments. In each determination, we first prepared a standard curve for each of the purified proteins (GFP and Spo0A) and determined the range for which there is a linear relationship between protein concentration and intensity of immunostaining (Fig. 4). Using appropriate amounts of cell extracts, the analysis for each protein was repeated three times, always in parallel with

the respective purified protein for comparison. The molar ratios of KinA, Spo0F, Spo0B and Spo0A increased approximately 10-fold from early to late sporulation (from hour 0 to 2.5 h after suspension in SM medium) (Fig. 1b). To calculate the concentrations of phosphorelay components per cell, we measured the number of cells and the cell volume based on the measurement of cell dimension (Table 2). By using these parameters, we estimated approximate mean concentrations of each component in a

Table 1. CVs for the levels of transcription and protein of the phosphorelay components

Component	CV at time:			
	0 h	1 h	2 h	3 h
Transcription				
<i>kinA</i>	0.2477	0.2183	0.2188	0.2376
<i>spo0F</i>	0.2181	0.4858	0.3923	0.4066
<i>spo0B</i>	0.1801	0.1353	0.1766	0.1911
Protein	1 h	1.5 h	2 h	2.5 h
KinA	0.2319	0.2308	0.1331	0.1530
Spo0F	0.2957	0.3745	0.3025	0.3095
Spo0B	0.1538	0.1458	0.1441	0.1448

cell population, as summarized in Table 2. We should note that the physiological level of Spo0B (Table 2) is estimated to be one-fifth (20%) of the actual value because a mutated MF1377 expressing approximately fivefold higher levels of Spo0B than the native MF3675 (Supplementary Fig. S1) was used for the measurement.

To determine the levels of the phosphorelay components in individual cell types in a culture population, it is important to establish a correlation between the fluorescence intensities and the concentrations in individual cells. For this purpose, we used the same series of fluorescence images showing an isogenic population distribution for each functional GFP fusion under identical sporulation culture conditions as shown in Fig. 2. Since GFP-tagged Spo0A is not functional, microscope images for Spo0A were not available in this study. Distributions of GFP intensities for KinA, Spo0B and Spo0F in randomly selected cells (more than 200 cells) were quantified for

images at 1, 1.5, 2 and 2.5 h after the onset of sporulation. It is natural to assume that average population fluorescence intensities are linearly related to the corresponding protein concentrations. Therefore, we conducted a least linear regression to determine the proportionality coefficients between the protein concentrations and normalized-by-background fluorescence intensities (see Methods). With this coefficient, we were able to convert the distribution of fluorescence intensities for GFP-fused KinA, Spo0F and Spo0B into the distribution of intercellular concentrations (Fig. 5). Thus, we successfully determined the distributions of concentrations for each component in a cell population during early sporulation (Fig. 3, histograms). We note that the GFP fusion proteins used in this study might have activities or stabilities altered from those of the native forms of these proteins. Therefore, the exact numerical values obtained here for protein concentrations may not correspond exactly to those in the wild-type situation. At present, this is an inherent technical limitation that we

Table 2. Cell number, cell volume and concentration of each phosphorelay component

Time after sporulation (min)	Cell number $\times 10^8$ (ml ⁻¹)	Cell volume (fl)	Concentration (μ M)*			
			KinA	Spo0F	Spo0B†	Spo0A
0	2.8	2.6	0.2	0.5	0.03	4.4
15	3.1	2.2	0.2	0.7	0.03	4.7
30	3.5	1.9	0.2	1.0	0.06	4.8
45	3.8	1.8	0.6	1.0	0.06	12.0
60	4.1	1.8	0.6	1.3	0.10	11.5
75	4.3	1.8	0.9	1.3	0.14	17.5
90	4.5	1.8	0.9	1.4	0.18	18.2
105	4.7	1.8	1.2	1.6	0.21	24.6
120	4.8	1.8	1.2	2.2	0.20	26.4
135	4.7	1.8	1.5	2.4	0.19	30.0
150	4.5	1.8	1.8	2.3	0.19	37.4

*Calculated as monomer.

†Values are converted to the estimated physiological levels according to the difference between strains MF1377 and MF3675 (Supplementary Fig. S1). More details can be found in the text.

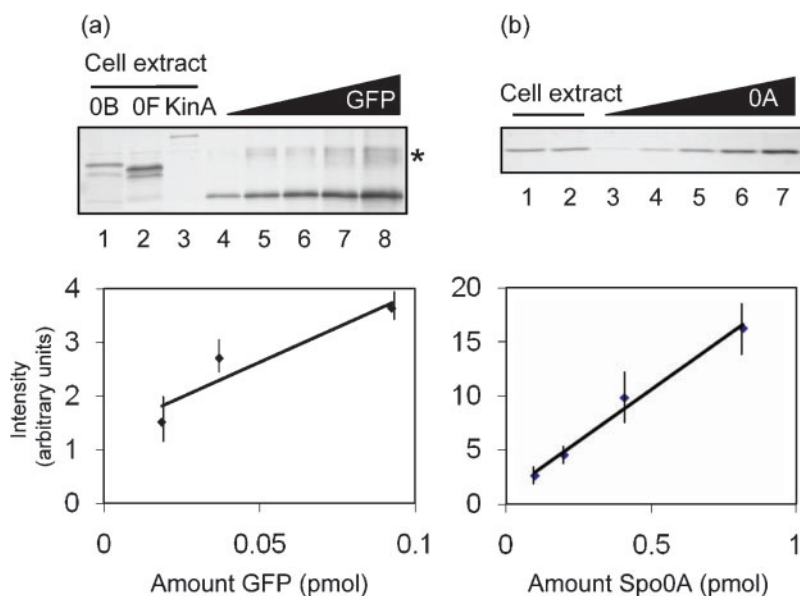


Fig. 4. Standard curves for determination of the purified proteins. Varying amounts of purified GFP (a) and Spo0A (b) were subjected to SDS-PAGE followed by immunoblot analysis with anti-GFP and anti-Spo0A antibodies, respectively. Shown are representative blot images from one of three independent experiments. The intensity of each band was quantified as described in Methods. The asterisk indicates a non-specific band.

cannot overcome. However, strains containing these fusions show a timing and frequency of sporulation nearly identical to those obtained in the wild-type. These results suggest that the parameters and distributions measured are still sufficiently close to those of the natural situation to allow a proper assessment of the functionality of the sporulation initiation network.

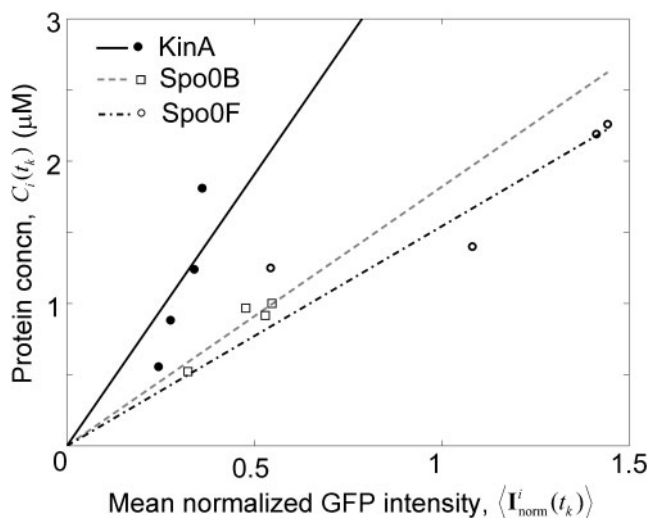


Fig. 5. Use of linear regression to relate population mean fluorescence intensities to immunoblot concentration measurements. The data points measured from the original fluorescence images shown in Fig. 3 are depicted as symbols, and least mean square fits are shown as lines. The regression coefficients were then used to compute the cell population distribution of concentrations from intensities in Fig. 3. More details can be found in Methods.

The heterogeneity of the phosphorelay components in a population is not mediated by genetic changes. Instead, such variations might arise from the stochasticity of protein expression, regulated mainly at the transcriptional level (Figs 2 and 3, Table 1). Presumably, the sum of the fluctuations in multiple components of the phosphorelay under the multiple feedback loops amplifies the noise in any particular cell in a population. Accordingly, we speculate that the net effect of the increased levels of each phosphorelay component is to generate a threshold level of Spo0A~P (Fujita *et al.*, 2005). Thus, once a critical level of Spo0A~P is achieved, the positive feedback loop serves as an amplifying cascade for the further generation of Spo0A~P (Fujita & Sadaie, 1998; Hoch, 1993; Strauch *et al.*, 1993). This feedback loop provides cells with a robust mechanism to achieve the expression of sporulation genes such as *spoIIA* and *spoIIG*, and ensures that the expression of these genes is maintained until the next stage of development (Fujita *et al.*, 2005).

Taken together, we propose that successful entry into the sporulation state is manifested by a particular level of each phosphorelay component: if one or more of the phosphorelay components is at less than the threshold level, sporulation is 'off'. Therefore, when a cell has the optimal quorum requirement of each of the components under sporulation conditions, the cell can proceed successfully to the completion of the sporulation process. However, remaining cells that do not reach the quorum do not sporulate. As a result, only a subpopulation of genetically identical cells enters sporulation, even under the same culture conditions.

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REFERENCES

- Antoniewski, C., Savelli, B. & Stragier, P. (1990). The *spoIIJ* gene, which regulates early developmental steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. *J Bacteriol* **172**, 86–93.
- Asai, K., Kawamura, F., Yoshikawa, H. & Takahashi, H. (1995). Expression of *kinA* and accumulation of σ^H at the onset of sporulation in *Bacillus subtilis*. *J Bacteriol* **177**, 6679–6683.
- Asayama, M., Yamamoto, A. & Kobayashi, Y. (1995). Dimer form of phosphorylated Spo0A, a transcriptional regulator, stimulates the *spo0F* transcription at the initiation of sporulation in *Bacillus subtilis*. *J Mol Biol* **250**, 11–23.
- Asayama, M., Saito, K. & Kobayashi, Y. (1998). Translational attenuation of the *Bacillus subtilis* *spo0B* cistron by an RNA structure encompassing the initiation region. *Nucleic Acids Res* **26**, 824–830.
- Bai, U., Lewandoski, M., Dubnau, E. & Smith, I. (1990). Temporal regulation of the *Bacillus subtilis* early sporulation gene *spo0F*. *J Bacteriol* **172**, 5432–5439.
- Bouvier, J., Stragier, P., Bonamy, C. & Szulmajster, J. (1984). Nucleotide sequence of the *spo0B* gene of *Bacillus subtilis* and regulation of its expression. *Proc Natl Acad Sci U S A* **81**, 7012–7016.
- Burbulys, D., Trach, K. A. & Hoch, J. A. (1991). Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. *Cell* **64**, 545–552.
- Chibazakura, T., Kawamura, F., Asai, K. & Takahashi, H. (1995). Effects of *spo0* mutations on *spo0A* promoter switching at the initiation of sporulation in *Bacillus subtilis*. *J Bacteriol* **177**, 4520–4523.
- Chung, J. D., Stephanopoulos, G., Ireton, K. & Grossman, A. D. (1994). Gene expression in single cells of *Bacillus subtilis*: evidence that a threshold mechanism controls the initiation of sporulation. *J Bacteriol* **176**, 1977–1984.
- de Jong, I. G., Veening, J. W. & Kuipers, O. P. (2010). Heterochronic phosphorelay gene expression as a source of heterogeneity in *Bacillus subtilis* spore formation. *J Bacteriol* **192**, 2053–2067.
- Dubnau, D. & Losick, R. (2006). Bistability in bacteria. *Mol Microbiol* **61**, 564–572.
- Eswaramoorthy, P., Guo, T. & Fujita, M. (2009). *In vivo* domain-based functional analysis of the major sporulation sensor kinase, KinA, in *Bacillus subtilis*. *J Bacteriol* **191**, 5358–5368.
- Ferrari, F. A., Trach, K. & Hoch, J. A. (1985). Sequence analysis of the *spo0B* locus reveals a polycistronic transcription unit. *J Bacteriol* **161**, 556–562.
- Fujita, M. (2000). Temporal and selective association of multiple sigma factors with RNA polymerase during sporulation in *Bacillus subtilis*. *Genes Cells* **5**, 79–88.
- Fujita, M. & Losick, R. (2002). An investigation into the compartmentalization of the sporulation transcription factor σ^E in *Bacillus subtilis*. *Mol Microbiol* **43**, 27–38.
- Fujita, M. & Losick, R. (2005). Evidence that entry into sporulation in *Bacillus subtilis* is governed by a gradual increase in the level and activity of the master regulator Spo0A. *Genes Dev* **19**, 2236–2244.
- Fujita, M. & Sadaie, Y. (1998). Feedback loops involving Spo0A and AbrB in *in vitro* transcription of the genes involved in the initiation of sporulation in *Bacillus subtilis*. *J Biochem* **124**, 98–104.
- Fujita, M., Gonzalez-Pastor, J. E. & Losick, R. (2005). High- and low-threshold genes in the Spo0A regulon of *Bacillus subtilis*. *J Bacteriol* **187**, 1357–1368.
- Gonzalez-Pastor, J. E., Hobbs, E. C. & Losick, R. (2003). Cannibalism by sporulating bacteria. *Science* **301**, 510–513.
- Gregory, J. A., Becker, E. C. & Pogliano, K. (2008). *Bacillus subtilis* MinC destabilizes FtsZ-rings at new cell poles and contributes to the timing of cell division. *Genes Dev* **22**, 3475–3488.
- Grossman, A. D. (1995). Genetic networks controlling the initiation of sporulation and the development of genetic competence in *Bacillus subtilis*. *Annu Rev Genet* **29**, 477–508.
- Hoch, J. A. (1993). Regulation of the phosphorelay and the initiation of sporulation in *Bacillus subtilis*. *Annu Rev Microbiol* **47**, 441–465.
- Kaern, M., Elston, T. C., Blake, W. J. & Collins, J. J. (2005). Stochasticity in gene expression: from theories to phenotypes. *Nat Rev Genet* **6**, 451–464.
- Kearns, D. B. & Losick, R. (2005). Cell population heterogeneity during growth of *Bacillus subtilis*. *Genes Dev* **19**, 3083–3094.
- Kubitschek, H. E. & Friske, J. A. (1986). Determination of bacterial cell volume with the Coulter Counter. *J Bacteriol* **168**, 1466–1467.
- Maamar, H. & Dubnau, D. (2005). Bistability in the *Bacillus subtilis* K-state (competence) system requires a positive feedback loop. *Mol Microbiol* **56**, 615–624.
- Perego, M. & Hoch, J. A. (2002). Two-component systems, phosphorelays, and regulation of their activities by phosphatases. In *Bacillus subtilis and its Closest Relatives: from Genes to Cells*, pp. 473–482. Edited by A. L. Sonneshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Pessi, G., Blumer, C. & Haas, D. (2001). *lacZ* fusions report gene expression, don't they? *Microbiology* **147**, 1993–1995.
- Piggot, P. J. & Losick, R. (2002). Sporulation genes and intercompartmental regulation. In *Bacillus subtilis and its Closest Relatives: from Genes to Cells*, pp. 483–518. Edited by A. L. Sonneshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Predich, M., Nair, G. & Smith, I. (1992). *Bacillus subtilis* early sporulation genes *kinA*, *spo0F*, and *spo0A* are transcribed by the RNA polymerase containing σ^H . *J Bacteriol* **174**, 2771–2778.
- Rudner, D. Z. & Losick, R. (2002). A sporulation membrane protein tethers the pro- σ^K processing enzyme to its inhibitor and dictates its subcellular localization. *Genes Dev* **16**, 1007–1018.
- Sharpe, M. E., Hauser, P. M., Sharpe, R. G. & Errington, J. (1998). *Bacillus subtilis* cell cycle as studied by fluorescence microscopy: constancy of cell length at initiation of DNA replication and evidence for active nucleoid partitioning. *J Bacteriol* **180**, 547–555.
- Sonenshein, A. L. (2000). Control of sporulation initiation in *Bacillus subtilis*. *Curr Opin Microbiol* **3**, 561–566.
- Stephenson, K. & Hoch, J. A. (2002). Evolution of signalling in the sporulation phosphorelay. *Mol Microbiol* **46**, 297–304.
- Sterlini, J. M. & Mandelstam, J. (1969). Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin resistance. *Biochem J* **113**, 29–37.
- Strauch, M. A., Trach, K. A., Day, J. & Hoch, J. A. (1992). Spo0A activates and represses its own synthesis by binding at its dual promoters. *Biochimie* **74**, 619–626.
- Strauch, M. A., Wu, J. J., Jonas, R. H. & Hoch, J. A. (1993). A positive feedback loop controls transcription of the *spo0F* gene, a component

of the sporulation phosphorelay in *Bacillus subtilis*. *Mol Microbiol* **7**, 967–974.

Trach, K. & Hoch, J. A. (1989). The *Bacillus subtilis* *spo0B* stage 0 sporulation operon encodes an essential GTP-binding protein. *J Bacteriol* **171**, 1362–1371.

Veening, J. W., Hamoen, L. W. & Kuipers, O. P. (2005). Phosphatases modulate the bistable sporulation gene expression pattern in *Bacillus subtilis*. *Mol Microbiol* **56**, 1481–1494.

Veening, J. W., Igoshin, O. A., Eijlander, R. T., Nijland, R., Hamoen, L. W. & Kuipers, O. P. (2008a). Transient heterogeneity in extracellular protease production by *Bacillus subtilis*. *Mol Syst Biol* **4**, 184.

Veening, J. W., Smits, W. K. & Kuipers, O. P. (2008b). Bistability, epigenetics, and bet-hedging in bacteria. *Annu Rev Microbiol* **62**, 193–210.

Veening, J. W., Stewart, E. J., Berngruber, T. W., Taddei, F., Kuipers, O. P. & Hamoen, L. W. (2008c). Bet-hedging and epigenetic

inheritance in bacterial cell development. *Proc Natl Acad Sci U S A* **105**, 4393–4398.

Vellanoweth, R. L. & Rabinowitz, J. C. (1992). The influence of ribosome-binding-site elements on translational efficiency in *Bacillus subtilis* and *Escherichia coli* *in vivo*. *Mol Microbiol* **6**, 1105–1114.

Wach, A. (1996). PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. *Yeast* **12**, 259–265.

Weir, J., Predich, M., Dubnau, E., Nair, G. & Smith, I. (1991). Regulation of *spo0H*, a gene coding for the *Bacillus subtilis* σ^H factor. *J Bacteriol* **173**, 521–529.

Youngman, P., Perkins, J. B. & Losick, R. (1984). Construction of a cloning site near one end of Tn917 into which foreign DNA may be inserted without affecting transposition in *Bacillus subtilis* or expression of the transposon-borne *erm* gene. *Plasmid* **12**, 1–9.

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