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## Heterochronic Phosphorelay Gene Expression as a Source of Heterogeneity in *Bacillus subtilis* Spore Formation

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## Supplementary Material

**Figure S1: Heterogeneous expression of the main phosphorelay components and *sigH*.** Snapshots of early, mid and late exponential phase from at least three time-lapse movies were selected per strain to create histograms displaying the normalized distribution of *gfp* expression during microcolony development. **(A)** wild type (168 *trp*<sup>+</sup>, not carrying a *gfp* construct), **(B)**  $P_{sigH}$ -*gfp* (IDJ001), **(C)**  $P_{kinA}$ -*gfp* (IDJ002), **(D)**  $P_{kinB}$ -*gfp* (IDJ003), **(E)**  $P_{spo0F}$ -*gfp* (IDJ004), **(F)**  $P_{spo0B}$ -*gfp* (IDJ005), **(G)**  $P_{spo0A}$ -*gfp* (IDJ006) and **(H)**  $P_{spollA}$ -*gfp* (IDJ007). The late exponential histograms were used for the insets in Fig. 2.

**Figure S2: Dynamic and variable expression of the main phosphorelay components and *sigH*.** Single cell trajectories of separate lineages leading to sporulating (left panel) and non-sporulating cells (right panel). **(A)** Wild type (no *gfp* expression), **(B)**  $P_{sigH}$ -*gfp* (strain IDJ001), **(C)**  $P_{kinA}$ -*gfp* (IDJ002), **(D)**  $P_{kinB}$ -*gfp* (IDJ003), **(E)**  $P_{spo0F}$ -*gfp* (IDJ004), **(F)**  $P_{spo0B}$ -*gfp* (IDJ005), **(G)**  $P_{spo0A}$ -*gfp* (IDJ006) and **(H)**  $P_{spollA}$ -*gfp* (IDJ007).

**Figure S3: Dynamic and variable expression of the main phosphorelay components and *sigH*.** Single cell trajectories of separate lineages leading to sporulating (left panel) and non-sporulating cells (right panel). **(A)**  $P_{spollA}$ -*gfp* (IDJ007), **(B)**  $P_{sigH}$ -*gfp* (strain IDJ001), **(C)**  $P_{kinA}$ -*gfp* (IDJ002), **(D)**  $P_{kinB}$ -*gfp* (IDJ003), **(E)**  $P_{spo0F}$ -*gfp* (IDJ004), **(F)**  $P_{spo0B}$ -*gfp* (IDJ005) and **(G)**  $P_{spo0A}$ -*gfp* (IDJ006). The GFP values of  $P_{sigH}$ -*gfp*,  $P_{kinB}$ -*gfp*,  $P_{spo0F}$ -*gfp*,  $P_{spo0B}$ -*gfp*,  $P_{spo0A}$ -*gfp* are lower compared to

the data shown in Fig. S2 due to different microscope settings. Nevertheless the data are valuable to determine differences in trajectories of sporulating and non-sporulating cells.

**Figure S4: Non-fluorescent controls for data shown in Fig. 4.** GFP (green circles) and mCherry (red squares) background levels of the *B. subtilis* 168 *trp+* wild type strain (does not contain a GFP or mCherry construct) were followed through time during microcolony development (Movie S10). After each cell division, indicated by gray vertical lines, one of the two resulting siblings was arbitrarily selected for further analysis. The single cell trajectories are from randomly selected sporulating **(A)** and non-sporulating cells **(B)**. The traces were stopped when a spore became visible in the corresponding movie.

**Figure S5: Phosphorelay is robust to noise in gene expression.** The effect of overproduction of the phosphotransferases Spo0F (IDJ023) and Spo0B (IDJ024) on the *spoIIA* promoter was analyzed after growing the strains in TY medium and inducing them with 1 mM IPTG at early exponential phase. 6 h after induction, samples were taken for flow cytometry. **(black)**  $P_{spoIIA}$ -*spo0F*, no induction, **(red)**  $P_{spoIIA}$ -*spo0F*, 1 mM IPTG, **(grey)**  $P_{spoIIA}$ -*spo0B*, no induction, **(blue)**  $P_{spoIIA}$ -*spo0B*, 1 mM IPTG.

**Figure S6: Positive feedback by SigH is essential for spore formation.** *Gfp* expression of the  $P_{spoIIA}$ -*gfp*  $\Delta sigH$  (IDJ020) microcolony shown in Fig. 8. This

histogram shows that SigH is not required for bimodal expression of  $P_{spoIIA-gfp}$ , since its distribution shows a long-tailed unimodal pattern.

**Movie S1.** Time-lapse sequence (snapshots taken at intervals of 8 minutes) of *B. subtilis* 168 trp+ wild type strain (no GFP). Bright field (left, 0.05 s exposure), GFP (right, 0.5 s exposure, 10% xenon light).

**Movie S2.** Time-lapse sequence (snapshots taken at intervals of 8 minutes) of  $P_{sigH-gfp}$  (IDJ001). Bright field (left, 0.05 s exposure), GFP (right, 0.5 s exposure, 10% xenon light).

**Movie S3.** Time-lapse sequence (snapshots taken at intervals of 8 minutes) of  $P_{kinA-gfp}$  (IDJ002). Bright field (left, 0.05 s exposure), GFP (right, 0.5 s exposure, 10% xenon light).

**Movie S4.** Time-lapse sequence (snapshots taken at intervals of 8 minutes) of  $P_{kinB-gfp}$  (IDJ003). Bright field (left, 0.05 s exposure), GFP (right, 0.5 s exposure, 10% xenon light).

**Movie S5.** Time-lapse sequence (snapshots taken at intervals of 8 minutes) of  $P_{spo0F-gfp}$  (IDJ004). Bright field (left, 0.05 s exposure), GFP (right, 0.5 s exposure, 10% xenon light).

**Movie S6.** Time-lapse sequence (snapshots taken at intervals of 8 minutes) of  $P_{spo0B}$ -*gfp* (IDJ005). Bright field (left, 0.05 s exposure), GFP (right, 0.5 s exposure, 10% xenon light).

**Movie S7.** Time-lapse sequence (snapshots taken at intervals of 8 minutes) of  $P_{spo0A}$ -*gfp* (IDJ006). Bright field (left, 0.05 s exposure), GFP (right, 0.5 s exposure, 10% xenon light).

**Movie S8.** Time-lapse sequence (snapshots taken at intervals of 8 minutes) of  $P_{spoIIA}$ -*gfp* (IDJ007). Bright field (left, 0.1 s exposure), GFP (right, 0.5 s exposure, 10% xenon light).

**Movie S9.** Time-lapse sequence (snapshots taken at intervals of 12 minutes) of *B. subtilis* 168 *trp+* wild type strain (no GFP, no mCherry production). Bright field (left, 0.05 s exposure), GFP (middle, 0.2 s exposure, 10% xenon light), mCherry (right, 0.8 s exposure, 32% xenon light).

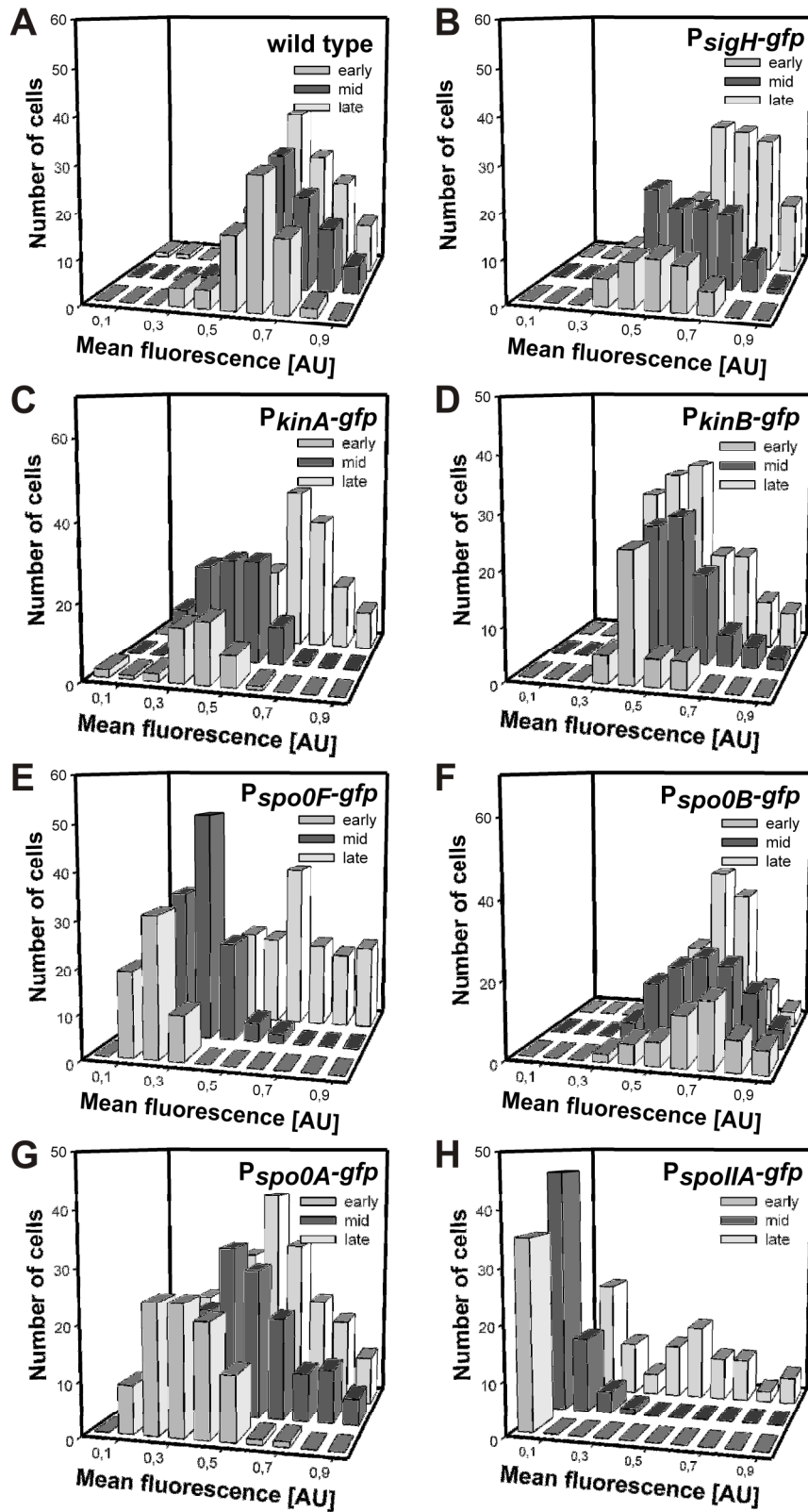
**Movie S10.** Time-lapse sequence (snapshots taken at intervals of 12 minutes) of  $P_{rapA}$ -*gfp*,  $P_{kinA}$ -*mCherry* (IDJ039). Bright field (left, 0.05 s exposure), GFP (middle, 0.2 s exposure, 10% xenon light), mCherry (right, 0.8 s exposure, 32% xenon light).

**Movie S11.** Time-lapse sequence (snapshots (0.1 s exposure) taken at intervals of 8 minutes) of  $P_{spank}$ -*kinA* (IDJ009).

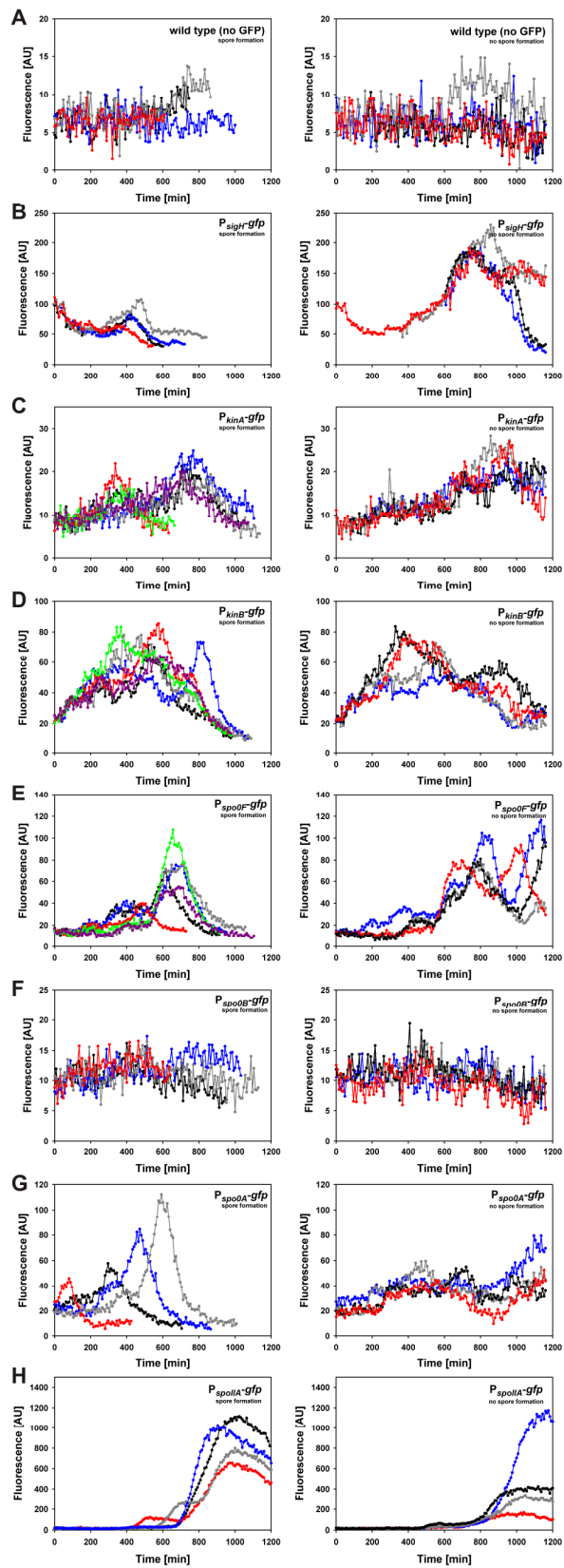
**Movie S12.** Time-lapse sequence (snapshots (0.1 s exposure) taken at intervals of 8 minutes) of  $P_{spank}$ -*kinB* (IDJ010).

**Movie S13.** Time-lapse sequence (snapshots (0.1 s exposure) taken at intervals of 8 minutes) of *B. subtilis* 168 trp+ wild type strain.

# de Jong\_FigS1

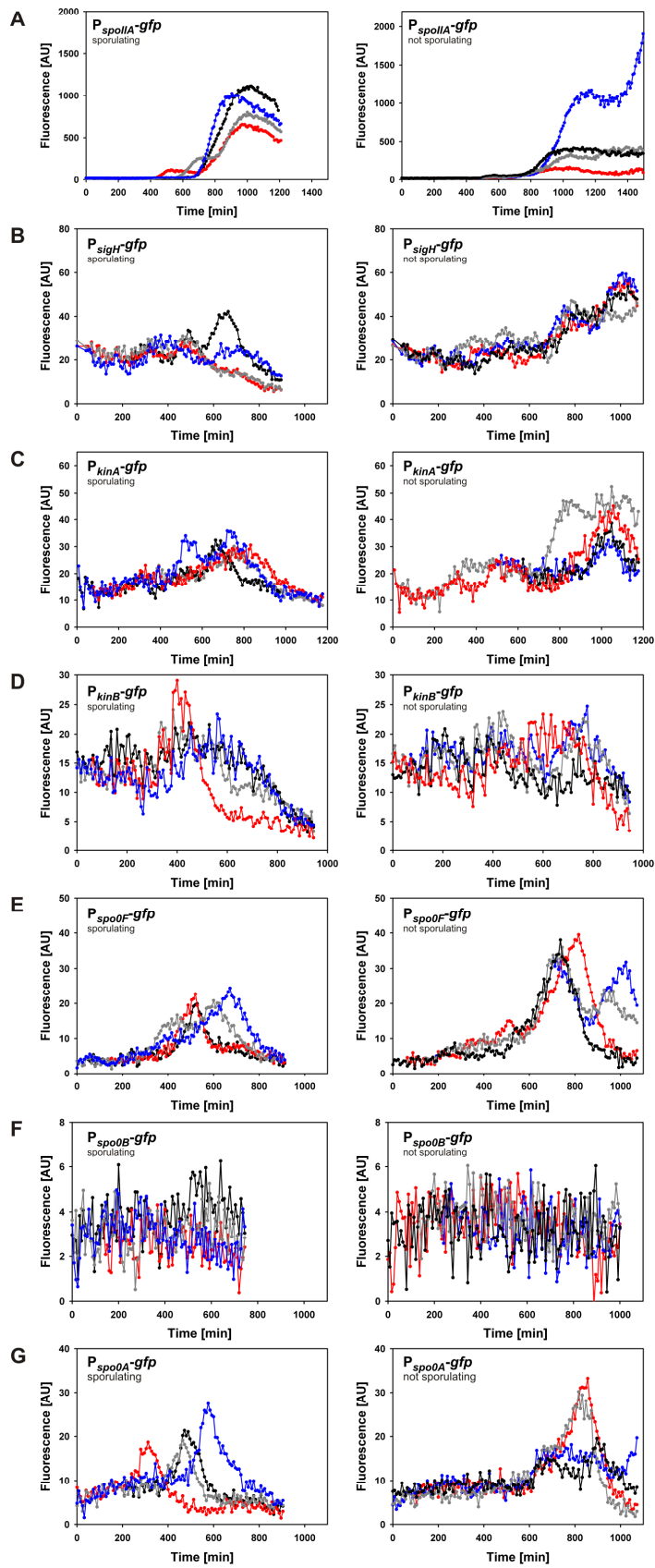


# de Jong\_Figure S2

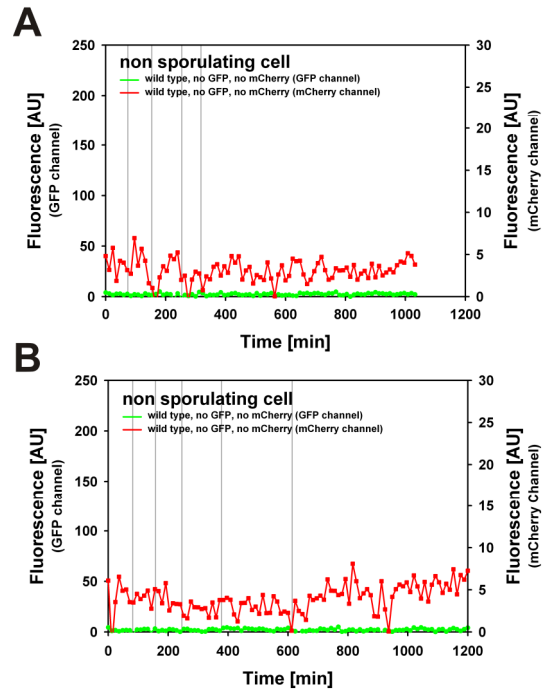




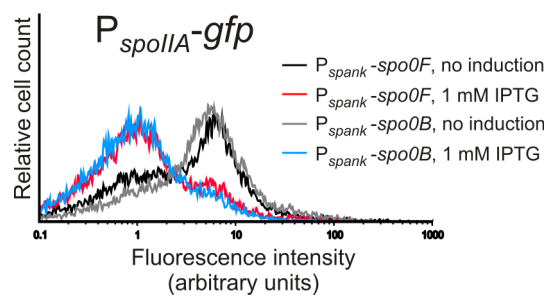
de Jong\_Fig. S3



# de Jong\_Figure S4



de Jong\_Figure S5



de Jong\_Fig. S6

