Supplementary material, Veening et al.

Supplementary results

In contrast to GFP, it was shown that Sda is a highly unstable protein since its uncharged C-terminus targets Sda to the ClpX/P proteolytic machinery (Ruvolo et al., 2006). In line with this, we noticed that a GFP fusion to the C-terminus of Sda, which stabilises Sda as judged by Western blotting (Fig. S5A) is sporulation deficient (data not shown). However, cells grown in DS medium and carrying an N-terminal GFP-Sda fusion, driven by the native sda promoter, was rapidly turned over and highly unstable with a half life less than 5 minutes as judged by Western blotting (Fig. S5A). This is similar to the half life of untagged Sda and consistent with data obtained using a IPTG inducible GFP-Sda construct (Ruvolo et al., 2006). To estimate the half life of our fusion protein in vivo, cells were grown in minimal medium and protein synthesis was blocked by spotting cells on agarose medium containing 250 μg/ml of chloramphenicol followed by time-lapse microscopy. This shows an approximate half life of 15 min for the GFP-Sda fusion while untagged GFP driven by the sda promoter did not show any signs of degradation during the course of the time lapse (Fig. S5B). However, the GFP signal did not entirely drop to background levels during the course of the experiment, likely because of accumulation of a degradation product that is still fluorescent after addition of chloramphenicol. However, this degradation product does not accumulate during steady state in untreated cells (Fig. S5A and Ruvolo et al., 2006), indicating that this product is normally rapidly turned over as well. Note that the Sda degradation rate increases with higher doubling rates (Ruvolo et al., 2006), consistent with the results described above. Taken together, these results indicate that fluorescence measured from a strain carrying the P<sub>sda</sub>-<i>gfp-sda</i> construct faithfully reflects <i>in vivo</i> Sda protein levels. Furthermore, the GFP-Sda was functional in complementing the Δsda mutant (data not shown).

Protein stability assays of cells grown using the two-step resuspension method showed an approximate half-life of 1h for GFP and approximately 30 min for CFP driven from the <i>abrB</i> promoter (data not shown). Note that the
P_{abrB}-gfp and P_{abrB}-cfp fusions contain the first three codons of abrB (Veening et al., 2006). Furthermore, to enhance translation, the cfp contains the first eight codons of comGA (Veening et al., 2004).

We note that other components of the replisome such as the clamp loader protein DnaN show a similar dynamic localisation pattern to DnaX and can be used a reporter for active replication.

**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). Restriction enzymes were obtained from Roche (Mannheim, GER) and all PCR’s were performed with Phusion (NEB, UK), unless stated otherwise. Oligonucleotides were purchased from Eurogentec (Liege, BEL). All constructs were sequence verified. *B. subtilis* was transformed as described before (Harwood and Cutting, 1990).

**Plasmids, bacterial strains and media**

Table S1 and S2 lists the plasmids and bacterial strains used. Nutrient agar (Oxoid, Basingstoke, UK) was used for routine selection and maintenance of both *B. subtilis* and *E. coli* strains. Spizizen minimal medium (Spizizen, 1958) was used for flow cytometry experiments. Sporulation medium was adapted from (Schaeffer et al., 1965) and contained dehydrated nutrient broth (0.8%; Difco), MgSO4 (1 mM), KCl (1 g/l), CaCl2 (1 mM) and MnSO4 (0.13 mM). When required, medium for *E. coli* was supplemented with ampicillin (Ap; 100 µg/ml) or spectinomycin (Sp; 50 µg/ml); media for *B. subtilis* were supplemented with chloramphenicol (Cm; 5 µg/ml), tetracycline (Tc; 10 µg/ml), spectinomycin (Sp; 50 µg/ml), kanamycin (Km; 5 µg/ml) or erythromycin and lincomycin (MLS; 0.5 µg/ml of erythromycin and 25 µg/ml of lincomycin). When indicated, IPTG (isopropyl-β-D-thiogalactosidase) or xylose was added to the medium.
Marker Frequency Analysis and extraction of spore DNA

Origin/terminus ratios were essentially determined as described before (Murray and Errington, 2008), but in brief, spore DNA was purified by first removing spore coat proteins followed by chloroform extraction. To do so, purified spores were incubated with urea (8M), tris-HCl pH 7 (15 mM), SDS (1%) and DTT (50mM) at 60°C for 90 min. Next, spores were washed three times in STE (tris-HCl 10mM pH 8, EDTA 10mM, NaCl 150mM) and one time in SE (NaCl 50mM, EDTA 100mM). The pellet was treated with lysozyme, Rnase, pronase and sarkosyl, before chloroform extraction and subsequent ethanol precipitation of the DNA. Power SYBR Green PCR Master Mix was used for PCR reactions (Applied Biosystems). Q-PCR was performed in a LightCycler 480 Instrument (Roche, Inc.). For quantification of the origin, the intergenic region between \textit{dnaA} and \textit{dnaN} was amplified using primers 5'-GATCAATCGGGAAAGTGTG-3' and 5'-GTAGGGCCTGTGGATTGTG-3'. For quantification of the terminus a region directly opposite from \textit{oriC} downstream of \textit{yocG} was amplified using primers 5'-TCCATATCCTCGCTCCTACG-3' and 5'-ATTCTGCTGATGTGCAATGG-3'. By use of crossing points and PCR efficiency a relative quantification analysis was performed using Light-Cycler Software version 4.0 (Roche, Inc.) to determine the ori/ter ratio of each sample.

Construction of plasmids

To construct plasmid pJWV017, carrying a promoterless \textit{gfp} allele (F64L, S65T, F99S, M153T, V163A) (Scholz et al., 2000; Zellmeier et al., 2006) flanked by a strong transcriptional terminator, a PCR with the primers gfp-R+FseI (5' GCTCAATGGCCGGCCTATTATTGGTAGAGCTCATCATGCCCATG 3') and ter-F+FseI (5' CGATAGGCCGGCCAATAAGATCCTAGACGCAAG 3') was performed, using plasmid pGFP_ssrA (Veening, unpublished; sequence available upon request) as a template. Note that although we use an ssrA-tagged \textit{gfp} as cloning intermediate, all experiments were performed with untagged, stable GFP. The amplified fragment was subsequently cleaved with FseI and self-ligated, resulting in plasmid pJWV017. The backbone of this new plasmid is based on plasmid pDG3661-Pveg (Krasny and Gourse, 2004).
To construct plasmid pGFP-rrnB, carrying the *B. subtilis* *rrnB* promoter region and a strong RBS fused with *gfp*\(^+\), a PCR with the primers *rrnB*-F+EcoRI (5' GCGCGAATTCCATCCCTACTAGCCAGCG 3') and *rrnB*-R+NheI (5' GCGCGCTAGCCATAGTTCCTCCTTACTAGTTACTAATACATTCAGC 3') was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with EcoRI and NheI and ligated into the corresponding sites of pJWV017, resulting in plasmid pGFP-rrnB.

To construct plasmid pJWV002, carrying the *B. subtilis* *spolI AA* promoter and RBS fused with *mCherry* (Shaner *et al.*, 2004) flanked by *amyE* regions, a PCR with the primers *spolI AA*-F+MunI (5' ATCCCGTGCAATTGAAGCGAAATATTGTCTGACTGC 3') and *spolI AA*-R+NheI (5' CGCGCTAGCCATGCTCATTCCTCCTGATATGATCG 3') was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with MunI and NheI and ligated into the EcoRI/NheI sites of pCherry-rrnB (Veening, unpublished; sequence available upon request), resulting in plasmid pJWV002.

To construct plasmid pJWV012, carrying the *B. subtilis* *spolI AA* promoter and RBS fused with *mCherry* flanked by *sacA* regions, a PCR with the primers *spolI AA*-F+MunI (5' ATCCCGTGCAATTGAAGCGAAATATTGTCTGACTGC 3') and construct-R+BglII (5' CGCGAGATCTGGATTTCC TTACGCGAAATACG 3') was performed, using chromosomal DNA of strain JWV002 as a template. The amplified fragment was subsequently cleaved with MunI and BglII and ligated into the EcoRI/BamHI sites of pSac-Kan (Middleton and Hofmeister, 2004), resulting in plasmid pJWV012.

To construct plasmid pJWV014, carrying the *B. subtilis* *sda* promoter region and a strong RBS fused with *gfp*\(^+\) flanked by *amyE* regions, a PCR with the primers *sda*-F+EcoRI (5' GCGCGAATTCTCATTGATATGCGGAGTTGG GAATCATTGATATGCGGAGTTGG 3') and *sda*-R+NheI (5' CGCGCTAGCCATAGTTCCTCCTTACTGTTACTAAATACG3') was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with EcoRI and NheI and ligated into the corresponding sites of pGFP-rrnB, resulting in plasmid pJWV014.
To construct plasmid pJWV021, carrying the *B. subtilis* sda promoter region and a strong RBS fused with mCherry flanked by sacA regions, a PCR with the primers sda-F+BglII (5’ GCGCAGATCTCCTCATTAAATGCGG TGTCC 3’) and sda-R+Nhel (5’GCGCGCTAGCCATAGTAGTCTCCTGTTAAAAGTTGGAATCATTAGATTTAAACGC3’) was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with BglII and Nhel and ligated into the corresponding sites of pJWV012, resulting in plasmid pJWV021.

To construct plasmid pJWV015, carrying the *B. subtilis* sda promoter region and the sda coding region with a flexible linker fused with gfp+ flanked by amyE regions, a PCR with the primers sda-F+EcoRI (5’ GCGCGAATTCTCATTAAATGCGG TGTCC 3’) and sda-R-link+Nhel (5’ GCTCGCTAGCTCCAGTCTTAGCAGCTCCTGCTCCACCAGATCCAGAAGA TACGGAAATAATATGTCCGAGCGGATC 3’) was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with EcoRI and Nhel and ligated into the corresponding sites of pGFP-rrnB, resulting in plasmid pJWV015.

To construct plasmid pJWV024, carrying the *B. subtilis* sda promoter region driving gfp+ with a flexible polylinker fused to the coding region of sda, a PCR with the primers sda-F+EcoRI (5’ GCGCGAATTCTCATTAAATGCGG TGTCC 3’)/gfp-R+XbaI (5’ GCGCTCTAGATTGTAG AGCTCATTCCATGCGGGGTGTTAAAGTCTCAGATCCTGATGCTGTTTAGAATATTGGGTTCCTGCTACATGGTTAGCATAAACCAGG 3’) and sda-F+XbaI+link (5’ GCGCTCTAGATTGTAG AGCTCATTCCATGCGGGGTGTTAAAGTCTCAGATCCTGATGCTGTTTAGAATATTGGGTTCCTGCTACATGGTTAGCATAAACCAGG 3’)/sda-R+FseI (5’ TGATGGCCGGCGCGCATA TACTGTTAGCATAAACCAGG 3’) was performed using JWV050 and 168 chromosomal DNA as template, respectively. The resulting fragments were digested with EcoRI/XbaI and XbaI/FseI and ligated in a 3-point ligation in the EcoRI/FseI sites of plasmid pGFP-rrnB.

To construct plasmid pJWV023, carrying the *B. subtilis* sda promoter and sda coding region flanked by amyE regions, a PCR with the primers sda-F+EcoRI (5’ GCGCGAATTCTCATTAAATGCGG TGTCC 3’)/sda-R+FseI (5’ TGATGGCCGGCGCGCATA TACTGTTAGCATAAACCAGG 3’) was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with EcoRI and Nhel and
ligated into the corresponding sites of pGFP-rrnB, resulting in plasmid pJWV023.

To construct plasmid pJWV022, plasmid pJWV014 was digested with NheI/FseI and the 725 bps fragment containing \( gfp^+ \) was ligated into NheI/FseI digested pJWV021.

To construct plasmid pJWV024-core, carrying the core \( B. \ subtilis \ sda \) promoter region (-35/-10 without any upstream regulatory regions) and \( gfp^+ -sda \), the BsmBI dovetail restriction enzyme was used as follows: A pcr using primers -35-F+BsmBI (5' GCGCCGTCTCATTTTATAT GCATTTCATGGTAG 3')/R-end+BsmBI (5' CGCGCGTCTCAACAAGATTTCT CATGTTTCAGCTTTATCATCG 3') was performed using pJWV024 as template. The PCR product was DpnI treated for 2h, purified and digested with BsmBI. After self-ligation, the plasmid was directly transformed to competent DH5\( \alpha \).

To construct plasmid pJWV024-no-dnaA_boxes, carrying the \( B. \ subtilis \ sda \) promoter region without the 5 major DnaA boxes fused to \( gfp^+ -sda \), a pcr using primers F+lexA+BsmBI (5' GCGCCGTCTCATTTTATATGCATTTCATGGTAG 3')/R+0A2+BsmBI (5' CGCGCGTCTCATTTTATATGCATTTCATGGTAG 3') was performed using pJWV024 as template. The PCR product was DpnI treated for 2h, purified and digested with BsmBI. After self-ligation, the plasmid was directly transformed to competent DH5\( \alpha \).

To construct plasmid pJWV024-sda1, carrying the \( B. \ subtilis \ sda1 \) mutant promoter (Burkholder et al., 2001) fused to \( gfp^+ -sda \), site directed mutagenesis (SDM) using primers sda1-F-SDM (5' GGGTTTCTTCACCAGTTATGGATAAATTTACACAC 3')/sda1-R (5' GTGTGTAAATTTATCCATACTGGTGAAGAAAACC 3') was performed using pJWV024 as template. SDM primers were designed using PrimerX (http://www.bioinformatics.org/primerx/) and PfuUltra was used for 12 cycles according to Stratagene’s QuikChange Site-Directed Mutagenesis Kit. The PCR product was DpnI treated for 2h and was directly transformed to competent DH5\( \alpha \).

To construct plasmid pJWV026-dnaN, carrying the 3’ end of \( B. \ subtilis \ dnaN \) (327 bps) fused to a flexible polylinker and \( mCherry \), a PCR with the primers dnaN-F+EcoRI (5’ CCTGATTCCGCAAGACGC 3’) and dnaN-
R+Xhol (5’ GCGCCTCGAGATAGG TTCTGACAGGAAGGATAAGC 3’) was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with EcoRI and Xhol and ligated into the corresponding sites of pUS-mCherry (kind gift of Wiep Klaas Smits, MIT, unpublished), resulting in plasmid pJWV026-dnaN.

To construct plasmid pJWV017-yvcD, carrying the *B. subtilis* yvcD promoter region and a strong RBS fused with *gfp* † flanked by *amyE* regions, a PCR with the primers yvcD-F+EcoRI (5’ GCGCGAATTCCGCCACATCTGAC AATGTTGC 3’) and yvdC-R+NheI (5’ GCGCGCTAGCCATAGTAGTTCC TCCTTAATTCTTTATGTATTATGATATTATC 3’) was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with EcoRI and Nhel and ligated into the corresponding sites of pGFP-rrnB, resulting in plasmid pJWV017-yvcD.

To construct plasmid pJWV017-ywlC, carrying the *B. subtilis* ywlC promoter region and a strong RBS fused with *gfp* † flanked by *amyE* regions, a PCR with the primers ywlC-F+EcoRI (5’ GCGCGAATTCCCGACAAAGCTTT ACGGCAAC 3’) and ywlC-R+NheI (5’ GCGCGCTAGCCATAGTAGTTCCTC CTTATATTCAAAACAAAGATTGTTTACGCGTGAATCC 3’) was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with EcoRI and Nhel and ligated into the corresponding sites of pGFP-rrnB, resulting in plasmid pJWV017-ywlC.

**Construction of bacterial strains**

*B. subtilis* strains JWV002, JWV050, JWV051, JWV112 and JWV110 were obtained by a double crossover recombination event between the *amyE* regions located on the pJWV002, pJWV014, pJWV015, pJWV024 and pJWV023 plasmids and the chromosomal *amyE* gene of strain 168, respectively. Transformants were selected on LB agar plates containing chloramphenicol (Cm) 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.
B. subtilis strains JWV048 and JWV073 were obtained by a double crossover recombination event between the sacA regions located on the pJWV012 and pJWV021 plasmids and the chromosomal sacA gene of strain 168, respectively. Transformants were selected on LB agar plates containing kanamycin (Km) after overnight incubation at 37°C. Correct integration into the sacA gene was tested and confirmed by lack of growth on plates containing sucrose as sole carbon source.

B. subtilis strain JWV017 was obtained by transformation of strain JWV002 with chromosomal DNA of strain KPL637 (Lemon and Grossman, 2000). Transformants were selected on LB agar plates containing Cm and spectinomycin (Sp), after overnight incubation at 37°C.

B. subtilis strain JWV029 was obtained by transformation of strain JWV002 with chromosomal DNA of strain HM160. Transformants were selected on LB agar plates containing Cm and Km, after overnight incubation at 37°C.

B. subtilis strain JWV054 was obtained by transformation of strain JWV048 with chromosomal DNA of strain JWV050. Transformants were selected on LB agar plates containing Cm and Km, after overnight incubation at 37°C.

B. subtilis strain JWV081 was obtained by transformation of strain JWV073 with chromosomal DNA of strain KPL637. Transformants were selected on LB agar plates containing Km and Sp, after overnight incubation at 37°C.

B. subtilis strain JWV071 was obtained by transformation of strain JWV054 with chromosomal DNA of strain PL20 (Meile et al., 2006). Transformants were selected on LB agar plates containing erythromycin, lincomycin and IPTG, after overnight incubation at 37°C.

B. subtilis strain JWV045 was obtained in the following way: plasmid pBEST501 (Itaya et al., 1989) was digested with PstI and XbaI and a 1.3 Kb fragment containing the neomycin resistance cassette was isolated by gel extraction. Two PCR’s with primer pairs spo0A-up-F (5’ GGATCACAAGTGCTGTGCATTAC 3’)/spo0A_up-R+PstI (5’ CGCGCTGCAG GTTTCTTCCTCCCCAAATGTAG 3’) and spo0A_down+XbaI (5’
GCGCTCTAGATAAACATGAGCTTATTAAGTGGTC 3')/spo0A\_down-R (5’ CTATGAGGATGGCTCTAATTGAGG 3’) was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragments were subsequently cleaved with PstI and XbaI, respectively, and ligated in a three point ligation together with the neomycin resistance cassette. The ligation mixture was directly transformed to strain JWV002, resulting in strain JWV045. Transformants were selected on LB agar plates containing Cm and Km after overnight incubation at 37°C. Correct integration and replacement of the *spo0A* gene was verified by PCR.

*B. subtilis* strain JWV067 was obtained by transformation of strain JWV050 with chromosomal DNA of strain JWV045. Transformants were selected on LB agar plates containing Cm and Km, after overnight incubation at 37°C.

*B. subtilis* strain JWV084 was obtained by transformation of strain JWV050 with chromosomal DNA of strain YK004. Transformants were selected on LB agar plates containing Cm, Tc and Sp, after overnight incubation at 37°C.

*B. subtilis* strain JWV089 was obtained by transformation of strain JWV084 with chromosomal DNA of strain JWV045. Transformants were selected on LB agar plates containing Sp and Km, after overnight incubation at 37°C.

*B. subtilis* strain JWV044 was obtained in the following way: plasmid pBEST309 (Itaya, 1992) was digested with BamHI and a 2 Kb fragment containing the tetracycline resistance cassette was isolated by gel extraction. Two PCR’s with primer pairs sda-up-F (5’ GCAAGTCCGCCAAATACAGTG 3’)/sda\_up-R+BamHI (5’ GCGTGATCCCCAATTAATGGGAGAGGCACC TCCT 3’) and sda\_down+BamHI (5’ GCGTGATCCCCGTATCTTA ATAGGAATTGG 3’)/ sda\_down-R (5’ CCATCATATCTCTGTGGG TCTG 3’) was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragments were subsequently cleaved with BamHI and ligated in a three point ligation together with the tetracycline resistance cassette. The ligation mixture was directly transformed to strain JWV002, resulting in strain JWV044. Transformants were selected on LB agar plates containing Cm and
Tc after overnight incubation at 37°C. Correct integration and replacement of the *sda* gene was verified by PCR.

*B. subtilis* strain JWV074 was obtained by transformation of strain JWV054 with chromosomal DNA of strain JWV044. Transformants were selected on LB agar plates containing Km and Tc, after overnight incubation at 37°C.

*B. subtilis* strain JWV075 was obtained by transformation of strain JWV064 with chromosomal DNA of strain JWV044. Transformants were selected on LB agar plates containing MLS, IPTG and Tc, after overnight incubation at 37°C.

*B. subtilis* strain JWV075 was obtained by transformation of strain JWV064 with chromosomal DNA of strain JWV044. Transformants were selected on LB agar plates containing MLS, IPTG and Tc, after overnight incubation at 37°C.

*B. subtilis* strain JWV075 was obtained by transformation of strain JWV063 with chromosomal DNA of strain JWV044. Transformants were selected on LB agar plates containing MLS, IPTG and Tc, after overnight incubation at 37°C.

*B. subtilis* strain JWV086 was obtained by transformation of strain JWV086 with chromosomal DNA of strain JWV044. Transformants were selected on LB agar plates containing MLS, IPTG and Tc, after overnight incubation at 37°C.

*B. subtilis* strain JWV086 was obtained by transformation of strain JWV086 with chromosomal DNA of strain JWV044. Transformants were selected on LB agar plates containing MLS, IPTG and Tc, after overnight incubation at 37°C.

*B. subtilis* strain JWV086 was obtained by transformation of strain JWV086 with chromosomal DNA of strain JWV044. Transformants were selected on LB agar plates containing MLS, IPTG and Tc, after overnight incubation at 37°C.

*B. subtilis* strain JWV094 was obtained by transformation of strain JWV094 with chromosomal DNA of strain JWV044. Transformants were selected on LB agar plates containing MLS and IPTG, after overnight incubation at 37°C.

*B. subtilis* strain JWV094 was obtained by transformation of strain JWV095 with chromosomal DNA of strain JWV044. Transformants were selected on LB agar plates containing MLS and IPTG, after overnight incubation at 37°C.

*B. subtilis* strain JWV094 was obtained by transformation of strain JWV095 with chromosomal DNA of strain JWV044. Transformants were selected on LB agar plates containing MLS and IPTG, after overnight incubation at 37°C.

B. subtilis strain JWV094 was obtained by transformation of strain JWV095 with chromosomal DNA of strain JWV044. Transformants were selected on LB agar plates containing MLS and IPTG, after overnight incubation at 37°C.

To obtain strain JWV115, the chloramphenicol resistance marker of strain JWV110 was replaced with a spectinomycin resistance marker using
plasmid pCm::Sp (Steinmetz and Richter, 1994). Transformants were selected for Sp resistance and Cm sensitivity.

*B. subtilis* strain JWV117 was obtained by transformation of strain JWV107 with chromosomal DNA of strain JWV115. Transformants were selected on LB agar plates containing Cm, Tc and Sp, after overnight incubation at 37°C.

*B. subtilis* strain JWV097 was obtained by transformation of strain JWV048 with chromosomal DNA of strain JWV044. Transformants were selected on LB agar plates containing Km and Tc, after overnight incubation at 37°C.

*B. subtilis* strain JWV101 was obtained by transformation of strain JWV097 with chromosomal DNA of strain HM285. Transformants were selected on LB agar plates containing Tc, MLS and IPTG, after overnight incubation at 37°C.

*B. subtilis* strain JWV119 was obtained by transformation of strain JWV101 with chromosomal DNA of strain JWV112. Transformants were selected on LB agar plates containing MLS, Cm and IPTG, after overnight incubation at 37°C.

*B. subtilis* strain JWV120 was obtained by transformation of strain JWV112 with chromosomal DNA of strain JWV044. Transformants were selected on LB agar plates containing Cm and Tc, after overnight incubation at 37°C.

*B. subtilis* strain JWV121 was obtained by transformation of strain JWV112 with chromosomal DNA of strain JWV045. Transformants were selected on LB agar plates containing Cm and Km, after overnight incubation at 37°C.

*B. subtilis* strain JWV122 was obtained by transformation of strain abrB-gfp with chromosomal DNA of strain JWV045. Transformants were selected on LB agar plates containing Cm and Km, after overnight incubation at 37°C.

*B. subtilis* strain JWV123 was obtained by transformation of strain HM263 with plasmid pJWV022. Transformants were selected on LB agar plates containing Km and 1% xylose, after overnight incubation at 37°C.
B. subtilis strain JWV129 was obtained by transformation of strain JWV120 with chromosomal DNA of strain YK04. Transformants were selected on LB agar plates containing Cm and Sp, after overnight incubation at 37°C.

B. subtilis strains JWV140 and JWV147 were obtained by transformation of strain JWV073 or JWV086 with chromosomal DNA of strain GFP-DnaN (kind gift of Masa Suetsugu, CBCB, unpublished). Transformants were selected on LB agar plates containing Cm, after overnight incubation at 37°C.

B. subtilis strain JWV128 was obtained by transformation of strain 168 with chromosomal DNA of strain JWV044. Transformants were selected on LB agar plates containing Tc, after overnight incubation at 37°C.

B. subtilis strains JWV150, JWV151 and JWV153 were obtained by transformation of strain JWV128 with plasmids pJWV024-core, pJWV024-no-dnaA_boxes and pJWV024-sda1, respectively. Transformants were selected on LB agar plates containing Tc and Cm, 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.

B. subtilis strain JWV158 was obtained by a single crossover recombination event between the dnaN regions located on the pJWV026-dnaN plasmid and the chromosomal dnaN gene of strain 168. Transformants were selected on LB agar plates containing Sp after overnight incubation at 37°C and correct integration into the dnaN gene was confirmed by PCR.

B. subtilis strain JWV159 was obtained by transformation of strain JWV120 with chromosomal DNA of strain JWV158. Transformants were selected on LB agar plates containing Cm and Sp, after overnight incubation at 37°C.

To obtain strain JWV168, the chloramphenicol resistance marker of strain JWV120 was replaced with a erythromycin/lincomycin (MLS) resistance marker using plasmid pCm::Er (Steinmetz and Richter, 1994). Transformants were selected for MLS resistance and Cm sensitivity.

B. subtilis strain JWV170 was obtained by transformation of strain JWV168 with chromosomal DNA of strain JWV158. Transformants were
selected on LB agar plates containing MLS and Sp, after overnight incubation at 37°C.

*B. subtilis* strain JWV171 was obtained by transformation of strain JWV170 with chromosomal DNA of strain abrB-icfp (Veening *et al.*, 2004). Transformants were selected on LB agar plates containing Cm and Sp, after overnight incubation at 37°C.

To obtain strain JWV203, the chloramphenicol resistance marker of strain JWV150 was replaced with a erythromycin/lincomycin (MLS) resistance marker using plasmid pCm::Er (Steinmetz and Richter, 1994). Transformants were selected for MLS resistance and Cm sensitivity.

*B. subtilis* strain JWV206 was obtained by transformation of strain JWV203 with chromosomal DNA of strain JWV158. Transformants were selected on LB agar plates containing MLS and Sp, after overnight incubation at 37°C.

*B. subtilis* strain JWV207 was obtained by transformation of strain JWV206 with chromosomal DNA of strain abrB-icfp (Veening *et al.*, 2004). Transformants were selected on LB agar plates containing Cm and Sp, after overnight incubation at 37°C.

*B. subtilis* strain JWV109 was obtained by transformation of strain 168 with plasmid pJWV022. Transformants were selected on LB agar plates containing Km, after overnight incubation at 37°C.

*B. subtilis* strain JWV160 was obtained by transformation of strain JWV109 with chromosomal DNA of strain JWV158. Transformants were selected on LB agar plates containing Km and Sp, after overnight incubation at 37°C.

*B. subtilis* strains JWV169, JWV184, JWV185 and JWV186 were obtained by transformation of strains abrB-gfp (Veening *et al.*, 2006), A::Phy_spank-gfp (Veening, unpublished), JWV107 and JWV122, respectively, with chromosomal DNA of strain JWV158. Transformants were selected on LB agar plates containing Cm and Sp, after overnight incubation at 37°C.

*B. subtilis* strain JWV199 was obtained by transformation of strain 168 with chromosomal DNA of strain HM466 (Murray, unpublished).
Transformants were selected on LB agar plates containing Cm after overnight incubation at 37°C.

*B. subtilis* strain JWV205 was obtained by transformation of strain JWV199 with chromosomal DNA of strain HM285 (Murray and Errington, 2008). Transformants were selected on LB agar plates containing Cm, MLS and IPTG, after overnight incubation at 37°C.

*B. subtilis* strain JWV201 was obtained by transformation of strain JWV048 with chromosomal DNA of strain GFP-DnaN. Transformants were selected on LB agar plates containing Cm and Km, after overnight incubation at 37°C.

*B. subtilis* strain JWV202 was obtained by transformation of strain JWV201 with chromosomal DNA of strain JWV044. Transformants were selected on LB agar plates containing Cm and Tc, after overnight incubation at 37°C.

*B. subtilis* strains JWV195 and JWV196 were obtained by transformation of strains JH642 and BB668 (Burkholder et al., 2001), respectively, with chromosomal DNA of strain LR69 (Rahn-Lee et al., 2009). Transformants were selected on LB agar plates containing Tc, after overnight incubation at 37°C.

*B. subtilis* strain JWV212 was obtained by transformation of strain JH642 with chromosomal DNA of strain JWV086. Strain JWV213 was obtained by transformation of strain BB668 with chromosomal DNA of strain JWV086. Strain JWV214 was obtained by transformation of strain JWV195 with chromosomal DNA of strain JWV086. Strain JWV215 was obtained by transformation of strain JWV196 with chromosomal DNA of strain JWV086. The absence/presence of *sda/sirA* in strains JWV212-215 was verified by PCR (not shown).

*B. subtilis* strains JWV217 and JWV218 were obtained by a double crossover recombination event between the *amyE* regions located on the pJWV017-ywlC and pJWV017-yvdC plasmids and the chromosomal *amyE* gene of strain JWV158, respectively. Transformants were selected on LB agar plates containing Cm and Sp after overnight incubation at 37°C. Correct
integration into the \textit{amyE} gene was tested and confirmed by lack of amylase activity upon growth on plates containing 1% starch.

\begin{table}[h]
\centering
\caption{Plasmids used in this study\textsuperscript{a}}
\begin{tabular}{lll}
\hline
Plasmids & Relevant genotype & Reference \\
\hline
pBEST309 & \textit{bla}, \textit{tet} & (Itaya, 1992) \\
pBEST501 & \textit{bla}, \textit{neo} & (Itaya \textit{et al.}, 1989) \\
pPSL10 & \textit{hutM::tetO}, \textit{kan} & (Lee and Grossman, 2006) \\
pRSET-mCherry & \textit{bla}, \textit{P}_{\text{TT-mCherry}} & (Shaner \textit{et al.}, 2004) \\
pSac-Kan & \textit{bla}, \textit{SacA}, \textit{kan} & (Middleton and Hofmeister, 2004) \\
pCm::Sp & \textit{bla}, \textit{cat::spec} & (Steinmetz and Richter, 1994) \\
pCm::Er & \textit{bla}, \textit{cat::ery} & (Steinmetz and Richter, 1994) \\
pCherry-rrnB & \textit{spec}, \textit{amyE}, \textit{P}_{\text{rrnB-mCherry}}, \textit{cat} & \text{This study} \\
pGFP-rrnB & \textit{spec}, \textit{amyE}, \textit{P}_{\text{rrnB-gfp+}}, \textit{cat} & \text{This study} \\
pJWV002 & \textit{spec}, \textit{amyE}, \textit{P}_{\text{spoIIA-mCherry}}, \textit{cat} & \text{This study} \\
pJWV012 & \textit{bla}, \textit{SacA}, \textit{P}_{\text{spoIIA-mCherry}}, \textit{kan} & \text{This study} \\
pJWV014 & \textit{spec}, \textit{amyE}, \textit{P}_{\text{sda-gfp+}}, \textit{cat} & \text{This study} \\
pJWV015 & \textit{spec}, \textit{amyE}, \textit{P}_{\text{sda-sda-gfp+}}, \textit{cat} & \text{This study} \\
pJWV017 & \textit{spec}, \textit{amyE}, \textit{gfp+} (\text{F64L, S65T, F99S, M153T, V163A}), \textit{cat} & \text{This study} \\
pJWV017-yvdC & \textit{spec}, \textit{amyE}, \textit{P}_{\text{yvdC-gfp+}}, \textit{cat} & \text{This study} \\
pJWV017-ywlC & \textit{spec}, \textit{amyE}, \textit{P}_{\text{ywlC-gfp+}}, \textit{cat} & \text{This study} \\
pJWV021 & \textit{bla}, \textit{SacA}, \textit{P}_{\text{sda-mCherry}}, \textit{kan} & \text{This study} \\
pJWV022 & \textit{bla}, \textit{SacA}, \textit{P}_{\text{sda-gfp}}, \textit{kan} & \text{This study} \\
pJWV023 & \textit{spec}, \textit{amyE}, \textit{P}_{\text{sda-sda}}, \textit{cat} & \text{This study} \\
pJWV024 & \textit{spec}, \textit{amyE}, \textit{P}_{\text{sda-gfp+sda}}, \textit{cat} & \text{This study} \\
pJWV024-core & \textit{spec}, \textit{amyE}, \textit{P}_{\text{sda-core-gfp+sda}}, \textit{cat} & \text{This study} \\
pJWV024-no-dnaA_boxes & \textit{spec}, \textit{amyE}, \textit{P}_{\text{sda-no-dnaA_boxes-gfp+sda}}, \textit{cat} & \text{This study} \\
pJWV024-sda1 & \textit{spec}, \textit{amyE}, \textit{P}_{\text{sda1-gfp+sda}}, \textit{cat} & \text{This study} \\
pJWV026-dnaN & \textit{bla}, \textit{spec}, \textit{\dagger_{\text{dnaN-mCherry}}} & \text{This study} \\
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<td>(Veening et al., 2006)</td>
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<td>P_{abrB}-icfp, Cm′</td>
<td>(Veening et al., 2004)</td>
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Δsda::tet, Tc’

hutM::tetO, Km’, amyE::P_xyrtetR-mCherry, Sp’, Δsda::tet, Tc’, Pspac-dnaAS326L, MLS’

This study

JWV097

sacA::PスポリA-mCherry, Km’, Δsda::tet, Tc’

This study

JWV101

sacA::PスポリA-mCherry, Km’, Δsda::tet, Tc’, Pspac-dnaAS326L, MLS’

This study

JWV107

Pабв-gfp, Cm’, Δsda::tet, Tc’

This study

JWV110

amyE::Pспад-gfp-sda, Cm’

This study

JWV112

amyE::Pспад-gfp-sda, Cm’

This study

JWV115

amyE::Pспад-sda, Sp’

This study

JWV117

Pабв-gfp, Cm’, Δsda::tet, Tc’, amyE::Pспад-sda, Sp’

This study

JWV119

sacA::PスポリA-mCherry, Km’, Δsda::tet, Tc’, Pspac-dnaAS326L, MLS’, amyE::Pспад-gfp-sda, Cm’

This study

JWV120

amyE::Pспад-gfp-sda, Cm’, Δsda::tet, Tc’

This study

JWV121

amyE::Pспад-gfp-sda, Cm’, Δsp00A::neo, Km’

This study

JWV122

Pабв-gfp, Cm’, Δsp00A::neo, Km’

This study

JWV123

JH642 (trpC2, pheA1), ΔdnaA-N::spec, Sp’, amyE::P_xyrtetR-mCherry, Sp’, P_dnaN-gfp-dnaN, Cm’,

This study

sacA::Pспад-gfp, Km’

This study

JWV128

Δsda::tet, Tc’

This study

JWV129

amyE::Pспад-gfp-sda, Cm’, Δsda::tet, Tc’, ΔlexA::spec, Sp’, ΔyneA-yneB-ynzC::tet, Tc’

This study

sacA::Pспад-mCherry, Km’, P_dnaN-gfp-dnaN, Cm’

This study

JWV140

hutM::tetO, Km’, amyE::P_xyrtetR-mCherry, Sp’, P_dnaN-gfp-dnaN, Cm’

This study

JWV147

Δsda::tet, Tc’, amyE::Pспад-core-gfp-sda, Cm’

This study

JWV150

Δsda::tet, Tc’, amyE::Pспад-core-gfp-sda, Cm’

This study

JWV151

Δsda::tet, Tc’, amyE::Pспад-no-dnaA_boxes-gfp-sda, Cm’

This study

JWV153

Δsda::tet, Tc’, amyE::Pспад1-gfp-sda, Cm’

This study

JWV159

amyE::Pспад-gfp-sda, Cm’, Δsda::tet, Tc’, P_dnaN-mCherry, Sp’

This study

JWV160

sacA::Pспад-gfp, Km’, P_dnaN-dnaN-mCherry, Sp’

This study

amyE::Pспад-gfp-sda, MLS’, Δsda::tet, Tc’

This study

JWV168

amyE::Pспад-gfp-sda, MLS’, Δsda::tet, Tc’, P_dnaN-mCherry, Sp’

This study

JWV170

amyE::Pспад-gfp-sda, MLS’, Δsda::tet, Tc’, P_dnaN-mCherry, Sp’

This study

JWV171

amyE::Pспад-gfp-sda, MLS’, Δsda::tet, Tc’, P_dnaN-mCherry, Sp’, Pабв-lcfp, Cm’

This study

amyE::Phyper_spank-gfp, Cm’, P_dnaN-dnaN-mCherry, Sp’

This study

JWV184

amyE::Phyper_spank-gfp, Cm’, P_dnaN-dnaN-mCherry, Sp’

This study

JWV185

Pабв-gfp, Cm’, Δsda::tet, Tc’, P_dnaN-dnaN-mCherry, Sp’

This study

Pабв-gfp, Cm’, Δsda::tet, Tc’, P_dnaN-dnaN-mCherry, Sp’

This study

JWV201

sacA::PスポリA-mCherry, Km’, P_dnaN-gfp-dnaN, Cm’

This study

JWV202

sacA::PスポリA-mCherry, Km’, P_dnaN-gfp-dnaN, Cm’, Δsda::tet, Tc’

This study

JWV205

amyE::Pспад-core-gfp, Cm’, Pspac-dnaAS326L, MLS’

This study

JWV207

Δsda::tet, Tc’, amyE::Pспад-core-gfp-sda, MLS’, dnaN-mCherry, Sp’, Pабв-lcfp, Cm’

This study
This study

References


\(^{a}\)All strains constructed in this study are derivatives of strain 168, unless stated otherwise.


**Supplementary figures and legends**

![Graph](image)

**Fig. S1.** Sporulation initiates primarily in cells that have completed ongoing rounds of DNA replication. Cells were scored for either the presence or absence of a DnaX-YFP focus after approximately 9 hours into microcolony development. Bins show the $P_{spolA}$-mCherry signals from individual cells.
Fig S2. Properties of the promoter constructs used in this study. All promoter fusions (PF) were integrated via double crossover events at non-essential loci (e.g. sacA or amyE). Since the flanking region of sacA or amyE are in opposing direction of the PF, a transcriptional terminator (rrnO) was inserted downstream of the PF and another terminator (rrnB) was inserted downstream of the flanking gene. Shown is a schematic representation of the constructs present in strain JWV054 (A) and the P_{sda}-gfp-sda construct present in strain JWV120 (B). Note that some strains used in this study carry mCherry at amyE or gfp at sacA (Table S1).
Fig. S3. Cell-to-cell variability of sda expression under microcolony conditions. Single cell traces of 6 different lineages of strain JWV054 ($P_{sda^-}$-gfp, $P_{spoIIA^-}$mCherry) from Movie S2 are shown. For clarity, cell division events are not depicted.
**Fig. S4.** Dynamic expression of \( P_{sda} \)-GFP. Single cells of strain JWV160 (\( P_{sda} \)-gfp, DnaN-mCherry) were followed by time-lapse microscopy after nutritional downshift. The mean GFP fluorescence is plotted against time. Fluorescence is indicated in arbitrary units (AU) above background. After cell division, one of the two resulting siblings is arbitrarily selected for further analysis. **(A)** Red vertical dotted lines indicate a replication event as judged by the appearance of a DnaN-mCherry focus. The grey shaded area represents the time a DnaN-mCherry focus is present. A focus cannot be seen in the white areas. **(B)** Six representative traces are shown. The final cells from lineages 4 and 5 will go on to sporulate. Under these conditions, after approximately 600 min after resuspension, typically, 50% of all cells have initiated sporulation.
Fig. S5. GFP-Sda is rapidly degraded in vivo. (A) Cells of strains JWV050 (P\textsubscript{sda-gfp}), JWV051 (P\textsubscript{sda-sda-gfp}) and JWV112 (P\textsubscript{sda-gfp-sda}) were grown in DS medium and protein synthesis was inhibited at mid-exponential growth (T0) by the addition of chloramphenicol (250 \( \mu \text{g/ml} \)). Samples were taken at the indicated times after addition of chloramphenicol and analyzed by SDS-PAGE and Western blotting using GFP specific polyclonal antibodies. * indicates the degradation product that accumulates after addition of chloramphenicol, but is not present before addition of chloramphenicol. (B) Cells were grown in MM and treated the same as in panel A, but immediately transferred to an agarose slide containing chloramphenicol (250 \( \mu \text{g/ml} \)). Representative single cell trajectories are shown. Non-GFP expressing cells were mixed with GFP expressing cells and the fluorescence signals were collected. The normalized GFP fluorescence of the GFP expressing cell over the non-expressing cell (cellular autofluorescence) is shown. Fluorescence is indicated in arbitrary units (AU). Representative traces are shown.
Fig. S6. Cyclic expression of Sda. Single cells of strain JWV120 (\(P_{sda}\)-gfp-\(sda\)) were followed by time-lapse microscopy after nutritional downshift. The mean GFP fluorescence is plotted against time. Fluorescence is indicated in arbitrary units (AU) above background. For clarity, cell division events are not indicated, but in all cases a peak of GFP-Sda occurs between cell division events. After cell division, one of the two resulting siblings is arbitrarily selected for further analysis. Five separate lineages are shown. The final cell from lineage 4 will go on to sporulate.
Fig. S7. Cyclic expression of Sda is not correlated to cell division. Single cells were followed by time-lapse microscopy after nutritional downshift. (A) Still frames from a time-lapse experiment of strain JWV120 ($P_{sda}$-gfp-$sda$). The top panel shows FM5-95 membrane staining and the bottom panel shows GFP-Sda fluorescence. Arrows indicate a cell that shows a pulse of GFP-Sda fluorescence during its cell-cycle. (B) Single cell trajectory of strain JWV120. The mean GFP fluorescence is plotted against time. Fluorescence is indicated in arbitrary units (AU) above background. Black vertical dotted lines indicate a cell division event as judged by the appearance of a septum based on FM5-95 membrane staining. After cell division, one of the two resulting siblings is arbitrarily selected for further analysis. Representative trajectories are shown (also see Figs. S4, S6 and Movie S4). Micrographs of the time period between 290 and 520 minutes plotted here are shown in panel A and a cell of the analyzed lineage is marked with an arrow.
Fig. S8. Evidence for a defined cell cycle after nutritional downshift. T0 approximates the time when initiation of replication occurs. Still frames from time-lapse experiments are shown. The indicated time is relative to the replication initiation event. Arrows point to a cell that demonstrates a typical cell-cycle under these conditions. (A) An overlay between phase contrast (green cells) and TetR-mCherry (red foci, origin label) from strain JWV086 (hutM::tetO, P<sub>xyl</sub>-TetR-mCherry) is shown. (B) An overlay between DnaX-YFP (green foci) and FM5-95 (red membrane stain) from strain JWV017 (P<sub>dnaX</sub>-dnaX-yfp) is shown. (C) An overlay between GFP-DnaN (green foci) and FM5-95 (red membrane stain) from strain JWV140 (P<sub>dnaN</sub>-GFP-DnaN) is shown. (D) An overlay between GFP-DnaN (green foci), TetR-mCherry (red foci) and phase contrast (dark blue cells) of strain JWV147 (P<sub>dnaN</sub>-GFP-DnaN, hutM::tetO, P<sub>xyl</sub>-TetR-mCherry) is shown (also see Movie S5).
Fig. S9. The pulse of GFP-Sda correlates with initiation of replication. Single cells of strain JWV159 ($P_{sda}$-gfp-sda, DnaN-mCherry) were followed by time-lapse microscopy after nutritional downshift. Four representative traces are shown. A vertical red dotted line is placed between the time points during which a DnaN-mCherry focus first appears. The grey shaded area represents the time a DnaN-mCherry focus is present. A focus cannot be seen in the white areas. The mean GFP fluorescence is plotted against time. Fluorescence is indicated in arbitrary units (AU) above background. The final cell from lineage 3 will go on to sporulate.
**Fig. S10.** Cell cycle dependent oscillations in the levels of Spo0A~P. Single cells were followed by time-lapse microscopy after nutritional downshift. The mean GFP fluorescence is plotted against time. Fluorescence is indicated in arbitrary units (AU) above background. For clarity cell division and replication events are not indicated. After cell division, one of the two resulting siblings is arbitrarily selected for further analysis. Six separate lineages of each strain are shown. (A) Lineages of strain $P_{abrB-gfp}$ or strain JWV169 ($P_{abrB-gfp}$, DnaN-mCherry). The final cells from lineages 2 and 6 will go on to sporulate. The trajectory of lineage 4 is from an $\Delta sda$ mutant strain complemented with an ectopic copy of $sda$ (strain JWV117: $P_{abrB-gfp}$, $\Delta sda$, $amyE::P_{sda}\cdot sda$). (B) Lineages of strain JWV186 ($P_{abrB-gfp}$, DnaN-mCherry, $\Delta spo0A$). (C) Lineages of strain JWV185 ($P_{abrB-gfp}$, DnaN-mCherry, $\Delta sda$). The final cells from lineages 2 and 5 will go on to sporulate.
Fig. S11. Single cell trajectories of strain JWV171 (P\textsubscript{abrB-cfp}, \(\Delta sda\), P\textsubscript{sda-gfp-sda}, DnaN-mCherry) after resuspension. Red dotted lines indicates the first appearance of a replisome and the grey shaded area represents the time a DnaN-mCherry focus is present. After cell division, one of the two resulting siblings is arbitrarily selected for further analysis.
Fig. **S12.** Spo0A is mainly responsible for the increase in GFP-Sda expression in the stationary growth phase. Comparative time-lapse microscopy of strains JWV120 (P_{sda}-GFP-Sda) and JWV121 (P_{sda}-GFP-Sda, Δspo0A) after resuspension. The mean fluorescence, normalized to the frame with the lowest signal, of all cells each time frame is plotted as a function of time.
Fig. S13. Cells were grown and analyzed by time-lapse microscopy after resuspension as described in the main text. Vertical dotted lines indicate a cell division event. Single cell trajectory of strain JWV123 (P$_{sda}$-gfp, ΔdnaA). Strain JWV123 was grown in the presence of 0.1 % xylose and because ΔdnaA cells poorly express sda, this experiment was performed using the stable GFP transcriptional reporter. The irregular timing of cell division is caused by the filamentous nature of this strain.
Fig. S14. A constitutively active promoter does not pulse. Single cells of strain JWV184 (P$_{hyper-spank}$-gfp, DnaN-mCherry) were followed by time-lapse microscopy after nutritional downshift. A single representative trace is shown. A vertical red dotted line is placed between the time points during which a DnaN-mCherry focus first appears. The grey shaded area represents the time a DnaN-mCherry focus is present. A focus cannot be seen in the white areas. The mean GFP fluorescence is plotted against time. Fluorescence is indicated in arbitrary units (AU) above background. Note that the expression pattern of GFP from the hyper_spank promoter differs with expression of the abrB promoter in a spo0A mutant background (Fig. 3C), indicating that abrB might be under more complex control.
**Fig. S15.** Aberrant sporangial morphologies and perturbed copy number control in the absence of the Sda checkpoint. Time-lapse analysis of strain JWV094 after nutritional downshift. The asterisk (*) indicates a cell which will form an endospore containing two origin regions. The arrow indicates an endospore with three or four origin regions.
Fig. S16. Transmission electron microscopy images of purified spores from strains JWV054 (wild type), JWV074 (Δsda) and JWV075 (Δsda, P<sub>spac</sub>-dnaAS326L). Strains were grown in Schaeffers sporulation medium supplemented with 1 mM IPTG at 37 °C for 24h after inoculation (A600nm: 0.05). Spores were purified by removing remaining vegetative cells by incubating with lysozyme (1.5 mg/ml) at 37 °C for 1h followed by the addition of SDS (4% final concentration) for another 30 min. Spores were washed at least 4 times with water before storing at 4 °C.
Fig. S17. Perturbed initiation of sporulation in the absence of the sda checkpoint. Comparative time-lapse microscopy of strains JWV201 ($P_{spoIIA}$-mCherry, GFP-DnaN) and JWV202 ($P_{spoIIA}$-mCherry, GFP-DnaN, Δsda). Still frames 5h after resuspension were analyzed for each strain and cells were scored for either the presence or absence of a GFP-DnaN focus and the mean mCherry fluorescence was recorded. Bins show the $P_{spoIIA}$-mCherry signals from individual cells. For each strain at least 500 cells were measured.
Fig. S18. Aberrant sporangial morphologies and perturbed copy number control in the absence of Sda and SirA. Strains JWV212 (JH642; hutM::tetO, tetR-mCherry) (A), JWV214 (JH642; hutM::tetO, tetR-mCherry, ΔsirA) (B), JWV213 (JH642; hutM::tetO, tetR-mCherry, Δsda) (C) and JWV215 (JH642; hutM::tetO, tetR-mCherry, Δsda, ΔsirA) (D) were grown for 24h in Schaeffers sporulation medium and collected for fluorescence microscopy. Arrows point to aberrant sporangia. Panel D was also used for Fig. 6E. It should be noted that these experiments were performed in the B. subtilis JH642 background in contrast to the other results, which were performed in the B. subtilis 168 background (Table S2). We observed that even in the JH642 wild type, mother cells sometimes contain multiple origins, in contrast to wild type 168 cells (Fig. S17A). The molecular basis for this strain to strain difference is unknown.
Movie legends

**Movie S1.** Time-lapse movie of a growing and differentiating *B. subtilis* microcolony (strain JWV017; dnaX-yfp, P_{spolIIA}-mCherry). The movie is comprised of an overlay of YFP and mCherry images. Images were acquired automatically every 12 min for 17.5 hours. Scale bar is in μM.

**Movie S2.** Time-lapse analysis of strain JWV054 (P_{sda-gfp}, P_{spolIIA}-mCherry). The movie is comprised of an overlay of GFP and mCherry images.

**Movie S3.** Time-lapse analysis of strain JWV081 (P_{sda-mCherry}, dnaX-yfp). The movie is comprised of an overlay of YFP and mCherry images.

**Movie S4.** Time-lapse analysis of strain JWV160 (P_{sda-gfp}, dnaN-mCherry). The movie is comprised of an overlay of GFP and mCherry images.

**Movie S5.** Time-lapse analysis of strain JWV120 (P_{sda-gfp-sda}). The movie is comprised of an overlay of GFP and FM5-95 images.

**Movie S6.** Time-lapse analysis of strain JWV147 (tetO @ ori, TetR-mCherry, GFP-DnaN). The movie is comprised of an overlay between GFP-DnaN (green foci), TetR-mCherry (red foci) and phase contrast (dark blue cells).

**Movie S7.** Time-lapse analysis of strain JWV159 (P_{sda-gfp-sda}, DnaN-mCherry). The movie is comprised of an overlay of GFP and mCherry images.

**Movie S8.** Time-lapse analysis of strain JWV169 (P_{abrB-gfp}, DnaN-mCherry). The movie is comprised of an overlay of GFP and mCherry images.

**Movie S9.** Time-lapse analysis of strain JWV171 (P_{abrB-cfp}, P_{sda-gfp-sda}, DnaN-mCherry). The movie is comprised of an overlay of CFP, GFP and mCherry images.
**Movie S10.** Comparative time-lapse analysis of strains JWV171 (P\textsubscript{abrB-cfp}, P\textsubscript{sda-gfp-sda}, DnaN-mCherry) and JWV207 (P\textsubscript{abrB-cfp}, P\textsubscript{sda-core-gfp-sda}, DnaN-mCherry). The movie shows the CFP images.

**Movie S11.** Comparative time-lapse analysis of strains JWV120 (P\textsubscript{sda-gfp-sda}) and JWV150 (P\textsubscript{sda-core-gfp-sda}). The two strains were grown side by side in the same experiment and the same fluorescence scaling is shown for both movies. The movies are comprised of overlays between GFP and FM5-95 images. Notice the lack of pulsing from strain JWV150.

**Movie S12.** Time-lapse analysis of strain JWV217 (P\textsubscript{ywIC-gfp}, DnaN-mCherry). The movie is comprised of an overlay of GFP and mCherry images.

**Movie S13.** Time-lapse analysis of strain JWV218 (P\textsubscript{yvdC-gfp}, DnaN-mCherry). The movie is comprised of an overlay of GFP and mCherry images.

**Movie S14.** Time-lapse analysis of strain JWV184 (P\textsubscript{hyper_spank-gfp}, DnaN-mCherry). The movie is comprised of an overlay of GFP and mCherry images.

**Movie S15.** Time-lapse analysis of strain JWV094 (tetO @ ori, TetR-mCherry, Δsda, P\textsubscript{spac-dnaAS326L}). The movie is comprised of an overlay between phase contrast (green cells) and TetR-mCherry (red foci).

**Movie S16.** Comparative time-lapse analysis of purified spores from strains JWV054 (wildtype, left), JWV074 (Δsda) and JWV075 (Δsda, P\textsubscript{spac-dnaAS326L}). Spores were inoculated after a heat shock treatment (70 °C for 30 min) on an agarose slide (LB medium supplemented with 25 μM IPTG and 0.4 mg/ml L-alanine) next to each other within the same slide (thus identical conditions) at 37 °C. Phase-contrast images were recorded every 8 min for a total time of 3 hours and 19 min.