

SirA enforces diploidy by inhibiting the replication initiator DnaA during spore formation in *Bacillus subtilis*

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Summary

How cells maintain their ploidy is relevant to cellular development and disease. Here, we investigate the mechanism by which the bacterium *Bacillus subtilis* enforces diploidy as it differentiates into a dormant spore. We demonstrate that a sporulation-induced protein SirA (originally annotated YneE) blocks new rounds of replication by targeting the highly conserved replication initiation factor DnaA. We show that SirA interacts with DnaA and displaces it from the replication origin. As a result, expression of SirA during growth rapidly blocks replication and causes cell death in a DnaA-dependent manner. Finally, cells lacking SirA over-replicate during sporulation. These results support a model in which induction of SirA enforces diploidy by inhibiting replication initiation as *B. subtilis* cells develop into spores.

Introduction

In eukaryotes, replication origins are licensed to fire once and only once per cell cycle regardless of growth rate. By contrast, in bacteria, origin licensing is tightly linked to nutritional status. Fast-growing bacteria like *Escherichia coli* and *Bacillus subtilis* have a generation time of 20–30 min but require 50–60 min to duplicate their DNA. Therefore, during rapid growth, replication initiation occurs more than once per cell division, resulting in partial diploidy and tetraploidy. *B. subtilis* responds to nutrient depletion by entering the developmental process of sporulation, during which it maintains two and only two copies of the chromosome. One chromosome is destined for the dormant spore, the other for the ‘mother’ cell that prepares it for dormancy. Here we show that the sporulating cell does not rely on nutritional status to enforce

diploidy. Instead, it specifically activates a developmental regulator that blocks new rounds of replication by inhibiting the replication initiation protein DnaA.

Upon entry into sporulation, *B. subtilis* first remodels its replicated chromosomes into a serpentine structure (called the axial filament) that extends from one cell pole to the other (Ryter *et al.*, 1966; Ben-Yehuda *et al.*, 2003) (Fig. 1A). Then, an asymmetric cell division generates two cellular compartments: the larger is referred to as the mother cell and the smaller cell is the prospective spore (called the forespore). The polar division plane traps approximately a quarter of one chromosome in the forespore compartment (Wu and Errington, 1994; Sullivan *et al.*, 2009). The remaining three quarters are pumped from the mother cell into the forespore by a DNA translocase (Wu and Errington, 1994). The second chromosome is retained in the mother cell. Next, mother cell and forespore-specific gene expression drive the engulfment of the forespore in a phagocytic-like process that generates a cell within a cell. At this late stage, the mother packages the spore in a protective coat while the spore prepares for dormancy. Once the stress-resistant spore is mature, it is released into the environment through lysis of the mother cell (reviewed in Stragier and Losick, 1996; Errington, 2003; Hilbert and Piggot, 2004).

The decision to sporulate is largely a function of the master regulator Spo0A. In response to starvation signals, sensor kinases phosphorylate and activate this response-regulator transcription factor (Burbulys *et al.*, 1991). Spo0A, in turn, activates the expression of several key genes that set the developmental pathway of sporulation into motion (Fig. 1A) (reviewed in Stragier and Losick, 1996; Errington, 2003; Hilbert and Piggot, 2004). Specifically, Spo0A activates genes required for the switch from medial to polar division (Levin and Losick, 1996; Khvorova *et al.*, 1998; Ben-Yehuda and Losick, 2002), and the reorganization of the replicated chromosomes into the axial filament (Ryter *et al.*, 1966; Ben-Yehuda *et al.*, 2003). In addition, it turns on the genes encoding the first compartment-specific transcription factors and their regulators (Jonas *et al.*, 1988; Schmidt *et al.*, 1990).

Here, we show that the master regulator for entry into sporulation induces an additional key regulator (SirA) whose role is to maintain a diploid state during

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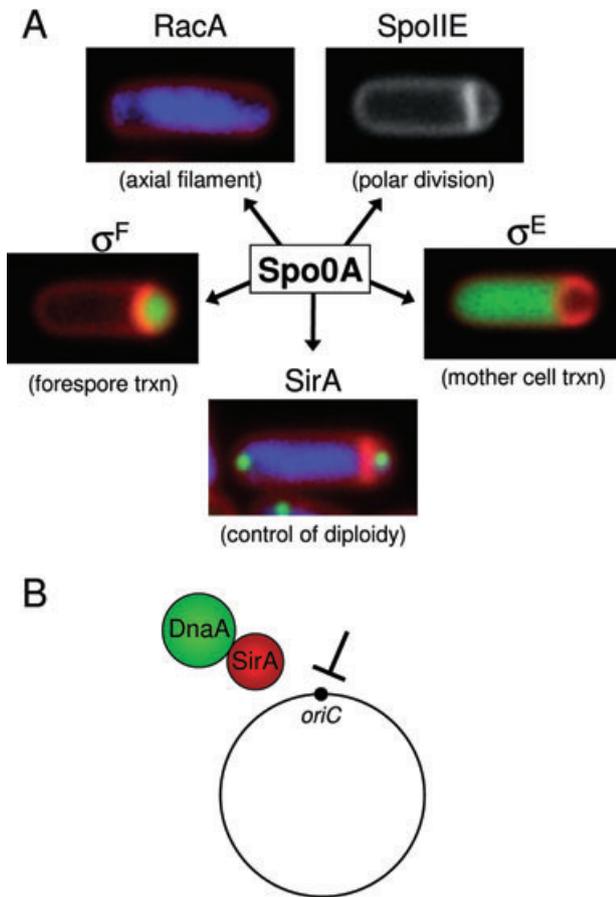


Fig. 1. A. The Spo0A transcription factor activates the key regulators that set the developmental pathway of sporulation into motion. These include SpoIIE, required for asymmetric cell division; RacA, responsible for reorganization of the replicated chromosomes into an axial filament; the first forespore (σ^F) and mother cell (σ^E) transcription factors and their regulators; and SirA, required to maintain diploidy. Representative sporulating cells are shown. The DNA was stained with DAPI (blue), the membranes with FM4-64 (red) or TMA-DPH (white). A forespore promoter (P_{spoIIQ}) or a mother-cell promoter (P_{spoIIA}) fused to the gene encoding GFP (green) shows compartment-specific transcription factor activity. TetR–GFP (green) bound to a *tetO* array inserted adjacent to *oriC* shows that the sporulating cell has only two origins, one in each compartment (B) SirA enforces diploidy in sporulating cells by inhibiting DnaA, thereby preventing new rounds of initiation at *oriC*.

development. We show that SirA (for sporulation inhibitor of replication A) inhibits new rounds of replication by targeting the replication initiator DnaA. Induction of SirA during vegetative growth rapidly blocks replication initiation and phenocopies cellular depletion of DnaA. Moreover, a strain that does not require DnaA to initiate replication is immune to SirA. We further show that SirA interacts directly with DnaA and that SirA disrupts DnaA localization at the replication origin (*oriC*). Low levels of SirA form faint foci at *oriC* that are themselves lost upon depletion of DnaA, suggesting that SirA acts on DnaA bound to *oriC*. Finally, we show that cells lacking SirA

over-replicate during sporulation. These results support a model in which SirA enforces diploidy by inhibiting DnaA after cells have entered the sporulation pathway.

Results

SirA (*YneE*) expression is lethal to vegetatively growing cells

In a screen for factors that are required for proper chromosome organization during sporulation, we identified a Spo0A-regulated gene (Fawcett *et al.*, 2000; Molle *et al.*, 2003; Fujita *et al.*, 2005) of unknown function *yneE* (*sirA*) that when deleted had a subtle defect in the organization of the axial filament (L. Mulcahy and D.Z. Rudner, unpublished). As a first step in the characterization of *sirA*, we investigated the consequences of artificial expression during vegetative growth. We placed *sirA* under the control of an IPTG-inducible promoter ($P_{hyperspank}$), and examined the cells pre and post induction. Strikingly, induction of SirA resulted in the inability to produce colonies on agar plates (Fig. 2A). Analysis of cells expressing SirA in liquid culture revealed a dramatic change in the appearance of the chromosome mass (called the nucleoid). After 40–60 min of induction, the nucleoids rounded up and the spacing between them increased (Fig. 2B and Fig. S1). As cell division continued unabated, SirA induction eventually led to the production of many anucleate cells (Fig. 2B and Fig. S1). In addition, some of the cells contained nucleoids guillotined by division septa. These phenotypes were strikingly similar to cells depleted of the DNA replication initiator protein, DnaA (Simmons *et al.*, 2007 and Fig. 2B).

SirA inhibits DNA replication at the initiation step

SirA induction during vegetative growth closely phenocopied DnaA depletion; the nucleoid rounded up and cells continued to divide even though the chromosomes appeared to have stopped replicating. To directly test whether ongoing replication had ceased, we used a marker for replication: a fusion of the *tau* subunit of DNA polymerase to the yellow fluorescent protein (DnaX–YFP). Cells harbouring DnaX–YFP that are actively replicating their DNA contain fluorescent foci that colocalize with the nucleoid (Fig. 3A) (Lemon and Grossman, 1998). Consistent with the idea that SirA blocks replication, the number of DnaX–YFP foci diminished over an induction time-course and by 60 min, all cells in the field had diffuse DnaX–YFP fluorescence (Fig. 3A and Fig. S2). Immunoblot analysis indicates that the diffuse fluorescent signal was not a consequence of proteolytic release of YFP (Fig. 3D). Replisome foci were similarly lost after 100 min of depletion of DnaA (data not shown).

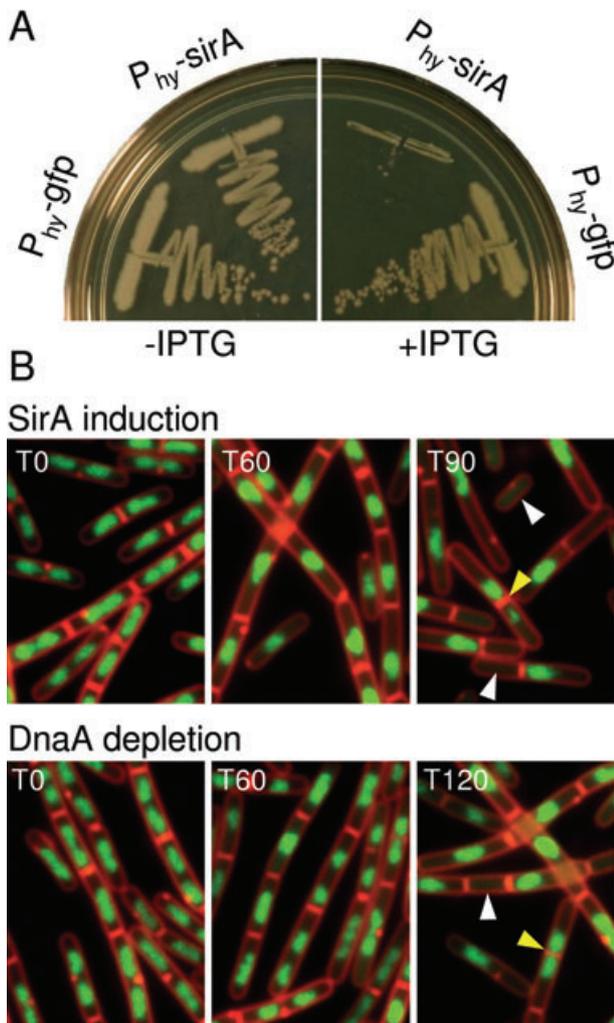


Fig. 2. SirA expression is lethal to vegetatively growing cells. A. Cells harbouring an IPTG-inducible promoter ($P_{hyperspank}$) fused to *sirA* ($P_{hy-sirA}$; strain BJW38) or *gfp* (P_{hy-gfp} ; strain BDR1003) were streaked on LB agar plates with and without IPTG. B. Phenotypic consequences of SirA induction (strain BJW38) and DnaA depletion (strain LAS223) in liquid CH medium. Time (in minutes) after induction or depletion is indicated. Membranes were stained with the fluorescent dye FM4-64 (red) and DNA was stained with DAPI (false-coloured green). Examples of anucleate cells (white carets) and guillotined nucleoids (yellow carets) are indicated.

Similar results were obtained with another marker for ongoing replication: a GFP fusion to the single-strand binding protein (SSB–GFP) (Fig. S3A).

As an independent assay to examine the replication status of the chromosome, we labelled a chromosomal locus near the origin of replication (-7° on the genetic map; 78 kb from *oriC*) by introducing a tandem array of 25 *tet* operators (*tetO*) and the *tet* repressor fused to GFP (TetR–GFP) (Michael, 2001; Burton *et al.*, 2007). We then examined the number of TetR–GFP foci within the cell before and after induction of SirA (Fig. 3B, Figs S2 and S3B). Prior to SirA induction, cells typically contained two

to four TetR–GFP foci per cell, representing two to four copies of the -7° locus per nucleoid (Fig. 3B). This number is consistent with the chromosomal content of *B. subtilis* grown under our conditions, where cell-doubling time is ~30 min (Haeusser and Levin, 2008). In support of the idea that SirA inhibits replication initiation, following 60 min of induction, the number of foci was reduced to one per DNA mass (Fig. 3B and Fig. S3B). Similar results were obtained in cells depleted of DnaA (data not shown). To rule out the possibility that the origins had duplicated but could not be resolved by fluorescence microscopy, we used a strain in which the *tetO* array was inserted at -35° (406 kb away from *oriC*). After 60 min of SirA induction, a single TetR–GFP focus was associated with each DNA mass (data not shown). These results are consistent with the idea that SirA inhibits replication initiation.

To directly assess whether replication was inhibited at the initiation step following SirA induction, we performed genomic microarray analysis. The genomic DNA isolated from cells (before and after SirA induction) was normalized to a reference sample in which the ratio of origin to terminus DNA (*oriC/ter*) was 1.0, and was then used to probe a *B. subtilis* microarray (see *Experimental procedures*). Prior to the induction of SirA, the microarray profile was similar to what was observed previously for vegetatively growing cells (Wang *et al.*, 2007) and the *oriC/ter* ratio was 1.6 (Fig. 3C). Following 60 min of SirA induction, the *oriC/ter* ratio was reduced to 1.02. As a control, we examined the *oriC/ter* ratio of cells depleted of DnaA. Following 100 min of depletion, the profiles looked similar to the SirA induction (Fig. 3C) and the *oriC/ter* ratio was 1.1. Collectively, these results are most consistent with SirA inhibiting the initiation of DNA replication.

SirA inhibits DnaA-dependent replication

We hypothesized that SirA might inhibit initiation by disrupting the activity of the initiator protein DnaA. If SirA is acting on DnaA, then a DnaA-independent strain should be immune to the growth-inhibitory (or lethal) effects of SirA induction. To test this, we took advantage of a strain in which a plasmid origin (*oriN*) that does not require DnaA to initiate replication was inserted into the *B. subtilis* chromosome. This strain can support growth and replication in the absence of *oriC* and DnaA. (Hassan *et al.*, 1997). The plasmid origin was inserted at 359° (~11 kb from the native origin) (Berkmen and Grossman, 2007) in a strain that contains a deletion in the *oriC* region, which disrupts DnaA-based replication. Accordingly, the matched control strain contained *oriC* inserted at this same position. Consistent with the idea that SirA inhibits DnaA-dependent replication, the cells utilizing *oriN* for replication initiation were immune to SirA induction and

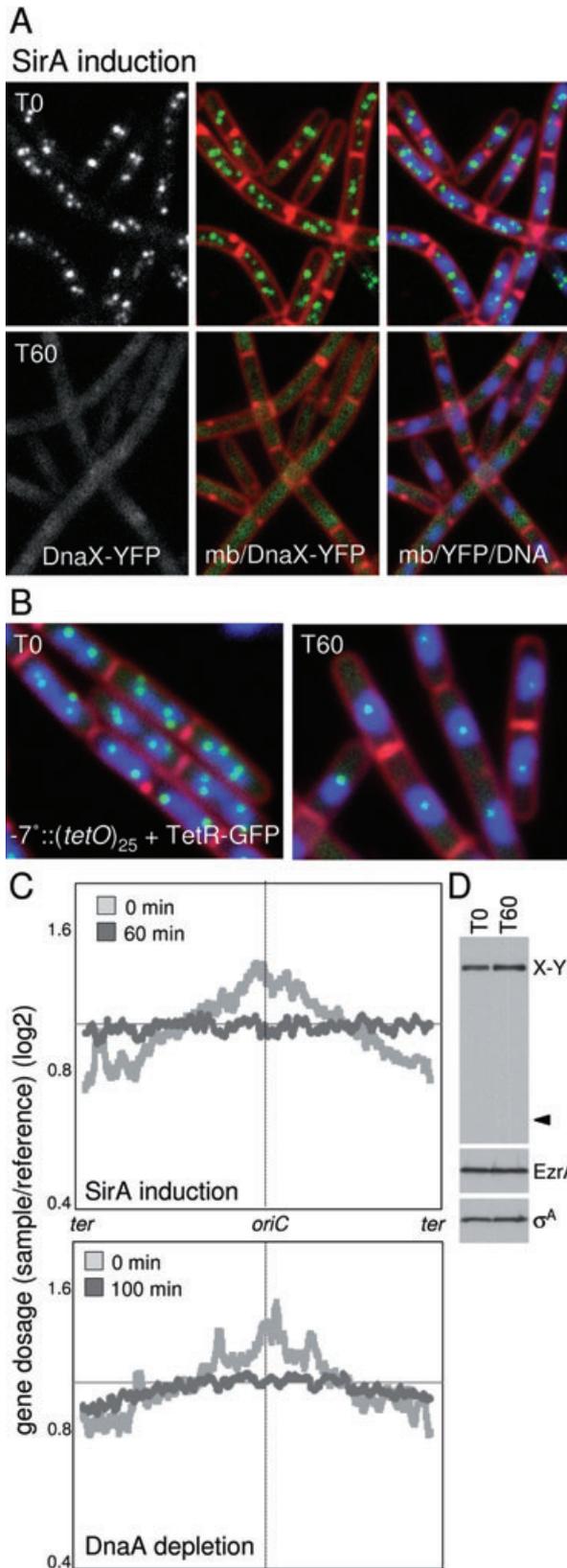


Fig. 3. SirA inhibits DNA replication at the initiation step.

A. Cells were analysed for ongoing replication using a DnaX–YFP fusion. Images show cells (strain BJW84) before and after SirA induction. Time (in min) after the addition of IPTG is indicated. Prior to SirA induction, DnaX–YFP (false-coloured green) is present in replisome foci. After SirA induction, DnaX–YFP foci are lost. Membranes (mb) were stained with FM4-64 (red) and DNA was stained with DAPI (blue).

B. Cells were analysed for origin content using TetR–GFP bound to a *tetO* array adjacent to *oriC*. Images show cells (strain BJW141) before and after SirA induction. Prior to SirA induction, most cells have two or four origin foci per DNA mass. After SirA induction, cells have a single origin focus per nucleoid.

C. Genomic DNA microarray analysis following SirA induction and DnaA depletion. The top graph shows the average gene dosage before (light grey) and after (dark grey) 60 min of SirA induction (strain BJW38). The bottom graph shows the average gene dosage before (light grey) and after (dark grey) 100 min of DnaA depletion (strain LAS223). All the probed genes in the *B. subtilis* chromosome arranged from -172° to $+172^\circ$ (*ter-oriC-ter*) are represented on the x-axis. The y-axis represents the gene dosage (\log_2) relative to a reference DNA with an *oriC/ter* ratio of 1 (see *Experimental procedures*). The smoothed line was generated by plotting the average gene dosage of the 25 genes before and 25 genes after each gene probed.

D. Immunoblot analysis shows that DnaX–YFP remains intact after SirA induction. DnaX–YFP was analysed using anti-GFP antibodies and the caret identifies the predicted size of free GFP. EzrA and σ^A were used to control for loading.

formed colonies in the presence of IPTG (Fig. 4). By contrast, the matched control strain could only grow in the absence of inducer. Analysis of the *oriN*-containing strain by fluorescence microscopy revealed no difference in nucleoid morphology in the presence or absence of SirA (data not shown). These results support the idea that SirA acts on *oriC* and/or DnaA.

SirA disrupts DnaA–GFP localization

If SirA is acting on DnaA, then expression of SirA might disrupt DnaA localization at *oriC*. To investigate this possibility, we used a strain harbouring wild-type DnaA and a

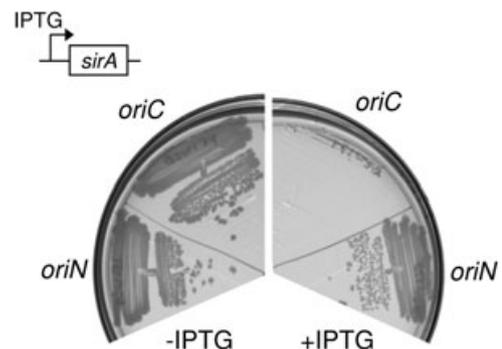


Fig. 4. Cells that initiate replication in a DnaA-independent manner are immune to SirA induction. Cells harbouring a DnaA-independent origin of replication (*oriN*, strain BJW173) or a DnaA-dependent origin (*oriC*, strain BJW174) were streaked on plates with and without IPTG. Both strains contain an IPTG-inducible promoter ($P_{hyperspank}$) fused to *sirA*.

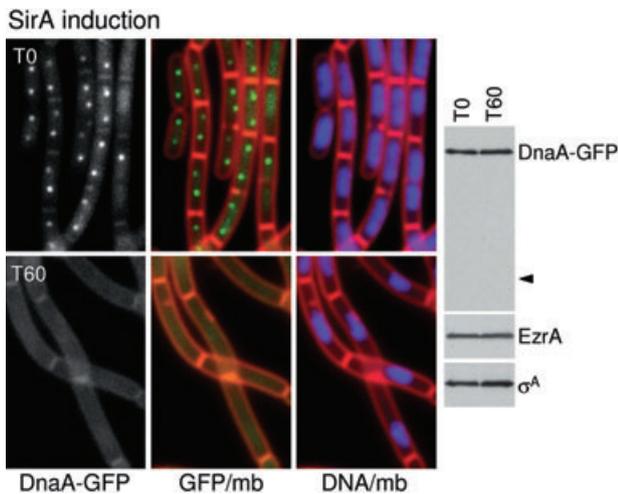


Fig. 5. SirA disrupts DnaA-GFP localization. A. Localization of DnaA-GFP (green) before and after SirA induction (strain BJW218). Time (in min) after the addition of IPTG is indicated. Membranes (mb) were stained with FM4-64 (red) and DNA was stained with DAPI (blue). Immunoblot analysis shows that DnaA-GFP remains intact after SirA induction. DnaA-GFP was analysed using anti-GFP antibodies and the caret identifies the predicted size of free GFP. EzrA and σ^A were used to control for loading. The apparent septal membrane association of DnaA-GFP is due to bleed through from the membrane dye.

DnaA-GFP fusion inserted at an ectopic chromosomal locus. Cells expressing this fusion contained two DnaA-GFP foci per DNA mass, one near each cell quarter, or a diffuse fluorescent haze that appeared to colocalize with the DAPI-stained DNA (Fig. 5). DnaA-YFP exhibited a similar localization pattern as DnaA-GFP, and the foci colocalized with an origin proximal marker (data not shown), suggesting that these fluorescent foci reflect DnaA bound to *oriC*. When SirA was induced, the DnaA-GFP foci became diffuse after 15 min of induction and were completely lost after 60 min. At this time, the DNA had taken on its characteristic rounded-up appearance (Fig. 5). Immunoblot analysis indicates that the diffuse fluorescent signal was not a consequence of proteolytic release of GFP (Fig. 5). Recent studies from Graumann and colleagues suggest that DnaA tracks with the replisome (Soufo *et al.*, 2008). Although our results suggest that DnaA-GFP foci colocalize with *oriC*, our data do not rule out the possibility that some of the DnaA-GFP is replisome-associated. In either case, our results indicate that SirA disrupts DnaA-GFP foci and suggest that SirA acts on DnaA.

DnaA is required for SirA localization

We hypothesized that if SirA targets DnaA, then SirA localization would be dependent on DnaA. We first examined the localization of an IPTG-inducible GFP-SirA fusion. The GFP fusion was a less potent inhibitor of cell

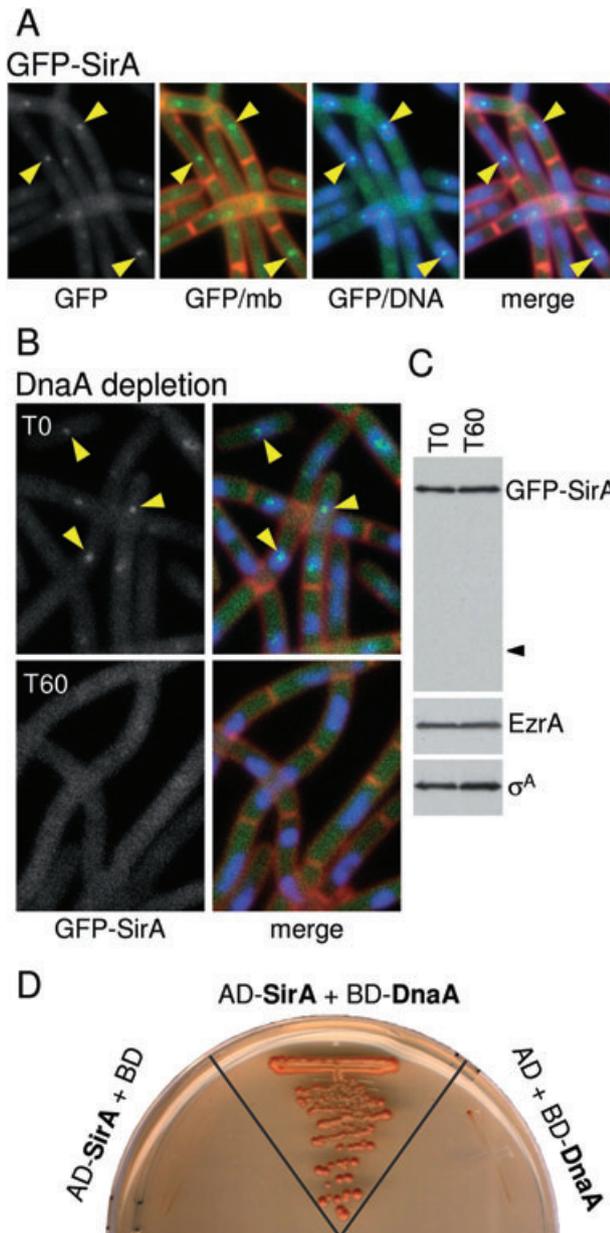
growth (Fig. S4), but analysis by fluorescence microscopy revealed rounded-up DNA and anucleate cell phenotypes (Fig. S5A) similar to the untagged fusion. Examination of GFP-SirA revealed faint foci early during an induction time-course, but within 20 min the fusion appeared as a diffuse haze (Fig. S5A). As the kinetics of foci loss were similar to what we observed for DnaA-GFP following SirA induction, we wondered whether GFP-SirA first localized to DnaA bound to *oriC*, and then became diffuse as DnaA was displaced from this site. To investigate this, we generated a xylose-inducible promoter fusion to *gfp-sirA* that produces approximately 10-fold less protein than the IPTG-inducible version (Fig. S5B). At this low level of GFP-SirA, the cells were not growth-inhibited although nucleoid morphology was somewhat aberrant (Fig. 6A). Importantly, 10–20% of the cells contained one or two faint foci. These foci colocalized with the DNA mass (Fig. 6A and Fig. S5A) and an origin marker (Fig. S6). Upon depletion of DnaA, the GFP-SirA foci were lost (Fig. 6B). Immunoblot analysis shows that the diffuse signal was not a result of degradation of SirA and release of free GFP (Fig. 6C). These results indicate that GFP-SirA localization requires DnaA and suggest that SirA acts on DnaA.

SirA and DnaA interact directly

We found that SirA disrupts DnaA foci, and that SirA foci are dependent on DnaA. A simple model incorporating these reciprocal results is that SirA acts on DnaA bound to *oriC*, releasing it from the origin, in turn preventing replication initiation. To determine if SirA and DnaA interact directly, we performed a yeast two-hybrid assay. SirA was fused to the DNA binding domain of GAL4, and DnaA was fused to the GAL4 activation domain. When both fusions were expressed in yeast a target gene (*ADE2*) was activated resulting in the ability to grow in the absence of adenine (Fig. 6D). Importantly, when the GAL4-SirA fusion was coexpressed with the empty GAL4 activation domain or the empty GAL4 DNA binding domain was coexpressed with the GAL4-DnaA fusion, neither was able to activate expression of the target gene and restore adenine prototrophy (Fig. 6D). Attempts to detect an interaction between GFP-SirA and DnaA by immuno-affinity purification from *B. subtilis* lysates were unsuccessful, suggesting that the interaction between these proteins is weak or transient.

SirA inhibits replication during sporulation

Collectively, our data indicate that SirA disrupts the interaction between DnaA and the origin of replication, and that this leads to an inhibition of DNA replication. However, the replication inhibition phenotypes we



observed resulted from the artificial induction of a sporulation-specific protein during vegetative growth. To determine whether SirA functions to inhibit DNA replication during sporulation, we assayed the replication status of wild type and the SirA mutant after the cells had committed to sporulation. As a marker for replication, we again utilized DnaX-YFP. Cells were induced to sporulate by resuspension in a nutrient-poor medium and analysed after 1.5 and 2 h of sporulation. In both wild type and the SirA mutant, DnaX-YFP foci were present in all cells at the time of resuspension (not shown). At hour 1.5, 6.9% ($n = 303$) of the wild-type sporulating cells (defined by the presence of a polar septum) had one or

Fig. 6. DnaA is required for GFP-SirA localization.

A. Localization of GFP-SirA (green) produced at low levels during vegetative growth (strain BJW196). Membranes (mb) were stained with FM4-64 (red) and DNA was stained with DAPI (blue). Yellow carets highlight nucleoid-associated GFP-SirA foci.

B. GFP-SirA localization before and after depletion of DnaA (strain BJW197). Time (in min) after the removal of IPTG (to deplete DnaA) is indicated.

C. Immunoblot analysis shows that GFP-SirA remains intact after DnaA depletion. GFP-SirA was analysed using anti-GFP antibodies and the caret identifies the predicted size of free GFP. EzrA and σ^A were used to control for loading.

D. SirA and DnaA interact in the yeast two-hybrid assay. A yeast strain harbouring the Gal4 activation domain fused to SirA (AD-SirA) and the Gal4 DNA binding domain fused to DnaA (BD-DnaA) was able to activate transcription of the *GAL2* promoter fused to the *ADE2* gene. Growth on medium lacking adenine indicates a positive interaction between the hybrid proteins. Strains containing the AD-SirA fusion and the unfused DNA binding domain (BD) or the BD-DnaA fusion and the unfused activation domain (AD) did not activate transcription and were unable to grow in the absence of adenine.

more DnaX-YFP foci (Fig. 7A). By contrast, 31% ($n = 202$) of the SirA mutant had DnaX-YFP foci at this time point (Fig. 7A). Thirty minutes later, at hour two, most of the wild-type cells had completed replication as evidenced by the loss of DnaX-YFP foci. At this time point only 0.6% ($n = 344$) of the sporulating cells had replisome foci. However, 12.2% ($n = 500$) of the SirA mutant cells still had ongoing replication. Strikingly, even SirA mutant cells that had completed engulfment (a late stage in the developmental pathway) contained replication foci. At this stage, all wild-type sporulating cells had diffuse DnaX-YFP signal. These results indicate that SirA contributes to the inhibition of replication during sporulation.

Although it would be inconsistent with the vegetative results, it was possible that the DnaX-YFP foci in the SirA mutant represent replisomes that were stalled during sporulation rather than an actual increase in replication initiation. To determine if the SirA mutant cells have an increased frequency of initiation relative to wild type, we labelled an origin-proximal region of the chromosome using a *tetO* array and TetR-GFP, and quantified the number of foci per sporulating cell. In wild-type cells at hour two of sporulation, two copies of the -7° region were present in 98.2% ($n = 393$) of the sporulating cells, while only 1.3% possessed more than two foci. By contrast, in the SirA mutant 4.5-fold more sporulating cells had greater than two foci (5.8%, $n = 380$). We could not detect a substantial increase in the *oriC/ter* ratio during sporulation using genomic microarrays (data not shown); this result was not surprising, as only a subset of cells in the population appear to over-replicate under these sporulation conditions. In sum, these results are consistent with the idea that SirA helps maintain diploidy during sporulation.

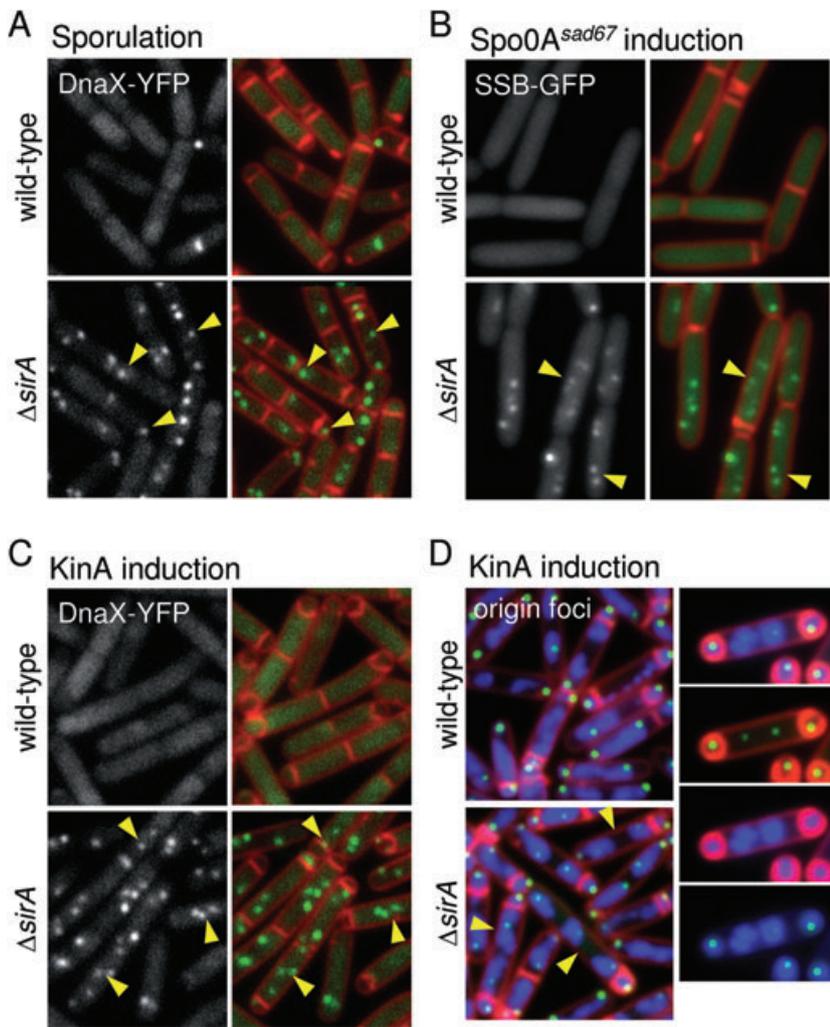


Fig. 7. Sporulating cells over-replicate in the absence of SirA.

A. Cells induced to sporulate by resuspension in defined minimal medium were analysed for ongoing replication using a DnaX–YFP fusion. The images show wild type (strain BKM1585) and the SirA mutant (strain BJW91) at hour 1.5 of sporulation. DnaX–YFP (false-coloured green) and membranes stained with TMA-DPH (false-coloured red) are shown. Yellow carets highlight sporulating cells with replisome foci.

B. Cells expressing a constitutively active allele of Spo0A (Spo0A^{sad67}) for 60 min were analysed for ongoing replication using an SSB–GFP fusion. The images show wild type (strain BJW271) and the SirA mutant (strain BJW275). Yellow carets highlight cells with ongoing replication as assessed by SSB–GFP foci.

C. Cells sporulated in rich medium by induction of the sensor kinase KinA were analysed for ongoing replication using a DnaX–YFP fusion. The images show wild type (strain BJW230) and the SirA mutant (strain BJW232) after 2 h of KinA induction. Yellow carets highlight sporulating cells with replisome foci.

D. Cells sporulated in rich medium by induction of the sensor kinase KinA were analysed for origin content using TetR–GFP bound to a *tetO* array adjacent to *oriC*. The images show wild type (strain BJW279) and the SirA mutant (strain BJW279) after 2 h of KinA induction. Yellow carets highlight cells with more than two TetR–GFP (green) foci. Membranes were stained with FM4-64 (red) and DNA was stained with DAPI (blue). An example of a mother cell (with two chromosomes) and twin forespores after 2.5 h of KinA induction is shown on the right.

Spo0A inhibits replication in a SirA-dependent manner

Our data indicate that only a subset of sporulating cells initiate new rounds of replication in the absence of SirA. This suggests that additional factors help enforce diploidy as cells initiate sporulation. A good candidate is Spo0A itself, which binds near *oriC* and prevents the formation of open replication complexes *in vitro* (Castilla-Llorente *et al.*, 2006). To investigate whether Spo0A directly inhibits replication *in vivo*, we took advantage of a constitutively active allele of Spo0A (called Spo0A^{sad67}) (Ireton *et al.*, 1993). It has been shown previously that Spo0A^{sad67} binds to *oriC* *in vivo* (Molle *et al.*, 2003) and is able to prevent replication *in vitro* (Castilla-Llorente *et al.*, 2006). Induction of Spo0A^{sad67} during growth results in the activation of the Spo0A regulon; the terminal phenotype is cell lysis. However, at early times after induction, it is possible to study cell physiology (Levin and Losick, 1996). To monitor ongoing replication upon Spo0A^{sad67} induction, we used a

fusion of the single-strand binding protein to GFP (SSB–GFP) (for reasons that we were unable to determine, the *spo0A*^{sad67} allele was unstable in the presence of the DnaX–YFP fusion.) Prior to induction of Spo0A^{sad67}, all cells were actively replicating their chromosomes as evidenced by the presence of SSB–GFP foci (for example, see Fig. S3A). However, within 60 min of induction, 81% ($n = 336$) of the cells had diffuse SSB–GFP signal (Fig. 7B). Those with active replisomes (19%) usually had a single focus. This result indicates that either Spo0A or genes under its control can efficiently inhibit replication. To determine the contribution of SirA to this replication block, we examined SSB–GFP foci upon induction of Spo0A^{sad67} in the SirA mutant. Strikingly, 94% ($n = 361$) of the cells had foci after 60 min of Spo0A^{sad67} induction (Fig. 7B). These results indicate that activated Spo0A is incapable of directly inhibiting replication initiation or does so inefficiently. It further suggests that SirA is the principal regulator of replication initiation under Spo0A control.

SirA plays a more critical role in replication inhibition during fast growth

We observed that, at most, 31% of SirA mutant cells have an over-replication phenotype during sporulation. These results indicate that SirA cannot be the only factor maintaining diploidy during sporulation. When bacteria enter stationary phase or slow down growth, they co-ordinately reduce replication (Haeusser and Levin, 2008). Accordingly, we wondered whether the conditions in which we induce sporulation in the laboratory (resuspension in minimal medium) might result in reduced replication, thus masking the importance of SirA. To test this, we took advantage of a strain in which efficient sporulation can be induced during vegetative growth. Fujita and Losick have shown that a small increase in expression of the sensor kinase KinA leads to a gradual accumulation of active Spo0A and efficient entry into sporulation (Fujita and Losick, 2005). We induced low levels of KinA in wild-type or SirA mutant cells in early exponential growth and monitored ongoing replication using the DnaX–YFP fusion. After 1.5 h of induction, virtually all cells had initiated sporulation as evidenced by the formation of polar septa. Under these conditions, 11% ($n = 607$) of the wild-type sporulating cells had DnaX–YFP foci (compared with 6.9% of the cells that had replisome foci when sporulation was induced by resuspension in minimal medium). By contrast, in cells lacking SirA, 73% ($n = 532$) of sporulating cells had replisome foci (Fig. 7C). Similar results were obtained monitoring TetR–GFP bound to a *tetO* array at -7° (Fig. 7D and Fig. S7A). Specifically, 11% ($n = 290$) of wild-type sporulating cells had more than two origin foci, while 54% ($n = 396$) had more than two foci in the SirA mutant. Over-replication in the sporulating cells lacking SirA could be observed in both the mother cell and forespore (Fig. 7C and D and Fig. S7A). Moreover, in a subset of cells (~2%) we could detect ‘twin spores’ (P. Piggot and M. Elowitz, pers. comm.). These sporulating cells have a single mother with one or two chromosomes and two spores, one at each pole (Fig. 7D and Fig. S7). These results indicate that the sporulation conditions can have a major impact on the importance of SirA in preventing new rounds of replication. They further suggest that nutrient status and SirA are the major enforcers of diploidy during spore formation.

Discussion

The Spo0A transcription factor is the master regulator governing entry into sporulation (Fig. 1A) (reviewed in Stragier and Losick, 1996; Errington, 2003; Hilbert and Piggot, 2004). Spo0A controls the genes responsible for the switch from medial to polar cell division (Khvorova *et al.*, 2000; Ben-Yehuda and Losick, 2002), the remodel-

ling of the replicated chromosomes into an axial filament (Ben-Yehuda *et al.*, 2003), and forespore- and mother-specific gene transcription (Jonas *et al.*, 1988; Schmidt *et al.*, 1990). Here we have shown that Spo0A also enforces diploidy on the sporulating cell. Spo0A appears to accomplish this primarily via the induction of the regulatory protein SirA, rather than through direct binding to the origin of replication. Our data indicate that SirA maintains diploidy by inhibiting new rounds of replication, specifically by targeting the initiator protein DnaA (Fig. 1B). The interaction between SirA and DnaA could prevent binding to *oriC*. Alternatively, SirA could function like *E. coli* Hda (Su’etsugu *et al.*, 2008), by stimulating the nucleotide hydrolysis of ATP bound to DnaA. SirA has no apparent sequence motifs (and is only found in endospore formers) providing no clues to its function. Experiments aimed at directly testing these models *in vitro* have been hampered by the insolubility of recombinant SirA. That SirA is an inhibitor of DNA replication was independently discovered by R. Losick and colleagues (pers. comm.) (Rahn-Lee *et al.*, 2009).

SirA and nutrient status enforce diploidy

Our results indicate that SirA inhibition of DnaA is not the sole mechanism for preventing new rounds of DNA replication as cells enter sporulation. SirA plays a more significant role in enforcing diploidy when cells are engineered to sporulate in the presence of nutrients than under nutrient-depleted conditions. Accordingly, these data are most consistent with the idea that nutrient status (and co-ordinately controlled replication) serves as the other mechanism that maintains diploidy. It is likely that *B. subtilis* typically sporulates under conditions of nutrient deprivation and slow growth, where the rate of replication initiation is also co-ordinately slow. Nutrient status should therefore dictate that most cells do not initiate new rounds of replication. Why then does the sporulating cell also induce a protein that specifically inhibits replication initiation? Differentiation into a dormant spore is an energy-intensive process, requiring the synthesis of hundreds of sporulation-specific proteins (Eichenberger *et al.*, 2004; Wang *et al.*, 2006). Therefore, cells must enter this developmental pathway prior to the complete exhaustion of nutrients. The induction of SirA as cells commence spore formation may serve as a fail-safe mechanism, ensuring that nutrient reserves for spore maturation are not further depleted by new rounds of inappropriate DNA replication. It is noteworthy that cells lacking SirA have only a modest defect in sporulation efficiency (15% reduction). However, in the context of the environment, it is likely that the stringent control of energy and cellular metabolites is critical to overall fitness.

An additional role for SirA could be to help commit the sporulating cell to its developmental fate. After polar division and the activation of the first mother cell and forespore transcription factors, the sporulating cell becomes irreversibly committed to sporulation (Dworkin and Losick, 2005). Even in the face of nutrient-rich conditions, the mother and forespore continue down their respective developmental pathways. It is possible that the inhibition of replication by SirA in both differentiating cell types is one arm of the control mechanism that prevents return to vegetative growth.

It had been previously proposed that the binding of active Spo0A to the sequences within *oriC* could serve to inhibit replication initiation (Castilla-Llorente *et al.*, 2006). Our data indicate that a constitutively active allele of Spo0A is not sufficient to inhibit replication in the absence of SirA, suggesting that Spo0A is not likely to play a major role in enforcing diploidy. However, these results do not exclude the possibility that Spo0A binding to *oriC* could function in tandem with SirA to help prevent replication initiation. Interestingly, the master transcriptional regulator (CtrA) in the bacterium *Caulobacter crescentus* has also been suggested to control the initiation of DNA replication (Quon *et al.*, 1998). In its absence, cells over-replicate. CtrA, like Spo0A, binds to sites around *oriC* and it has been proposed that this binding occludes DnaA (Quon *et al.*, 1998). However, mutations in the five CtrA binding sites in *C. crescentus oriC* did not measurably perturb replication control (Bastedo and Marczyński, 2009). By analogy to SirA, we hypothesize that a CtrA-controlled protein inhibits replication initiation, perhaps by directly targeting DnaA.

What prevents haploid cells from entering sporulation?

We have argued that nutrient status and SirA maintain and enforce diploidy during sporulation. What then prevents slow-growing cells with only one chromosome from prematurely entering this developmental pathway? A good candidate for this function is the checkpoint protein Sda. In response to blocks in replication initiation and/or elongation, Sda inhibits the initiation of sporulation by preventing the activation of Spo0A (Burkholder *et al.*, 2001). In addition to inhibiting sporulation when cells are experiencing replication stress, one of Sda's functions could be to prevent haploid cells from entering sporulation prior to a final round of DNA replication (Michael, 2001). Mechanistically, one way this regulation could occur is through the normal cycling of ATP- and ADP-bound DnaA. In *E. coli* (and presumably in *B. subtilis*) DnaA-ATP (the form of DnaA that promotes origin melting) accumulates during the cell cycle. Once DnaA-ATP achieves a critical concentration, it triggers the initiation of replication (Kurokawa *et al.*, 1999), resulting in ATP hydrolysis. Tran-

scription of the *sda* gene is regulated by DnaA; however, it is unclear how it carries out this function (Burkholder *et al.*, 2001). If the accumulation of DnaA-ATP promotes the expression of *sda* (and inhibition of sporulation), conditions that reduce DnaA-ATP levels (such as initiation itself) would lead to the loss of *sda* gene expression and ultimately to entry into sporulation.

Twin spores

Finally, we have found that one consequence of a failure to enforce diploidy during sporulation is the formation of 'twins', a phenotype identified by P. Piggot and M. Elowitz (pers. comm.) (Eldar *et al.*, 2009) in which a single mother (with one or two chromosomes) nurtures two spores, one at each cell pole. These spores appear to develop normally as judged by their grey appearance by phase-contrast microscopy (Fig. S7B) (Piggot and Coote, 1976). Symmetrical spore formation has been described previously in a cousin of *B. subtilis*, *Metabacterium polyspora* (Angert and Losick, 1998). Remarkably, these bacteria produce spores as part of their cell cycle, and the forespores themselves have been observed replicating DNA and undergoing division (Ward and Angert, 2008). We speculate that the energetic cost of raising two spores instead of one has been selected against in asymmetrical spore formers like *B. subtilis*. Indeed, *B. subtilis* has evolved elaborate regulatory pathways that normally ensure a one mother one spore family (Khvorova *et al.*, 1998; Eichenberger *et al.*, 2001; Zupancic *et al.*, 2001).

Experimental procedures

General methods

Unless otherwise noted, *B. subtilis* strains were derived from the prototrophic strain PY79 (Youngman *et al.*, 1983). *E. coli* strains were TG1 and DH5a. To visualize the localization of fluorescent fusions during vegetative growth, strains were grown in CH medium (Harwood and Cutting, 1990) at 37°C. Sporulation was induced by resuspension at 37°C according to the method of Sterlini-Mandelstam (Harwood and Cutting, 1990). A description of strains (Table S1) and plasmids (Table S2) and oligonucleotide primer sequences (Table S3) can be found in supplemental material.

Induction and depletion conditions

Unless otherwise indicated, fluorescence microscopy was performed on exponentially growing cells ($OD_{600} = 0.3-0.8$). SirA was induced with 1.0 mM IPTG or 10 mM xylose in liquid cultures and on LB agar plates. KinA and Spo0A^{sad67} were induced as described previously (Fujita and Losick, 2005) with 20 μM and 200 μM IPTG respectively. SSB-GFP and DnaA-GFP were induced with 10 mM xylose. The DnaA-GFP fusion was synthesized at levels below those required to

support growth in the absence of DnaA. For DnaA depletions, exponentially growing cells in CH medium supplemented with 0.5 mM IPTG and 10 $\mu\text{g ml}^{-1}$ tetracycline were washed three times with CH medium supplemented with 10 $\mu\text{g ml}^{-1}$ tetracycline but lacking IPTG. The washed cells were resuspended in CH supplemented with 10 $\mu\text{g ml}^{-1}$ tetracycline at an $\text{OD}_{600} = 0.1$ to begin the depletion time-course.

Genomic microarray

Genomic microarray analysis was carried as described previously (Wang *et al.*, 2007). Briefly, genomic DNA was isolated from the strains of interest and cleaved to completion with HaeIII. Reference DNA was from a temperature-sensitive replication initiation mutant (*dnaB134*) grown at the non-permissive temperature (42°C) for one hour to ensure that all rounds of replication were complete. The HaeIII-cleaved reference DNA was labelled with Cy3 (GE Healthcare) and the experimental samples were labelled with Cy5 (GE Healthcare). The labelled DNA was hybridized to an oligonucleotide microarray containing a 70mer for each gene in the *B. subtilis* genome. Scanning was carried out on a Genepix 4000B scanner (Software v. 5.1), and data were analysed in Microsoft Excel. Intensities from the 532 nm channel were normalized to the reference (635 nm channel) prior to subsequent analysis. Each data point on the smooth line in Fig. 3C represents an average of the normalized ratios of the 25 genes on either side of each locus. The *oriC/ter* ratios were calculated by dividing the average normalized intensities of the 25 genes on either side of *dnaA* (*oriC*) by the normalized intensities of 25 genes on either side of *rtp* (*ter*).

Immunoblot analysis

During logarithmic growth the OD_{600} was measured (for equivalent loading) and samples were collected by centrifugation. Whole-cell extracts were prepared by resuspension of cell pellets in 50 μl lysis buffer (20 mM Tris pH 7.0, 10 mM MgCl_2 , 1 mM EDTA, 1 mg ml^{-1} lysozyme, 10 $\mu\text{g ml}^{-1}$ DNase I, 100 $\mu\text{g ml}^{-1}$ RNase A, with protease inhibitors: 1 mM PMSF, 1 $\mu\text{g ml}^{-1}$ leupeptin, 1 $\mu\text{g ml}^{-1}$ pepstatin) and incubation at 37°C for 10 min followed by addition of 50 μl sodium dodecyl sulphate (SDS) sample buffer (0.25 M Tris pH 6.8, 4% SDS, 20% glycerol, 10 mM EDTA) containing 10% 2-Mercaptoethanol. Samples were heated for 5 min at 80°C prior to loading. Proteins were separated by SDS-PAGE on 15% polyacrylamide gels, electroblotted onto Immobilon-P membrane (Millipore) and blocked in 5% non-fat milk in phosphate-buffered saline-0.5% Tween-20. The blocked membrane was probed with anti-GFP (Rudner and Losick, 2002), anti-EzrA (Levin *et al.*, 1999) and anti- σ^A (Fujita, 2000) antibodies diluted into 3% BSA in phosphate-buffered saline-0.05% Tween-20. The primary antibodies were detected using horseradish peroxidase-conjugated goat, anti-rabbit immunoglobulin G (Bio-Rad) with the Supersignal Substrate as described by the manufacturer (Perkin Elmer).

Fluorescence microscopy

Fluorescence microscopy was performed as previously described (Doan *et al.*, 2005). Fluorescent signals were visu-

alized with a phase contrast objective UplanFLN 100 \times and captured with a monochrome CoolSnapHQ digital camera (Photometrics) using Metamorph software version 6.1 (Universal Imaging). Exposure times were typically 500–1000 ms for GFP and YFP protein fusions. Membranes were stained with either TMA-DPH or FM4-64 (Molecular Probes), at a final concentration of 0.02 mM and 3 $\mu\text{g ml}^{-1}$ respectively, and imaged with exposure times of 200 ms. DNA was stained with DAPI (Molecular Probes), at a final concentration of 2 $\mu\text{g ml}^{-1}$ and imaged with a typical exposure time of 200 ms. Fluorescence images were analysed, adjusted and cropped using Metamorph v 6.1 software (Molecular Devices).

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