Spo0A regulates chromosome copy number during sporulation by directly binding to the origin of replication in Bacillus subtilis†

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Introduction

Bacillus subtilis is widely used as a model organism for studying cell cycle progression and cellular differentiation. Under growth promoting conditions, B. subtilis divides symmetrically, giving rise to two identical cell types. When exposed to stress, such as nutrient starvation, a subpopulation of cells initiates sporulation. Sporulation is a costly and time-consuming process resulting in the development of two different cell types: the larger mother cell and the smaller forespore. Importantly, both cell types inherit a single copy of the chromosome during sporulation (Fig. 1) (Errington, 2003; Higgins and Dworkin, 2012). Tight co-ordination between DNA replication and sporulation results in correct chromosome copy number, and is a requirement for efficient sporulation (Murray and Errington, 2008; Eldar et al., 2009; Veening et al., 2009; Xenopoulos and Piggot, 2011). So far, two checkpoint factors have been identified that play a role in this: SirA (sporulation inhibitor of replication A) and Sda (suppressor of dnaA1). SirA prevents initiation of DNA replication in cells committed to sporulation. Transcription of sirA is directly activated by Spo0A–P, the master regulator of sporulation. SirA maintains the diploid state of sporulating cells by directly targeting the DNA replication initiator protein DnaA which consequently becomes displaced from the origin of replication (oriC) (Rahn-Lee et al., 2009; 2011; Wagner et al., 2009). While SirA prevents re-initiation of DNA replication in sporulating cells, the checkpoint function of Sda is to prevent sporulation initiation in replicating cells and during DNA repair (Burkholder et al., 2001; Veening et al., 2009). The intrinsically unstable Sda protein binds to the major sporulation kinases (KinA and KinB) and prevents their autophosphorylation (Burkholder et al., 2001; Rowland et al., 2004; Ruvolo et al., 2006; Whitten et al., 2007; Cunningham and Burkholder, 2009). KinA and KinB are proteins of the so-called phosphorelay, which regulates the level of phosphorylated Spo0A (the transcriptionally active, DNA-binding form of Spo0A) (Burbulys et al., 1991). Interestingly, pulses of Sda synthesis occur concomitantly with the initiation of DNA replication via transcriptional activation by DnaA. Following the burst of Sda expression,
targeted proteolysis of Sda reduces its concentration to generate a window of opportunity at the end of the replication cycle during which cells can enter sporulation as diploids. Cells that do not enter sporulation during that small time period have to undergo a new round of replication before getting the next opportunity to do so (Veening et al., 2009). Mother cells and endospores of a sda/sirA double mutant frequently contain more than one copy of the chromosome and polyploid spores show reduced fitness (Veening et al., 2009). Relevant for the current study is the fact that even in the sda/sirA double mutant, the majority of sporulating cells still show wild-type chromosome copy numbers and wild-type fitness. Hence, additional factors are likely at play to co-ordinate DNA replication with sporulation.

In order to initiate DNA replication, DnaA binds various perfect and imperfect DnaA-boxes (aA-boxes) present within the oriC region where it assembles into a large nucleoprotein complex. This results in denaturation of an AT-rich region at oriC, which is required for the assembly of a functional replisome (Moriya et al., 1999; Messer, 2002). Interestingly, Castilla-Llorente et al. (2006) noted that the aA-box sequence (consensus 5′-TGTGNATAA-3′; Fig. 2, orange) partially overlaps with the recognition sequence for Spo0A (the 0A-box, consensus 5′-TGTGCAA-3′; Fig. 2, blue, Strauch et al., 1990; Molle et al., 2003), and indeed the B. subtilis origin region contains many Spo0A binding sites that are specifically recognized by purified protein in vitro (Fig. 2; Castilla-Llorente et al., 2006). However, it remains unclear whether the binding of Spo0A at the B. subtilis origin is physiologically relevant for B. subtilis, as studies performed to show a possible direct effect of Spo0A on DNA replication have not led to conclusive results. On the one hand it was shown that Spo0A can inhibit DnaA-dependent DNA duplex unwinding in vitro (Fig. 2; Castilla-Llorente et al., 2006). On the other hand Spo0A does not seem to have a significant effect on DNA replication in vivo when sporulation is artificially induced in exponentially growing cells (Wagner et al., 2009). Spo0A-P also reaches high levels in the mother cell (Fujita and Losick, 2003) and recently the Piggot lab showed that Spo0A-P inhibits mother cell growth and DNA replication independently of SirA. The molecular mechanism for this Spo0A-dependent repression of replication is yet unknown, although a possible role for Sda in this has been suggested (Xenopoulos and Piggot, 2011).

Here, we have investigated the role of Spo0A as a direct inhibitor of DNA replication initiation during sporulation by mutating the 0A-boxes within oriC, which is the most decisive method to assess a possible direct role of Spo0A in the control of DNA replication. The results indicate that Spo0A directly controls chromosome copy number by binding to a number of specific Spo0A binding sites present within the oriC region and that this regulation is especially important in the absence of SirA and Sda or when sporulation is induced in actively replicating cells.
Results

Mutating 0A-boxes within the origin of replication

The *B. subtilis* oriC region contains several bona fide Spo0A binding sites (consensus sequence 5′-TGTCGAA-3′; Fig. 2A, blue) that partially overlap with functional DnaA binding sites (inverted consensus sequence 5′-TTATNCACA-3′; 5′-TGTGNATAA-3′; inverted consensus sequence 5′-TGTCGAA-3′). The promoter of DnaA lies in between aA-box 6 and 7 (Ogasawara *et al.*, 1985) and its approximate position is indicated by an arrow. Note that the graph is not drawn to scale.

B. Sequence of the native oriC (oriC\textsuperscript{nat}).

C. Sequence of the mutated oriC (oriC\textsuperscript{mut}).

0A-boxes are indicated in blue, aA-boxes in orange. The lighter the colour, the less similar the sequence is to its consensus. Arrows indicate the direction of these recognition sequences on the chromosome. Nucleotides shown in bold are used to introduce point mutations. ‘X’ indicates mismatches compared with the consensus sequences of Spo0A- and DnaA-boxes. Red ‘X’ indicates mismatches introduced by mutation.
ing consensus in Fig. 2B with Fig. 2C). In order to diminish the chance of negatively affecting DnaA binding to the oriC, existing recognition sequences of DnaA within the oriC region were used for the design when possible. The wild-type, native oriC region (oriCnat) was altered by allelic replacement using a synthetic oriC containing the mutated 0A-boxes (oriCmut) to obtain strain IDJ056 (see Experimental procedures). Strain IDJ056 (oriCmut) and its wild-type parental strain IDJ055 (oriCnat) exhibit similar doubling times and similar dnaA transcript levels and DnaA protein levels during steady-state growth (Fig. S1). To test whether DnaA binding to oriCmut is affected, we purified B. subtilis DnaA and performed electrophoretic mobility shift assays (EMSA). Since DnaA can bind to DNA in both ADP- and ATP-bound forms (Sekimizu et al., 1987), we carried out EMSAs in the presence of either nucleotide. As shown in Fig. 3, the purified DnaA protein bound similarly to both the wild-type and mutant DNA fragments. Taken together, these results indicate that DnaA-binding efficiency to the mutated oriC is not significantly altered.

Spo0A binds less efficiently to the mutated oriC region
To test whether Spo0A–P is able to bind oriCmut in vitro, we purified C-terminally his-tagged Spo0A variants and performed EMSAs. Spo0A consists of two domains, the C-terminal DNA-binding domain and the N-terminal phosphoacceptor domain. Phosphorylation of a conserved aspartic acid residue within the N-terminal domain results in a conformational change of Spo0A–P, upon which the protein dimerizes and becomes active (Ladds et al., 2003; Muchová et al., 2004). It has been reported that Spo0A purified from Escherichia coli is only active up to 40%, while other studies did not obtain active Spo0A without subsequent in vitro phosphorylation steps (Lewis et al., 2002; Ladds et al., 2003). Therefore, we also purified the DNA-binding domain of Spo0A alone (Spo0A-DB), which does not require phosphorylation for DNA binding (Molle et al., 2003). Full-length Spo0A (Spo0A-FL) and Spo0A-DB were incubated with [γ-32P]-labelled oriC DNA containing the 0A-boxes within incA, incB or both of the incA and incB regions that harbour the 0A-boxes of interest (Figs 2 and 4A). As a negative control, we used the srfAA promoter and as a positive control the abrB promoter (Molle et al., 2003). As shown in Fig. 4B, Spo0A-DB as well as Spo0A-FL bind the oriCnat DNA fragments at concentrations of 30–120 nM, whereas no or poor shifts were obtained after incubation with our synthetic oriCmut DNA, confirming that the introduced mutations are effective and reduce Spo0A binding. Interestingly, in contrast to Spo0A-FL, incubation of the oriCnat DNA with Spo0A-DB results in multiple retarded species. This difference may be due to the fact that whereas active Spo0A-FL binds DNA as a dimer, Spo0A-DB binds DNA as a monomer (Lewis et al., 2000). Therefore, different affinities of individual 0A-boxes may be reflected in different retarded species. Overall, the results presented in Figs 3 and 4 show that the mutations introduced and present in oriCmut affect the in vitro affinity of Spo0A to this region without significantly altering DnaA binding.

0A-boxes within the oriC function as DNA replication control elements during sporulation
Next, we set out to investigate the differences in chromosome copy number between cells in which Spo0A binding

Fig. 3. DnaA binds with similar efficiency to oriCmut compared with oriCnat in vitro. The incAB PCR product (474 bp, 36 nM) was incubated with twofold increasing concentrations, ranging from 94 nM to 12 μM, of DnaA-ADP (A) or DnaA-ATP (B). Nucleoprotein complexes were separated on a 1.5% agarose gel and visualized by staining with ethidium bromide.
to the oriC is unaffected (oriC\textsuperscript{nat}) or disturbed (oriC\textsuperscript{mut}). In order to monitor the number of replication events we integrated an array of tet operator sites close to the oriC (at 345° of the circular chromosome) and a xylose-inducible tetR-mCherry gene at the non-essential amyE locus in both strains (oriC\textsuperscript{nat} and oriC\textsuperscript{mut}). Upon xylose induction, mCherry-labelled TetR binds to the tet operator sites near oriC, and the number of fluorescent foci will reflect the number of oriC regions present in the cell (see Veening et al., 2009, Fig. 5A). We also generated reporter strains that are mutated for sda, sirA or both sda and sirA to test for possible additive/synthetic effects of these known checkpoint mechanisms. Since the conditions that induce sporulation are important to observe replication checkpoint mechanisms (Wagner et al., 2009), we first triggered sporulation upon gradually depleting nutrients and increasing cell density by growing cells in Schaeffer’s sporulation medium (Schaeffer et al., 1965). Under these conditions, cells grow initially fast, similar to cells growing in LB medium. However, in contrast to LB medium, sporulation is efficiently initiated in a large subpopulation of cells once nutrients become limiting and cell densities high.

The TetR-mCherry reporter strains harboring either oriC\textsuperscript{nat} or oriC\textsuperscript{mut} in the different genetic backgrounds (wild type, Δsda, ΔsirA or Δsda/ΔsirA) were allowed to initiate sporulation and at least two biological replicates per strain were taken to determine the number of mCherry foci in the mother cell compartment of cells containing a phase bright

**Fig. 4.** Spo0A binds less efficiently to oriC\textsuperscript{mut} compared with oriC\textsuperscript{nat} in vitro.

A. Overview of the PCR fragments used for the EMSAs. Mismatches within the 0A-box are indicated as ‘X’. Red ‘X’ indicates mismatches introduced by mutation. 0A-boxes of interest are shown in blue.

B. Full-length Spo0A (Spo0A-FL) as well as the DNA-binding domain of Spo0A (Spo0A-DB) bind less efficiently to oriC PCR fragments harboring mutated 0A-boxes (oriC\textsuperscript{mut}) compared with oriC fragments in which no additional mismatches were introduced (oriC\textsuperscript{nat}). Protein concentrations: 30, 60, 90 and 120 nM. The promoter region of srfA (P\textsubscript{srfa}) was used as a negative control to which Spo0A\textsuperscript{~P} does not bind. As a positive control, we used the promoter region of abrB (P\textsubscript{abrB}), to which Spo0A–P binds with high affinity (Molle et al., 2003).
forespore (Fig. 5B–F). Due to strong autofluorescence of the phase-bright forespore compartment, it was not possible to detect oriC-mCherry foci in forespores. If replication and sporulation are co-ordinated correctly, mother cells (as well as forespores) are expected to contain exactly one chromosome (Fig. 1). As shown in Fig. 5B and D, wild-type mother cells (IDJ075, IDJ076) as well as ΔsirA mother cells (IDJ079, IDJ080) mainly have one oriC focus, irrespective of a native or mutated origin of replication. Interestingly, mother cell compartments with more than one fluorescent focus were frequently observed in the single sda mutant containing oriCmut (strain IDJ077) (Fig. 5C and F). Moreover, the fraction of Δsda mother cells containing more than one fluorescent foci was slightly elevated when it also contained the oriCmut region (strain IDJ078; average of 1.16 versus 1.18 foci; n > 450) (Fig. 5C and F). However, this difference is not statistically significant (P = 0.244, Mann–Whitney U-test). Therefore, the obtained results indicate that, under the conditions tested, the co-ordination between DNA replication and sporulation is not significantly affected in oriCmut cells when tested in a wild-type, or in a single Δsda or ΔsirA mutant background.

However, the co-ordination between DNA replication and sporulation was significantly affected in cells containing oriCmut when this was tested in a Δsda/ΔsirA double-mutant background. The average origin copy number in mother cells was 1.48 and 1.26 (n > 430 cells; P < 0.001, Mann–Whitney U-test) in the case of strains IDJ082 (oriCmut) and IDJ081 (oriCmut) respectively (Fig. 5E and F).

These results demonstrate that the presence of bona fide Spo0A binding sites in the oriC region contributes to proper co-ordination of DNA replication and sporulation. In addition, the results show that, at least under the conditions tested, sda is the locus that mostly contributes to proper co-ordination between replication and sporulation since no significant defect in this co-ordination is observed in a sirA or oriCmut strain as long as sda is not affected.

Induction of sporulation in actively replicating cells leads to increased chromosome copy numbers in oriCmut strains

In the previous section, the effect of oriCmut was tested under conditions in which sporulation is rather gradually induced upon starvation and high cell density. However, in nature the growth conditions can change rapidly, and apparently redundant mechanisms may contribute differently under different conditions, as also suggested by Xenopoulos and Piggott (2011). Therefore, we examined functionality of Spo0A binding sites within oriC when sporulation was induced in actively growing and replicating cells, which was achieved by conditionally inducing kinA from an ectopic locus. KinA is the major sporulation kinase and induction of KinA in exponentially growing and actively replicating cells initiates sporulation by gradually increasing the intracellular levels of Spo0A–P (Fujita and Losick, 2005). The kinA gene was placed under control of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter

Fig. 5. Cells carrying the mutated oriC (oriCmut) over-initiate replication in the absence of both the SirA and the Sda checkpoints when sporulation is induced in slowly growing cells. Replication initiation events are monitored by fluorescence microscopy of sporulating cultures of strains harbouring a xylose-inducible tetR-mCherry fusion and tet operator sites near the oriC. A. Example of cells with different numbers of oriC-mCherry foci representing variations in replication initiation events (arrows). B–E. Number of oriC-mCherry foci in mother cells of cultures grown in Schaeffer medium (number of cells in %). Error bars represent standard errors of the mean. (B) Wild type (IDJ075, oriCmut; IDJ076, oriCmut), (C) Δsda (IDJ077, oriCmut; IDJ078, oriCmut), (D) ΔsirA (IDJ079, oriCmut; IDJ080, oriCmut), (E) and Δsda/sirA double mutant (IDJ081, oriCmut; IDJ082, oriCmut). F. Comparison of the average numbers of mCherry foci per cell counted for wild type (IDJ075, IDJ076), Δsda (IDJ077, IDJ078), ΔsirA (IDJ079, IDJ080) and Δsda/sirA double mutant mother cells (IDJ081, IDJ082) with oriCmut and oriCmut genetic backgrounds respectively. Significant differences (P < 0.001), according to a Mann–Whitney U-test, are marked with an asterisk.
Pspank, and the resulting construct was introduced into the oriCnat and oriCmut TetR-mCherry strains. In addition, Pspank-kinA in the oriCmut background was combined with single or double mutation in sda and sirA. The resulting strains, MB003–MB010, were grown in Schaeffer’s sporulation medium containing 1 mM IPTG and samples were taken for fluorescence microscopy during exponential growth and during sporulation when phase bright forespores became visible. Strikingly, independent of the genetic background tested, induction of sporulation in exponentially growing cells caused a significant increase in fluorescent foci when they contained oriCmut (P < 0.005 Mann–Whitney U-test, n > 470, in all cases tested). Differences were observed both in exponentially growing cells and in mother cells during sporulation (Figs 6 and 7 and Table 1). These results show that initiation of sporulation in actively replicating cells significantly affects chromosome copy number when the OA-boxes are removed, even in the presence of the SirA and Sda checkpoints.

It is interesting to note that under these conditions the defects in proper copy number regulation in cells containing oriCmut becomes particularly evident in the ΔsirA mutant background (on average 1.07 versus 1.45 oriC foci per mother cell in ΔsirA/Pspank-kinA; Table 1). Whereas the results presented above showed that Sda was most prominent in the proper control of mother cell copy number when sporulation is induced as a consequence of nutrient starvation and high cell density, the latter results indicate that SirA plays a particularly important role in DNA replication control when sporulation is initiated in actively growing cells, as was shown previously (Wagner et al., 2009).

To determine whether the effect of kinA overexpression on oriC copy numbers is indeed the result of increased levels of Spo0A–P, spo0A was deleted in the oriCnat (MB003) and oriCmut (MB004) Pspank-kinA strains. Cultures were induced with 1 mM IPTG upon inoculation in Schaeffer’s sporulation medium and samples were taken only during mid-exponential growth, since cells lacking spo0A do not sporulate (Hoch, 1976). We found no significant difference between the oriCnat and oriCmut Pspank-kinA strains in the absence of spo0A (Fig. 8), demonstrating that the effect of KinA overproduction on replication depends on Spo0A–P and is not an artefact of kinA overexpression.

In summary, our data provide strong evidence that, especially when sporulation is induced in fast-growing cells, Spo0A–P contributes significantly in the control of

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Fig. 6. Initiation of sporulation by kinA induction in fast-growing cells results in an increased chromosome copy number when Spo0A binding at oriC is reduced in vegetative cells. Cells were grown in the presence of 1 mM IPTG and harvested for fluorescence microscopy analysis at early-exponential growth after 3 h of induction (from the start of inoculation).

A. Wild type (MB003, Pspank-kinA, oriCnat; MB004, Pspank-kinA, oriCmut).
B. ∆sda (MB005, Pspank-kinA, oriCnat; MB006, Pspank-kinA, oriCmut).
C. ∆sirA (MB007, Pspank-kinA, oriCnat; MB008, Pspank-kinA, oriCmut).

The error bars represent the standard deviation. The difference in oriC copy number is significant for all strains (Mann–Whitney U-test) (Table 1). Pie charts show the fraction of cells with a certain number of origins.
DNA replication initiation and hence proper co-ordination with sporulation by binding to its cognate binding sites in the oriC region.

Discussion

Spo0A, the master regulator of sporulation, has been shown to indirectly inhibit DNA replication by regulating genes involved in replication and cell division (Molle et al., 2003; Rahn-Lee et al., 2009; 2011; Wagner et al., 2009). Whether Spo0A also directly regulates DNA replication of B. subtilis in vivo is debated in the literature (see, e.g. Molle et al., 2003; Castilla-Llorente et al., 2006; Wagner et al., 2009; Xenopoulos and Piggot, 2011). For instance, induction of a constitutively active allele of Spo0A (called Spo0A-sad67; Ireton et al., 1993) in the B. subtilis PY79 genetic background showed no downregulation of initiation of DNA replication in the absence of SirA, indicating that SirA rather than Spo0A is the major factor controlling DNA replication under these experimental conditions (Wagner et al., 2009). However, Spo0A-sad67 is highly toxic to the cells (Ireton et al., 1993) and the sad67 allele was also shown to be unstable in the presence of a

**Table 1.** The fraction of cells with abnormal chromosome copy numbers increases in the absence of specific Spo0A binding sites at oriC.

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<sup>*</sup>Significance was calculated using a Mann–Whitney U-test.

(A) Number of oriC foci in exponentially growing or in mother cells (B) of cultures induced to produce KinA.
DnaX–YFP fusion indicating pleiotropic consequences of its induction (Wagner et al., 2009). Furthermore, as shown here, the growth conditions and timing of sporulation initiation relative to replication status appear to be crucial to observe the direct effects of Spo0A–P on chromosome copy number (Figs 5–7).

The oriC of B. subtilis harbours a number of bona fide Spo0A binding sites that overlap with functional DnaA binding sites (Fig. 2, blue), and binding of Spo0A to these sequences has been shown in vitro (Castilla-Llorente et al., 2006). In addition, Castilla-Llorente et al. showed that Spo0A can inhibit DnaA-mediated open complex formation in vitro (Castilla-Llorente et al., 2006). Interestingly, they also showed that Spo0A binds the origins of the virulent B. subtilis phage Φ29 in vivo, thereby preventing amplification of Φ29 DNA after infection which forces the phage genome to remain dormant in cells with active Spo0A (Castilla-Llorente et al., 2006). In Φ29, binding of Spo0A–P prevents formation of the replication initiation nucleoprotein complex by preventing and displacing the replication initiator protein p6 from the origins (Castilla-Llorente et al., 2006). Inspired by these findings, we examined whether B. subtilis Spo0A–P also affects DNA replication in vivo by constructing strains that lack the particular 0A-boxes to which Spo0A–P binds with high affinity within the oriC region (Fig. 2C and Castilla-Llorente et al., 2006). Electrophoretic mobility shift assays confirmed that Spo0A binds less efficiently to our mutated oriCmut compared with the native oriC in vitro, while DnaA still binds at near wild-type efficiency (Figs 3 and 4).

To investigate a possible direct effect of Spo0A on replication in vivo, we monitored origin copy number by means of imaging TetR-mCherry foci that localize near the oriC (Fig. 5A). Interestingly, we found that the contribution of Spo0A–P in proper co-ordination of replication and sporulation varies upon the growth conditions that induce sporulation. A rather small effect of oriC-located Spo0A binding sites on DNA replication control was observed when sporulation was induced in cells in which growth has been slowed down, which happens during nutrient depletion in Schaeffers medium at the end of the exponential growth phase. Under these conditions, the effect was only significant in the Δsda/ΔsirA double mutant background (Fig. 5E). These results imply that under these conditions, both SirA and Sda are sufficient to ensure proper regulation of DNA replication in sporulating cells. The observation that proper control of replication is lost in Δsda, but not ΔsirA single mutant cells indicates that Sda is the most important locus for co-ordinating replication and sporulation under these conditions.

Strikingly, the role of Spo0A–P in properly co-ordinating DNA replication and sporulation is much more pronounced when rapidly growing cells were suddenly induced to sporulate. Under these conditions, oriCmut has significant effects on the regulation of DNA replication even in an otherwise wild-type background (Figs 6 and 7 and Table 1). Interestingly, whereas Sda turned out to be most important when sporulation was induced in cells having slowed down their growth, SirA appeared most important when sporulation was induced in rapidly growing cells in the oriCmut background.

The fraction of cells with increased numbers of oriC foci in wild-type mother cells is only slightly (but significantly) elevated in the absence of SirA upon KinA induction in Schaeffers medium in our genetic JH642 background (Table 1). Wagner et al. reported a much larger impact of the absence of SirA when KinA was induced using the Phyper-spank promoter (which is stronger than the here used Pspank) in LB medium in the PY79 genetic background, and more than 54% of sporulating cells had more than two foci (Wagner et al., 2009). Besides the genetic and experimental differences between the two studies, it should also be noted that chromosome copy number was assessed at an earlier stage of sporulation (when the asymmetric septum became visible using a membrane stain) thus also accounting for chromosomes destined for the forespore compartment, while we assessed only the chromosome copy number in the mother cell when the forespore was already phase bright.

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Importantly, deletion of spo0A in our strains that over-express kinA did not result in a significant difference between oriC<sup>ss</sup> and oriC<sup>md</sup>, indicating that the observed effect is not an artefact of KinA overproduction (Fig. 8). Together, our results suggest that Spo0A directly inhibits initiation of DNA replication by binding to the origin of replication. In addition, these findings may also explain the recently observed Spo0A-dependent inhibition of growth and DNA replication in mother cells (Xenopoulos and Piggot, 2011).

It is interesting to note that regulation of DNA replication by two-component response regulators, such as Spo0A, is not unique and might be a more common mechanism in bacteria. For instance, in the asymmetrically dividing α-proteobacterium Caulobacter crescentus, DNA replication is controlled by a two-component response regulator called CtrA to ensure correct coordination between DNA replication and cell differentiation. It has been proposed that CtrA competes with C. crescentus DnaA for binding to the origin and in that way regulates chromosomal replication (for a recent review see Collier, 2012). The mechanism by which B. subtilis Spo0A directly inhibits DNA replication still remains to be elucidated. It has been shown in vitro that Spo0A prevents DnaA-dependent open complex formation within incC (Castilla-Llorente et al., 2006). This might be caused either by directly impeding DnaA binding to the oriC sequences where Spo0A is bound, or by altering the ability of DnaA to assemble into its active ATP-dependent nucleoprotein complex. It is noteworthy to mention that the region of the origin with the highest DnaA affinity (in the incB region; Moriya et al., 1988; Fukuoka et al., 1990) also contains four consecutive OA-boxes, of which one is a perfect match to the consensus sequence. However, Krause et al. (1997) have indicated, using a plasmid system, that DnaA-dependent open complex formation can still take place in the absence of the incA and incB regions (Krause et al., 1997), thus, clearly more research is required to establish the underlying molecular mechanism.

In nature, cells encounter ever fluctuating conditions in which growth can be rapidly inhibited and thereby rapidly inducing sporulation. Consequently, we speculate that in their natural environment, B. subtilis cells encounter fitness disadvantages if the regulatory pathway of Spo0A binding to the oriC is defective since under rapidly changing conditions Spo0A plays a significant role in coordinating replication with sporulation (Figs 6 and 7, Table 1). Interestingly, even in the most extreme case investigated in this study (P<sub>spanA-kinA</sub> in the sda/sirA double mutant harbouring oriC<sup>md</sup>), 54% of cells were seemingly still able to correctly co-ordinate DNA replication with sporulation (Fig. 7D). This suggests that additional, yet unknown factors contribute to the co-ordination of DNA replication with sporulation. For B. subtilis it is known that the nutritional status also controls replication (Wang et al., 2007). This occurs by the production of the small nucleotides ppGpp and pppGpp upon nutrient starvation. These so-called ‘alarmones’ directly inhibit primase, an essential component of the replication machinery (Wang et al., 2007). This control might indirectly be responsible for correct copy number control during sporulation. Whether this or other mechanisms are at play remains to be investigated.

**Experimental procedures**

**Oligonucleotides, plasmids, strains and media**

Oligonucleotides, plasmids and bacterial strains are listed in Tables S1, S2 and S3 respectively. B. subtilis strains were grown in TY or Schaeffer medium at 37°C (see below). E. coli DH5α was used as host for cloning and grown in TY medium at 37°C. When required, the growth media were supplemented with antibiotics at the following concentrations: 5 μg ml<sup>−1</sup> chloramphenicol, 5 μg ml<sup>−1</sup> kanamycin, 100 μg ml<sup>−1</sup> spectinomycin, 6 μg ml<sup>−1</sup> tetracycline, 0.5 μg ml<sup>−1</sup> erythromycin + 12.5 μg ml<sup>−1</sup> lincomycin (MLS) for B. subtilis and 100 μg ml<sup>−1</sup> ampicillin or 150 μg ml<sup>−1</sup> erythromycin for E. coli. Agar (1.5%) was included for solid medium.

**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, GERM) and all PCRs were performed with Phusion (NEB, UK), unless stated otherwise. Oligonucleotides were purchased from Biologeo (Nijmegen, the Netherlands). All constructs were sequence-verified. B. subtilis was transformed as described before (Harwood and Cutting, 1990).

**Strain construction**

IDJ055, in which a chloramphenicol cassette is introduced between the rpmH gene and the dnaA promoter, was obtained as follows: the upstream region and downstream region of the oriC sequence of interest were amplified using B. subtilis 168 chromosomal DNA as a template in combination with prIDJ241 + prIDJ242 and prIDJ245 + prIDJ246. The chloramphenicol cassette was amplified from pUC19-c with prIDJ243 + prIDJ244 and inserted between the upstream and downstream region of the oriC via BamHI and NbH. Transformation of the ligation product to B. subtilis 168 resulted in strain IDJ055. IDJ056 was obtained as follows: the upstream region of the oriC region of interest and the chloramphenicol resistance cassette were amplified from IDJ055 with prIDJ233 + prIDJ243. Plasmid pIDJ070 harbouring the oriC region with the mutated 0A-boxes was synthesized by Mr Gene (Regensburg, Germany) and the 0A-boxes of interest were amplified from pIDJ070 with prIDJ245 and prIDJ274.
Both PCR fragments were ligated via NheI and transformed to *B. subtilis* 168 resulting in strain IDJ056. Strains IDJ075, IDJ077, IDJ079 and IDJ081 were obtained by transforming JWW212, JWW213, JWW214 and JWW215 with chromosomal DNA of IDJ056 and selecting for chloramphenicol resistance. Strains IDJ076, IDJ078, IDJ080 and IDJ082 were obtained by transforming JWW212, JWW213, JWW214 and JWW215 with chromosomal DNA of IDJ056 and selecting for chloramphenicol resistance. Correct integration was confirmed by PCR and integrated DNA was sequence verified in all cases.

To construct plasmid pMB001, which contains P*spank*-kinA and thrC flanking regions, the lacI-P*spank*-kinA genes were cut from plasmid pDR110-kinA (de Jong et al., 2010) using BamHI and EcoRI and ligated into the corresponding sites of pDG1664 (Guéroult-Fleury et al., 1996). Strains MB003, MB004, MB005, MB006, MB007, MB009 and MB010 were created by transformation of IDJ075, IDJ076, IDJ077, IDJ078, IDJ079, IDJ080, IDJ081 and IDJ082, respectively, with plasmid pMB001 and selecting for MLS resistance. Correct integration of P*spank*-kinA into the thrC locus was verified by PCR.

MB015 was created by amplification of the upstream region and downstream region of spo0A with primIDJ179 + prMB017 and primIDJ182 + prMB018, respectively, using chromosomal DNA of strain *B. subtilis* 168 as template. The up- and downstream regions of spo0A and pBEST309 (Itaya, 1992) were digested with BamHI and ligated. The ligation mixture was transformed directly to *B. subtilis* 168 and transformants were selected on tetracycline. Strains MB016 and MB017 were created by transformation with MB015 genomic DNA. MB017 was sequenced at the oriC region to confirm that the mutations remained unaltered.

**Spo0A and DnaA purification**

*Escherichia coli* BL21 containing either pMF14 (Molle et al., 2003) or pETspo0AHIS (Fujita and Losick, 2003) were grown at 30°C in LB medium and induced with 1 mM IPTG for overproduction of full-length Spo0A (Spo0A-FL) or the DNA-binding domain of Spo0A (Spo0A-DB) at OD600 = 0.6. Cells were harvested 5 h after induction (10,000 rcf, 10 min, RT) and the cell pellets were stored at −80°C. After resuspension of the pellet in wash buffer (300 mM NaCl, 50 mM NaH2PO4 pH 8.0, 10 mM imidazole), the cells were disrupted by sonication (30 s ON, 30 s OFF, 60%, 6 min) and the supernatant containing the protein of interest was separated from the cell lysate by centrifugation (10,000 rcf, 10 min, 4°C, three times). The supernatant was incubated with 2 ml of Ni-NTA superflow (resin, Qiagen) and 5 ml of lysis buffer [300 mM NaCl, 50 mM NaH2PO4 pH 8.0, 20 mM imidazole, 5 mM [β-mercaptoethanol, 2 mM phenylmethanesulphonylfuoride (PMSF)] for 1 h on ice before being loaded to the column. The column was washed with wash buffer and Spo0A was eluted in elution buffer (300 mM NaCl, 50 mM NaH2PO4 pH 8.0, 50–250 mM imidazole) with increasing concentrations of imidazole. All protein isolation steps were performed at 4°C. Elution fractions were loaded on a SDS-PAGE gel to check for presence and purity of Spo0A. Spo0A concentrations were determined with a Bradford assay using bovine serum albumin (BSA) as a standard. DnaA was purified as previously described (Scholefield et al., 2012).

**EMSAs**

Spo0A-FL and Spo0A-DB (as C-terminal His6 fusions) were used directly after purification without additional phosphorylation treatment. All primer sequences used for the PCR products are listed in Table S1. The incA region was amplified with primers prIDJ247 and prIDJ300; the incB region with prIDJ276 and prIDJ301; the *incA-incB* region with prIDJ247 and prIDJ276; P*spank* with prIDJ004 and prIDJ281; and P*ubr* with prIDJ282 and prIDJ283. The corresponding fragments were labelled with [γ-32P]-ATP using T4 polynucleotide kinase (Fermentas). Various protein concentrations were incubated at 30°C for 20 min in the following reaction mix: 5000 cpm labelled DNA, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 0.5 mg ml−1 BSA. Fifteen microlitres of the mix was loaded onto a native polyacrylamide gel [4% (v/v) polyacrylamide, 1× TBE buffer, 0.125% (w/v) APS, 0.0125% (v/v) TEMED] and electrophoresed in 1× TBE buffer for 50 min at 100 V. Radioactive signals were detected at room temperature by autoradiography using Cyclone PhosphorImager (Packard Instruments, Meridian, CT) and Opti-Quant analysis software (Packard Instruments, Meridian, CT) after overnight incubation on phosphorscreens.

The EMSAs with DnaA were performed as previously described (Scholefield et al., 2012). In brief, the DnaA protein was serially diluted in twofold steps from 12 μM to 94 nM and incubated with either ATP or ADP (2 mM). The *incAB* PCR product (474 bp) was created with primers oGJS61/oGJS62, purified using a Qiagen PCR purification kit, and utilized at 36 nM. Nucleoprotein complexes were separated on a 1.5% agarose gel and visualized by staining with ethidium bromide.

**RT-QPCR**

Strains MB003, MB004, MB009 and MB010 were grown in 10 ml of LB containing kanamycin (10 μg ml−1) at 37°C and 200 r.p.m. using 100 ml bottles (Schott). The cultures were diluted to OD600 = 0.05 in 40 ml of Schaeffer’s sporulation medium containing 5 μg ml−1 kanamycin and *kinA* was induced with 1 mM IPTG. Cultures were grown at 37°C and 225 r.p.m. using 250 ml conical shake flasks. Ten millilitres of samples were taken between OD600 of 0.3 and 0.4 and centrifuged for 10 min at 1677 g. Samples were flash frozen in liquid nitrogen and stored at −80°C. Samples were thawed and dissolved in 400 μl of Diethyl-pyrocarbonate (DEPC)-treated TE buffer (10 mM TRIS-HCl 1 mM EDTA, pH 7.5). Samples were added to a 2 ml screw-cap tube on ice containing 500 mg of glass beads (75–150 μm), 175 μl of Macaloid, 50 μl of 10% SDS and 500 μl of phenol/chloroform mixture (1:1). Samples were placed in a Biospec Minibeadbeater-8 and beaten 2× 1 min with a 1 min interval on ice. Samples were centrifuged at 10 600 g for 10 min at 4°C. The upper phase was added to 500 μl of chloroform and centrifuged for 5 min at 10 600 g, 4°C. Further purification was with the Roche High Pure RNA isolation kit using 1 ml of binding/lysis buffer. After purification, a second 1 h DNase incubation step was performed with DNase from the Roche kit, and the sample was purified a second time. For cDNA preparation, 2 μl of random nonamers (1.6 μg ml−1) were annealed to 10 μg of total RNA in a total volume of 18 μl for 5 min at 70°C. Next, 12 μl of mastermix (6 μl of 5× First
Strand buffer, 3 μl of 0.1 M DTT, 25× dNTP mix (12.5 mM each dNTP), 1.8 μl of Superscript III reverse transcriptase was added and samples were incubated for 16 h at 42°C. To degrade RNA, samples were treated with 3.5 μl of 2.5 M NaOH for 15 min at 37°C. Fifteen microlitres of 2 M HEPES free acid was added and samples were purified using a Nucleospin Gel and PCR clean up kit. RT-QPCR was performed on a Bio-Rad iQ5 PCR machine using Bio-Rad iQ supermix. QPCR was performed comparing MB003 with MB004 and MB009 and MB010 dnaA transcription levels. The housekeeping gene gyra was used as a reference gene. Samples were diluted to 20 ng μl⁻¹. Efficiency of the primers prMB022, prMB023, prMB024, prMB025 was experimentally determined to lie between 1.95 and 2.05. The relative gene expression was calculated using the Livak (2^ΔΔCt) method (Livak and Schmittgen, 2001).

Microscopy

oriC-mCherry foci (Fig. 5). Pre-cultures were inoculated from −80°C stocks and grown overnight at 37°C in 10 ml of LB medium at 225 r.p.m. using blue capped 100 ml bottles (Schott-Duran) allowing for good aeration. The following morning, the cultures were diluted to OD₆₀₀ = 0.05 in 10 ml of Schaeffer medium (Scheff er et al., 1965) and grown for 9 h at 37°C at a speed of 250 r.p.m. using 100 ml bottles (Schott-Duran). For better visualization of the oriC-mCherry foci, 0.05% xylose was added to IDJ077, IDJ078, IDJ081 and IDJ082 cultures at mid-exponential phase. Samples for microscopy were taken at OD₆₀₀ = 0.5 in Schaeffer’s sporulation medium containing 5 μg ml⁻¹ kanamycin. Cultures (10 ml in 100 ml bottles) for exponential sampling were inoculated at mid-exponential phase with 1 mM IPTG upon inoculation in Schaeffer’s sporulation medium for approximately 3 h. Samples for microscopy were taken during early exponential growth at OD₆₀₀ 0.3–0.4 and washed with 1× Spizizen salts. Cultures to be harvested during sporulation were inoculated at mid-exponential phase with 1 mM IPTG to induce kinA expression and with 0.5% xylose 30 min before harvesting to produce TetR-mCherry (total of approximately 4 h of induction). Samples for microscopy were taken when phase bright endospores became visible. The samples were washed with 1× Spizizen salts and spotted on a Spizizen salts slide containing 1% agarose to be examined by fluorescence microscopy as described above.

Adaptations for oriC-mCherry foci in Δspo0A strains (Fig. 8). Overnight cultures were grown in 10 ml of LB containing kanamycin (10 μg ml⁻¹) at 37°C and 200 r.p.m. using 100 ml bottles (Schott-Duran). The cultures were diluted to OD₆₀₀ = 0.05 in Schaeffer’s sporulation medium containing 5 μg ml⁻¹ kanamycin and kinA was induced with 1 mM IPTG. Samples for microscopy were taken at OD₆₀₀ = 0.6–0.7 and microscopy was performed as described above.

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References


**Supporting information**

Additional supporting information may be found in the online version of this article.