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Spo0A, the key transcriptional regulator for entrance into sporulation, is an inhibitor of DNA replication

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The transcription factor Spo0A is a master regulator for entry into sporulation in *Bacillus subtilis* and also regulates expression of the virulent *B. subtilis* phage ϕ29. Here, we describe a novel function for Spo0A, being an inhibitor of DNA replication of both, the ϕ29 genome and the *B. subtilis* chromosome. Binding of Spo0A near the ϕ29 DNA ends, constituting the two origins of replication of the linear ϕ29 genome, prevents formation of ϕ29 protein p6-nucleoprotein initiation complex resulting in inhibition of ϕ29 DNA replication. At the *B. subtilis oriC*, binding of Spo0A to specific sequences, which mostly coincide with DnaA-binding sites, prevents open complex formation. Thus, by binding to the origins of replication, Spo0A prevents the initiation step of DNA replication of either genome. The implications of this novel role of Spo0A for phage ϕ29 development and the bacterial chromosome replication during the onset of sporulation are discussed.

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Introduction

The Gram-positive bacterium *Bacillus subtilis* belongs to a large family of bacteria that respond to nutritional stress by forming highly resistant endospores that can remain dormant for huge periods of time before germinating to resume growth. The multistage sporulation process has been and is widely used as a model system to study cell development. These studies have made of sporulation one of the best understood examples of cellular development and differentiation (for a review see, Piggot and Losick, 2002; Errington, 2003). The master regulator for entry into sporulation is the response regulator Spo0A (Hoch, 1993). Once activated by phosphorylation, Spo0A binds to a DNA sequence containing a so-called '0A-box' (Strauch *et al*, 1990), where it exerts its role by acting as a transcriptional activator or repressor. Besides being required for the onset of sporulation, Spo0A is also involved in the transcriptional regulation of various other stationary phase processes. Spo0A influences the expression of 520 *B. subtilis* genes showing that it has indeed a profound effect on the global gene expression pattern of *B. subtilis* (Fawcett *et al*, 2000; Liu *et al*, 2003). Of these 520 genes, 121 are under the direct control of Spo0A. Several of these encode proteins that themselves are directly or indirectly involved in transcriptional regulation, explaining the global effect of Spo0A on transcription (Molle *et al*, 2003, and references therein). The levels of Spo0A protein and activity increase gradually during the early stages of sporulation (Fujita and Losick, 2005) and the progressive increase of activated Spo0A explains the temporal fashion by which the low- and high-threshold Spo0A-regulated genes (Fujita *et al*, 2005) are activated or repressed.

Spo0A has been subject to extensive mutational analysis, which, together with the recently resolved 3D structures of its phospho-receptor (Spo0AN) (Lewis *et al*, 1999) and effector (Spo0AC) domain (Lewis *et al*, 2000; Zhao *et al*, 2002), have led to major advances in the understanding of its DNA-binding and gene activation properties at the molecular level. Thus, a combination of genetic, biochemical, functional and structural studies has made of Spo0A one of the best-studied response regulators.

The genome of ϕ29 consists of a linear double-stranded DNA (dsDNA) with a terminal protein (TP) covalently linked at each 5′ end (see Figure 1A for a genetic and transcriptional map). Phage ϕ29 transcription is divided into an early and a late stage (for a review, see Rojo *et al*, 1998; Meijer *et al*, 2001). All late genes are clustered in a single, centrally located, operon that is transcribed from the late promoter A3. The early-expressed genes, encoding all proteins required for phage DNA replication as well as the transcriptional regulator protein p4, are present in two operons. One, located at the right side of the genome, is under the control of the C2 promoter, and the other, located at the left side, is expressed from two tandemly organized promoters named A2b and A2c.

The ϕ29 genome contains six perfect 0A boxes (5′-TGTCGAA-3′). Whereas one of these (0A-4) is present within gene 8.5, the other five 0A boxes are located in the vicinity of ϕ29 promoters. Three 0A boxes (0A-1, 0A-2, and 0A-3) are present in the intergenic A2c–A3 promoter region and two (0A-5 and 0A-6) are located upstream of promoter C2 (Figure 1A). Binding of Spo0A to sequences containing the 0A boxes in the A2c–A3 promoter region causes repression of the early A2b and A2c promoters and prevents p4-mediated activation of the late A3 promoter. Spo0A also represses the early C2 promoter (Meijer *et al*, 2005).

Here, we describe a novel function for Spo0A. We show that Spo0A functions *in vitro* as an inhibitor of DNA replication of both, the ϕ29 genome and the *B. subtilis* chromosome,

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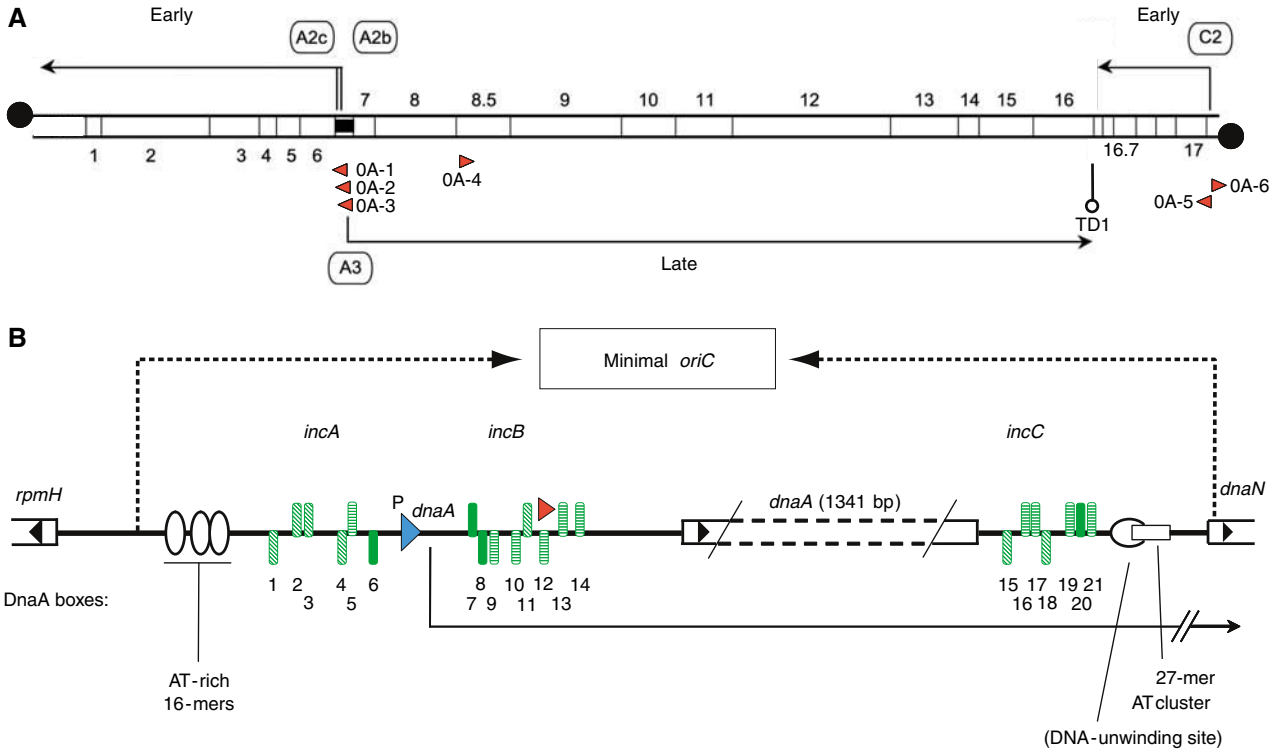

Figure 1 Physical, genetic and transcriptional maps of the ϕ29 genome and the *B. subtilis oriC* region. (**A**) Map of the ϕ29 genome. The direction of transcription and length of the transcripts are indicated by arrows. The positions of genes are indicated with numbers and those of the main early (A2c, A2b, C2) and late (A3) promoters are boxed. The positions and directionality of the perfect 0A-box sequences are indicated with red triangles. The divergently oriented 0A boxes 5 and 6 are separated by 55 bp. Note that imperfect 0A boxes are not indicated. The bidirectional transcriptional terminator TD1 is indicated with a hairpin structure. Black circles represent the terminal protein covalently attached to the 5′ DNA ends. A black box indicates the region spanning the early promoters A2b and A2c and the late A3 promoter. Adapted from Meijer *et al* (2005). (**B**) Map of the *B. subtilis oriC* region. Positions of perfect DnaA boxes (5′-TTATCCACA-3′; filled) and those having one (diagonal stripes) or two (horizontal stripes) mismatches are indicated by green rectangles and numbered 1–21. DnaA boxes on the upper and lower DNA strand are shown offset high or low, respectively. The *dnaA* upstream (*incA* and *incB*) and downstream (*incC*) clusters of DnaA boxes are indicated. The position of the single consensus 0A-box sequence is indicated with a red triangle. The white vertical ovals and the white horizontal oval represent the three AT-rich tandem repeats and the 27-mer AT cluster, respectively. The direction of transcription from the *dnaA* promoter (blue triangle) is indicated by a bent arrow. The divergently oriented *rpmH* and *dnaN* genes at the *oriC* borders are shown on the left and right side, respectively. Dashed bent arrows encompass the minimal *oriC* region.

by preventing the initiation step of DNA replication. Initiation of ϕ29 DNA replication occurs via a so-called protein-primed mechanism and starts at either side of the genome (reviewed by Salas, 1991, 1999). A schematic overview of the *in vitro* ϕ29 DNA replication mechanism is shown in Figure 2. DNA replication starts at either DNA end by recognition of the origins of replication, constituted by the TP-containing DNA ends, by a heterodimer formed by the ϕ29 DNA polymerase and TP. The essential ϕ29-encoded protein p6 functions as initiator of DNA replication by forming a nucleoprotein complex near the DNA ends required to activate *in vivo* ϕ29 DNA replication (Blanco *et al*, 1986; Serrano *et al*, 1994).

Initiation of the circular *B. subtilis* chromosome also requires the formation of an initiation complex. In this case, the host-encoded DNA-replication initiator protein DnaA binds to multiple (im)perfect DnaA boxes within the *oriC* region. The DnaA boxes are clustered in three regions, named *incA*, *incB* and *incC* (Moriya *et al*, 1988, see Figure 1B). All three DnaA box clusters are required for initiation of chromosomal replication (Moriya *et al*, 1992). DnaA bound to its cognate sites in the *incA* and *incB* regions interact with DnaA bound to the DnaA boxes in *incC* resulting in loop formation of the intervening region (reviewed by Moriya *et al*, 1999; Messer, 2002). This looped complex appears necessary for local unwinding of dsDNA close to the 27-mer AT cluster in *incC*, which provides entry for the DNA helicase and recruitment of additional factors to allow formation of a functional replisome.

Here we show that Spo0A binds specifically near both phage ϕ29 DNA ends, thereby preventing the formation of the p6–nucleoprotein complex and hence activation of ϕ29 DNA replication. Similarly, we show that Spo0A binds to sequences in the *incA* and *incB oriC* region of the *B. subtilis* chromosome and that Spo0A prevents DnaA-mediated *oriC* unwinding. Together, these results show for the first time that the well-studied transcriptional regulator protein Spo0A of *B. subtilis* acts directly as an inhibitor of DNA replication.

Results

Spo0A inhibits ϕ29 DNA replication

Phage ϕ29 0A box 6, which is divergently oriented with respect to 0A box 5, is located 50 bp from the right DNA end, which constitutes an origin of ϕ29 DNA replication. Binding of Spo0A to the 0A box 6 region might, therefore, interfere with phage ϕ29 DNA replication. Using two different

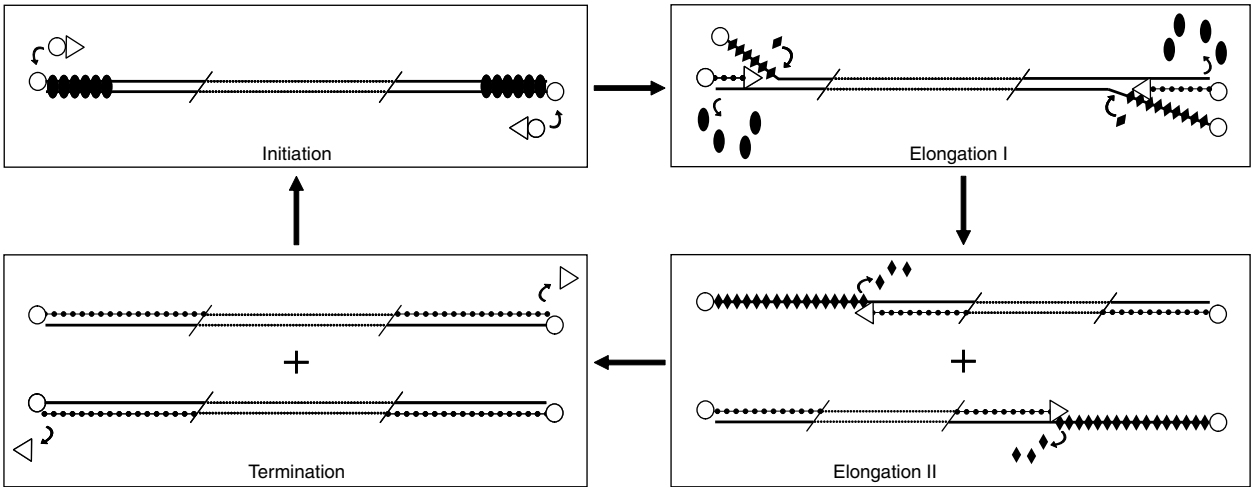

Figure 2 Schematic overview of *in vitro* ϕ29 DNA replication mechanism. Replication starts by recognition of the p6–nucleoprotein complexed origins of replication by a TP/DNA polymerase heterodimer. The DNA polymerase then catalyzes the addition of the first dAMP to the TP present in the heterodimer complex. Next, after a transition step (not shown), these two proteins dissociate and the DNA polymerase continues processive elongation until replication of the nascent DNA strand is completed. Replication is coupled to strand displacement. The ϕ29-encoded SSB protein p5 binds to the displaced ssDNA strands and is removed by the DNA polymerase during later stages in the replication process. Continuous polymerization results in the generation of two fully replicated ϕ29 genomes. Circles, TP; triangles, DNA polymerase; ovals, replication initiator protein p6; diamonds, SSB protein p5; *de novo* synthesized DNA is shown as beads on a string.

approaches, we studied whether Spo0A affects ϕ29 DNA replication *in vivo*. In the first approach, ϕ29 DNA replication was studied in wild-type *B. subtilis* 168 (168) and isogenic *spo0A*-deleted (168::Δ*spo0A*) cells infected under sporulation-inducing conditions. Thus, cells grown in Schaeffer's sporulation medium were infected with ϕ29 one hour after the end of logarithmical growth. The kinetics of ϕ29 DNA replication was then quantified by real-time PCR by determining the amount of intracellular ϕ29 DNA at different times after infection. The results presented in Figure 3A show that the level of intracellular ϕ29 DNA started to increase about 20 min after infection in 168::Δspo0A but not in wild-type cells. These results show that ϕ29 is able to replicate its DNA in stationary phase growing *spo0A* mutant cells, and indicate that ϕ29 DNA replication is inhibited in a *spo0A*-dependent way. In the second approach, ϕ29 DNA replication was analyzed in logarithmical phase cells with or without artificial induction of Spo0A. The activity of Spo0A is normally subject to complex regulatory pathways (for a review, see Hoch, 1993). To bypass the transcriptional circuits that control Spo0A expression and the requirement of the phosphorelay for its activation, we used for these experiments strain sad67::Tc in which a mutant *spo0A* gene, *spo0A–sad67*, is under the control of an IPTG inducible promoter (Veening *et al*, 2005). Spo0A–Sad67 does not require phosphorylation to be active (Ireton *et al*, 1993). Thus, real-time PCR was used to study *in vivo* ϕ29 DNA replication as a function of time using infected *B. subtilis* sad67::Tc cells grown in rich medium (LB) without or with Spo0A–Sad67 induction. As expected, ϕ29 DNA replication occurred in the absence of Spo0A–Sad67 induction. However, phage DNA replication was almost completely blocked in cells in which Spo0A–Sad67 was induced (Figure 3B).

Whereas these latter results confirm that ϕ29 DNA replication is inhibited in a Spo0A-dependent way, these approaches do not allow to distinguish whether Spo0A inhibits phage DNA replication directly or indirectly. This distinction can be made by *in vitro* approaches in which the input of all components is controlled. Thus, native Spo0A was purified from overexpressing *E. coli* cells as described before (Muchová *et al*, 2004). Spo0A forms dimers upon phosphorylation and Spo0A dimers constitute the active form of Spo0A (Asayama *et al*, 1995; Lewis *et al*, 2002; Ladds *et al*, 2003; Muchová *et al*, 2004). Ladds *et al* (2003) showed that a portion of wild-type Spo0A purified from *E. coli* is in its active phosphorylated dimeric form. Indeed, gel filtration experiments showed that 15–40% of our purified Spo0A

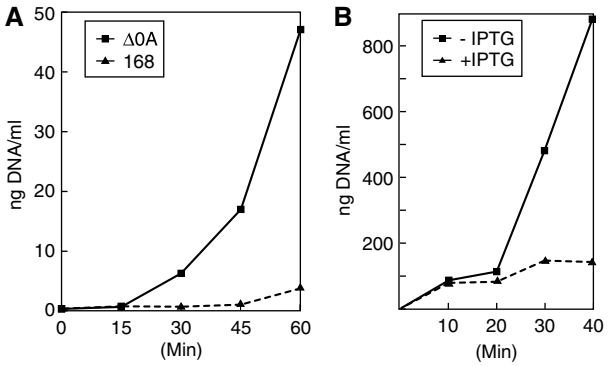

Figure 3 *In vivo* ϕ29 DNA replication is inhibited in a *spo0A*-depedent way. (**A**) Cultures of wild-type *B. subtilis* 168 (168) or the isogenic *spo0A*-deletion strain WM90 (Δ0A), grown in Schaeffer's sporulation medium, were infected with ϕ29 (multiplicity of 5) 1 h after entry into the stationary phase. At the indicated times after infection, the amount of viral DNA was measured by real-time PCR. Data are expressed as nanograms of ϕ29 DNA per ml of culture. (**B**) A culture of the *B. subtilis* sad67::Tc strain containing the *spo0A–sad67* gene under the control of an inducible IPTG promoter (Veening *et al*, 2005) was grown in LB medium. At the mid-log phase, the culture was split into two and in one of them Spo0A–Sad67 was induced. After 10 min, the cultures were infected with ϕ29 (multiplicity of 10). Next, at the indicated times after infection, the amount of viral DNA was measured by real-time PCR. Data are expressed as nanograms of ϕ29 DNA per ml of culture.

were dimers (not shown). The functionality of our purified Spo0A is demonstrated by the facts that (i) it produces highly similar footprints on the *B. subtilis* promoter *spoIIG*-associated 0A boxes to those published (Satola *et al*, 1992, not shown) and (ii) it has *in vitro* promoter activating and repressing activity (Meijer *et al*, 2005).

Thus, to study the possibility that Spo0A is directly responsible for the observed inhibition of ϕ29 DNA replication *in vivo*, we analyzed the effect of purified Spo0A in the *in vitro* ϕ29 DNA amplification system. This system, which allows the amplification of low amounts of ϕ29 DNA, requires four ϕ29-encoded proteins: DNA polymerase, TP, replication initiator protein p6, and the SSB protein p5 (Blanco *et al*, 1994, see Figure 2 for *in vitro* ϕ29 DNA replication scheme). Figure 4A shows that Spo0A directly inhibits ϕ29 DNA amplification; thus, more than 75% was inhibited in the presence of 5 μM Spo0A. As at most 40% of the Spo0A of the purified sample is in its active dimeric form, this inhibitory effect is exerted at a maximum concentration of 2 μM active Spo0A.

The observed Spo0A-mediated inhibition of ϕ29 DNA amplification is not due to non-specific binding of Spo0A to DNA because Spo0A did not affect the efficiency and/or velocity in M13 replication assays in which ϕ29 DNA polymerase drives continuous rounds of replication on primed circular M13 DNA (not shown).

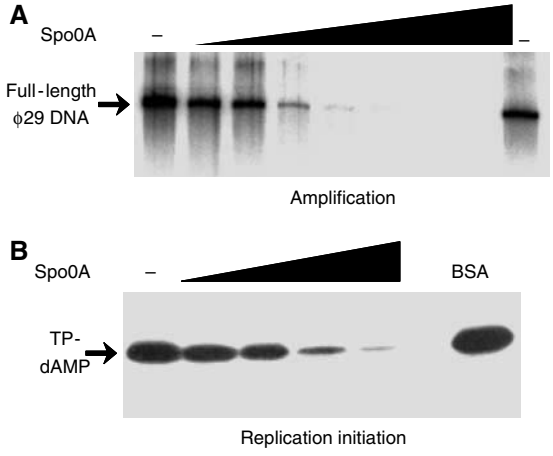

Figure 4 Spo0A inhibits *in vitro* ϕ29 DNA replication. (**A**) *In vitro* ϕ29 DNA amplification. Reaction mixtures contained ϕ29 DNA polymerase (6 nM), TP (6.5 nM), ϕ29 TP-DNA (32 pM), initiation protein p6 (33.3 μM), SSB protein p5 (24.0 μM), and no or increasing amounts of Spo0A. After incubation for 45 min at 30°C, reactions were stopped and subjected to alkaline agarose gel electrophoresis. The migration position of full-length ϕ29 DNA (19 285 bp) is indicated. Spo0A concentrations ranged from 1.25 to 80 μM (two-fold dilution steps). (**B**) *In vitro* protein-primed initiation of ϕ29 DNA replication (ϕ29 TP-dAMP formation). Protein-primed, TP-DNA-dependent replication initiation activity was measured as a function of Spo0A concentration. Reaction mixtures contained ϕ29 DNA polymerase (12 nM), TP (12.9 nM), initiation protein p6 (33.3 μM), ϕ29 TP-DNA template (1.6 nM), 0.1 μM [α-^{32}P]dATP (1 μCi), and no or increasing amounts of Spo0A. After incubation for 5 min at 30°C, the reactions were stopped, processed and analyzed by SDS–PAGE and autoradiography. The position of TP-dAMP initiation product is indicated. Spo0A concentrations ranged from 2 to 16 μM (two-fold dilution steps). Note that higher amounts of ϕ29 TP-DNA are used in initiation than in amplification assays.

Spo0A inhibits ϕ29 DNA replication at the initiation step

To study whether Spo0A exerts its inhibitory effect on ϕ29 DNA amplification at the initiation step, TP-DNA-directed *in vitro* ϕ29 DNA replication initiation assays were performed in the absence or presence of Spo0A. As efficient initiation of *in vitro* ϕ29 DNA replication requires the replication initiation protein p6, this protein was included in the reaction mixtures. Figure 4B shows that the initiation of TP-DNA replication is inhibited in a Spo0A-dependent way.

Low levels of TP-primed initiation activity can be obtained in the absence of protein p6 (Blanco *et al*, 1986). Under these conditions, Spo0A did not have a significant effect (not shown) indicating that Spo0A affects specifically the protein p6-mediated initiation of ϕ29 DNA replication (see also below).

Spo0A binds to the ϕ29 origins of replication in vitro, thereby preventing formation of a p6–nucleoprotein initiation complex

Phage ϕ29 DNA replication starts at either side of the genome. Spo0A almost fully prevented p6-stimulated initiation of DNA replication in reactions containing full-length ϕ29 DNA indicating that Spo0A affects the initiation reaction at either genome end. The possibility that Spo0A inhibits ϕ29 DNA replication by binding to the origins of replication was studied by DNase I footprinting using DNA fragments (202 bp) corresponding to the left or right side DNA sequences of the ϕ29 genome. Figure 5A and B show that Spo0A protects a specific region of about 45 bp near both DNA ends. Full protection of this region was observed using 1.8 μM of purified Spo0A. Thus, Spo0A binds at very similar positions near the left and right DNA ends. Interestingly, these Spo0A-protected DNA regions contain four adjacent 0A-box (like) sequences, each separated by a 3 bp spacer. Moreover, the positions of these boxes are located at exact equidistant positions from the extreme DNA ends (Figure 6).

Protein p6 binds preferentially at the ϕ29 DNA end regions, forming a specific nucleoprotein complex that highly stimulates ϕ29 DNA replication (Blanco *et al*, 1986; Serrano *et al*, 1989). We therefore studied whether binding of Spo0A at the ϕ29 origins prevents the formation of the p6–nucleoprotein complex. Lanes 8 of Figure 5A and B show the typical footprint generated by binding of protein p6 at the DNA ends (Serrano *et al*, 1989). However, the p6-induced footprint became lost when Spo0A bound to the regions near the DNA ends (Figure 5A and B, lanes 9 and 10), indicating that Spo0A prevents formation of the p6–nucleoprotein complex. Note that protein p6 generates a footprint that extends all along the DNA fragment used (Figure 5A and B, lanes 8). Binding of Spo0A to its confined sequences not only prevents p6 from binding to this region but also to sequences outside the Spo0A-binding region. Very similar results were obtained when Spo0A was added to the reaction after formation of the p6–nucleoprotein complex was allowed to take place (not shown), demonstrating that Spo0A can also annihilate a pre-existing replication initiation complex.

Together, these results show that specific binding of Spo0A near the ϕ29 DNA ends inhibits initiation of phage DNA replication by preventing the formation of the replication-initiation p6–nucleoprotein complex.

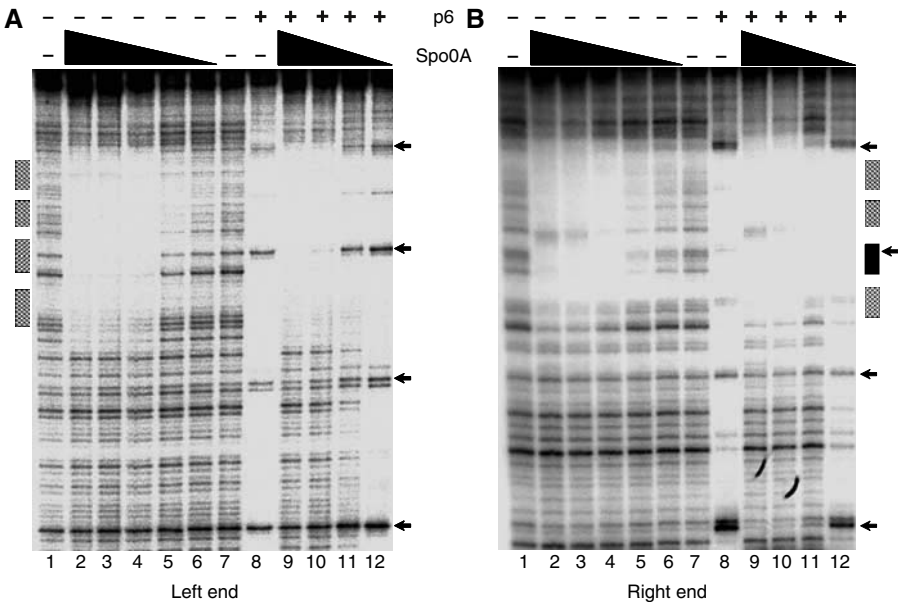

Figure 5 Spo0A prevents formation of p6–nucleoprotein initiation complexes at the ϕ29 origins of replication. Binding of Spo0A without (left panels) or with (right panels) the initiator protein p6 to the left (**A**) and right (**B**) ϕ29 origins of replication was analyzed by DNase I footprinting. DNA fragments corresponding to the 202 bp DNA ends of ϕ29, labeled at their 5′ ends, were incubated with the proteins as shown above the footprints. When indicated, 1 μg of initiator protein p6 was added 10 min after Spo0A addition. Spo0A concentrations ranged from 116 nM to 29.7 μM and 464 nM to 29.7 μM (four-fold dilution steps) in the left and right panels, respectively. Hatched boxes indicate the positions of imperfect 0A boxes. The single filled box indicates the position of the perfect 0A box sequence at the right ϕ29 origin (corresponding to 0A-6 in Figure 1A). Protein p6-induced hypersensitive sites are indicated with arrows. The top of the footprints correspond to the ϕ29 DNA ends.

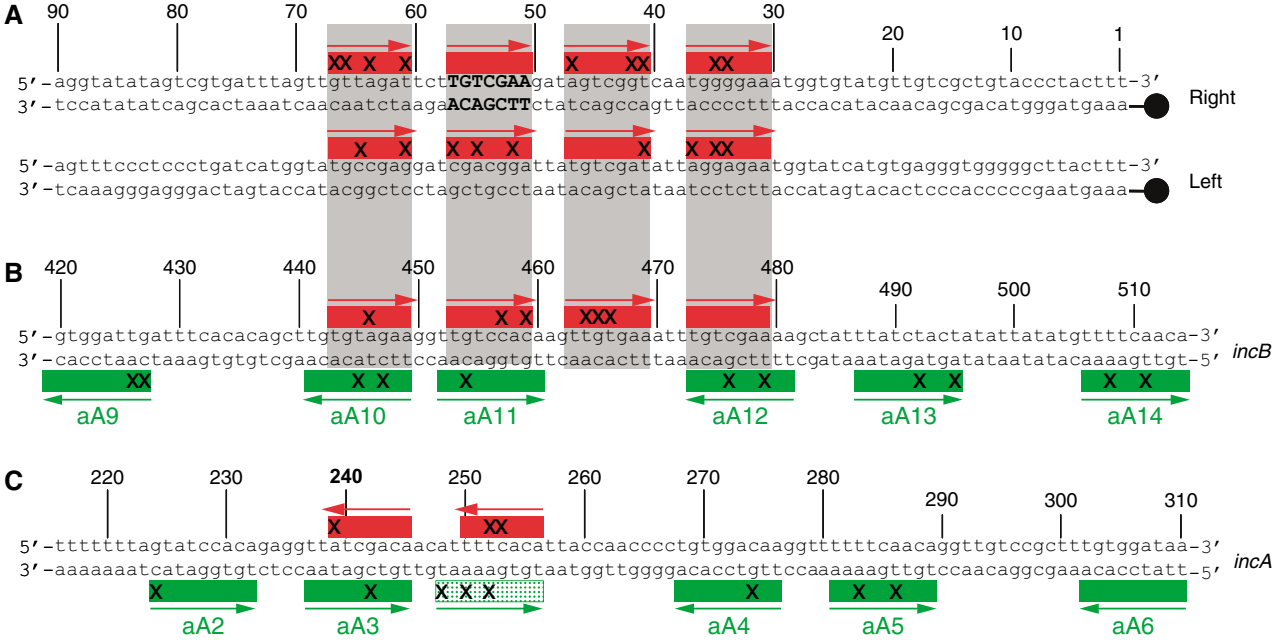

Figure 6 Organization of 0A boxes at the origins of replication of ϕ29 and the *B. subtilis* chromosome. The positions of 0A and DnaA boxes are indicated with red and green rectangles above and below the sequence, respectively. Mismatches with respect to their consensus sequence are indicated with crosses. (**A**) Right and left DNA sequences of the ϕ29 genome are shown in the upper and lower panel, respectively. Black circles represent TP bound to the 5′ DNA ends. The perfect 0A box at the right ϕ29 genome corresponds to 0A-6 in Figure 1A. (**B**) Sequence of the *incB* region of *B. subtilis oriC* containing the perfect 0A box. Note (i) that the organization of the 0A boxes is similar to that at the ϕ29 origins, (ii) that three 0A boxes coincide with imperfect DnaA boxes, and (iii) that the mismatches in the DnaA boxes result in (im)perfect 0A boxes (see text for details). (**C**) Sequence of the *incA* region of *B. subtilis oriC* containing two imperfect 0A boxes that coincide with imperfect DnaA boxes. Note that the stippled DnaA box containing three mismatches is not indicated in Figure 1. Numbering in (B) and (C) are according to Moriya *et al* (1992).

Spo0A binds to sequences within the B. subtilis oriC region

A perfect 0A box is located in the *incB oriC* region and, as assessed by gel mobility shift assays, the C-terminal DNA-binding domain of Spo0A, Spo0AC, binds to the *incB* region (Molle *et al*, 2003). In addition, the 0A box sequence (5′-TGTCGAA-3′) shares homology with the complementary strand of the perfect DnaA box sequence (5′-TGTGGATAA-3′). As Spo0A can bind to some imperfect 0A boxes, it might bind to DnaA boxes. If this were the case, Spo0A might affect initiation of replication of the *B. subtilis* chromosome.

DNase I footprinting was used to study possible binding of Spo0A to the *incA*, *incB*, and *incC* regions of *oriC*. Spo0A did not bind to the *incC* region (not shown). However, it did bind to sequences in the *incB* and *incA* regions (Figure 7A and B, respectively). In the case of the *incB* region, ~30 bp including the perfect 0A box was protected at low Spo0A concentrations (0.46 μM). At higher Spo0A concentrations, the footprint was enlarged by ~20 bp towards the 5′ direction. Indeed, the extended Spo0A-protected region contains, besides the perfect 0A box, three imperfect 0A boxes. These four 0A boxes have the same orientation and are all separated by a 3 bp spacer (see Figure 6B). Thus, the organization of the 0A boxes in this region is highly similar to that found at the ϕ29 origins of replication.

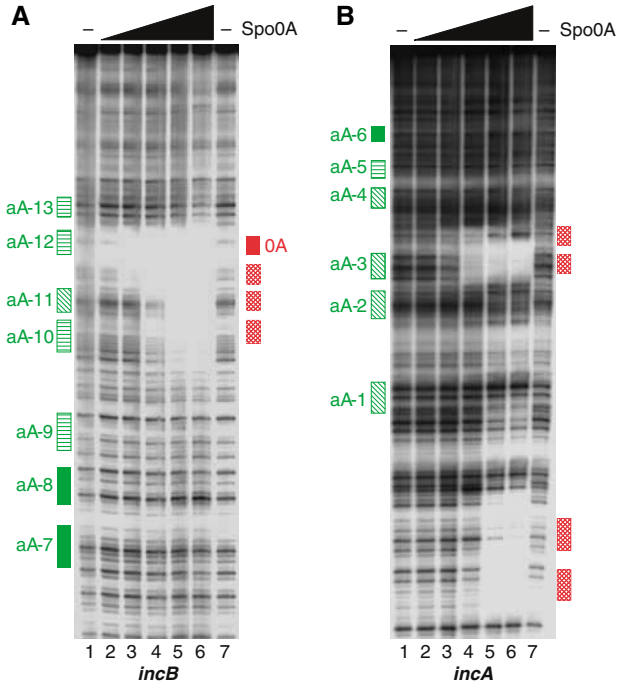

Figure 7 Spo0A binds to sequences in the *B. subtilis oriC incB* and *incA* regions. Binding of Spo0A to the *incB* (**A**) or *incA* (**B**) region of *B. subtilis oriC* was analyzed by DNase I footprinting. DNA fragments, containing *oriC* positions 314 till 626 (A) and 86 till 387 (B) according to Moriya *et al* (1992) and labeled at their 5′ upper strand, were incubated without or with increasing amounts of Spo0A before DNase I digestion. Filled and hatched red boxes indicate the positions of the perfect and imperfect 0A boxes, respectively. Green boxes indicate the positions of the DnaA boxes. The numbering and coloring scheme of Figure 1B is used to indicate perfect or imperfect DnaA boxes. Spo0A concentration ranged from 116 nM to 29.7 μM (four-fold dilution steps).

As shown in Figure 6B, three of the four 0A boxes coincide with imperfect DnaA boxes, which constitute *bona fide* binding sites for DnaA (Fukuoka *et al*, 1990). The perfect 0A box (5′-TGTCGAA-3′) corresponds to an imperfect DnaA box having two mismatches (5′-TTtTCgACA-3′) with respect to its consensus sequence (5′-TTATCCACA-3′). Interestingly, these two deviations result in the perfect 0A box sequence. A similar situation occurs for the other two upstream DnaA boxes that are protected by Spo0A; that is, the single deviation in the first upstream DnaA box (5′-TTgTCCACA-3′) and the two deviations in the second upstream DnaA box (5′-TTcTaCACA-3′) create 0A boxes that have two and one mismatches with respect to the consensus 0A box sequence, respectively. Thus, binding of Spo0A to this extended region overlaps with three functional DnaA-binding sites.

At *incA*, Spo0A protected two separate regions. The downstream protected region contains two imperfect 0A boxes (one and two mismatches) that are separated by a 4 bp spacer. Also in this case, the 0A boxes coincide with imperfect DnaA boxes (see Figure 6C). The upstream *incA* region on which Spo0A produced a footprint corresponds to the 45 bp spacer that separates the three 16-mer AT-rich tandem repeats from the *incA* DnaA cluster. Whereas Spo0A produced a clear footprint at the upstream half of this region, the downstream half became only partially protected even at high Spo0A concentrations. Inspection of this 45 bp sequence revealed that the well-protected upstream half contains two heptamers, separated by a 3 bp spacer, whose sequences have three mismatches with respect to the consensus 0A box sequence.

In summary, Spo0A binds to sequences present in the *incA* and *incB oriC* regions and, except for those in the 45 bp sequence downstream of the three 16-mer AT-rich tandem repeats, the Spo0A-binding sites overlap with functional DnaA-binding sites.

Spo0A prevents DnaA-mediated unwinding of oriC

The following approach was used to analyze whether Spo0A affects the initiation of DNA replication at *oriC*. Binding of DnaA to its cognate binding sites is required for initiation of DNA replication of the *B. subtilis* chromosome (Moriya *et al*, 1990). This binding causes local unwinding at or near an AT-rich region, which is a crucial step in the initiation of DNA replication. Krause *et al* (1997) precisely mapped the DnaA-mediated open complex to a short region that includes the left part of the 27-mer AT cluster and its 15 upstream bp (see Figure 1B).

We used the system developed by Krause *et al* (1997) to study whether Spo0A interferes with DnaA-mediated open complex formation (see Materials and methods). In brief, purified DnaA was added without or with increasing amounts of Spo0A to supercoiled plasmid pBsoriC4, which carries the entire *B. subtilis oriC* region. Samples were then treated with potassium permanganate to oxidize any unpaired pyrimidines. Next, after linearization and purification of the DNA, primer extension was used to probe for the level of open complex formation at *incC*. As expected, DnaA-dependent open complex formation at positions identical to those described before (Krause *et al*, 1997) was observed in the absence of Spo0A (lanes 3 of Figure 8A and B). Open complex formation was progressively inhibited, however, in the presence of increasing amounts of Spo0A (lanes 4–8 of Figure 8A and B); ~50% inhibition being obtained at 2 μM. This

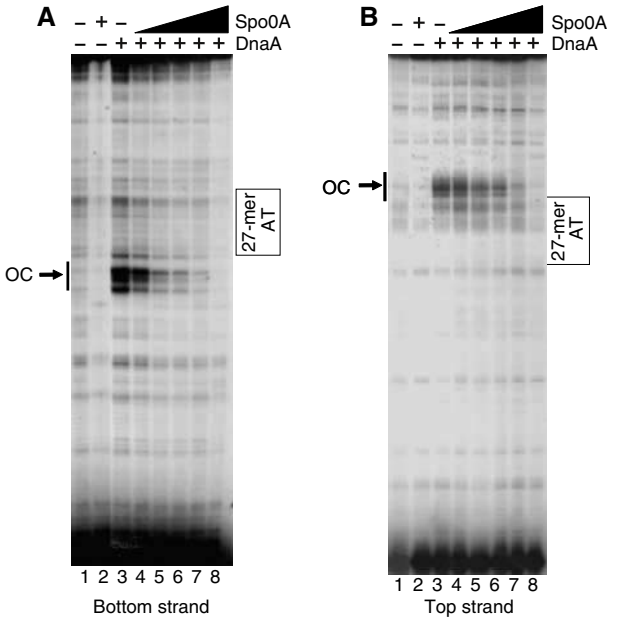

Figure 8 Spo0A inhibits DnaA-mediated open complex formation at *B. subtilis oriC*. DnaA-mediated open complex formation was probed by $KMnO_4$ footprinting on supercoiled pBsoriC4 plasmid in the presence of 100 ng HBsu protein as detailed in Materials and methods. The $KMnO_4$-sensitive positions in the bottom (**A**) and top (**B**) strand, and that of the 27-mer AT cluster are indicated. When indicated, fixed amounts of DnaA (262 nM) were used. Spo0A concentrations ranged from 0.48 to 7.9 μM (two-fold dilution steps). Quantification of three independent experiments revealed that the level of open complex formation was inhibited more than 70% in the presence of 1.98 μM of Spo0A. Taking into account that at most 40% of the Spo0A present in the purified protein sample was in its dimeric active form, this inhibitory effect is caused at a maximum concentration of 0.79 μM of active Spo0A. Lanes 2 contained 7.9 μM Spo0A.

indicates that Spo0A blocks the initiation of *B. subtilis* chromosomal DNA replication by preventing DnaA-mediated open complex formation at the *incC* region.

Discussion

Besides its extended effect on expression of the *B. subtilis* genome, the response regulator Spo0A also affects transcription of the virulent phage ϕ29 (Meijer *et al*, 2005). Here, we describe a novel function for Spo0A, that is, being an inhibitor of DNA replication of the ϕ29 genome as well as that of the *B. subtilis* chromosome. At both genomes, Spo0A exerts its *in vitro* inhibitory effect at the initiation step of DNA replication by binding to the origins of replication.

Phage ϕ29 is classified as a virulent phage and is optimized to complete its lytic cycle during the logarithmical phase of the infected cell. Indeed, the ϕ29 promoters are recognized by the host-encoded RNA polymerase containing the vegetative sigma factor A, and, when infected during this phase, the ϕ29 lytic cycle is completed in about 50 min generating up to 1000 phage progeny. However, cells initiating sporulation in response to nutrient limitation are not well suited for the production of phage progeny. Phage ϕ29, as well as other Bacillus phages, possesses the ability to adapt its infection strategy under these conditions by repressing its lytic cycle and arranging that its genome becomes trapped into the highly resistant spore. This strategy allows the phage to postpone its lytic cycle until spore germination occurs, when conditions have turned favorable for the production of phage progeny (reviewed by Sonenshein, 2006). Previously, it has been shown that, contrary to *spo0A* mutant strains, ϕ29 does not form large plaques on the wild-type *B. subtilis* strain 168, demonstrating that the lytic cycle of ϕ29 is suppressed in a *spo0A*-dependent way (Ito and Spizizen, 1972; Meijer *et al*, 2005). Using two different approaches we have extended this finding and show here that Spo0A inhibits *in vivo* ϕ29 DNA replication. Thus, on the one hand, we showed that, contrary to wild-type strain, ϕ29 is able to replicate its DNA in a *spo0A*-deletion strain grown under sporulation-inducing conditions, and, on the other hand, we showed that ϕ29 DNA replication was almost completely blocked in logarithmically growing cells in which the constitutively active Spo0A–Sad67 mutant was induced (see Figure 3). Thus, repression of the ϕ29 lytic life cycle is effectively coupled to the onset of sporulation by Spo0A.

Kawamura and Ito (1974) found (i) that the overall ϕ29 transcription decreased gradually during sporulation progression and (ii) that ϕ29 DNA replication stopped abruptly once infected cells had entered sporulation. These results suggest that replication is inhibited at the level of both phage DNA replication and transcription; the latter by repressing the genes encoding the phage replication proteins. The facts that (i) unphosphorylated Spo0A is unable to bind DNA (Ladds *et al*, 2003, see below), and (ii) that ϕ29 produces large plaques on a *spo0B* mutant strain, which is blocked in Spo0A phosphorylation (Meijer *et al*, 2005), imply that the activated phosphorylated form of Spo0A is responsible for inhibition of ϕ29 DNA replication.

The *in vivo* results do not allow to distinguish, however, whether Spo0A is directly responsible for these inhibiting effects or whether they are due to other protein(s) that are under the control of Spo0A. This distinction can be made by *in vitro* experiments. Recently, we demonstrated that Spo0A directly represses all three main early ϕ29 promoters and that it also prevents activation of the single late promoter and thus is at least in part responsible for the *spo0A*-mediated suppression of *in vivo* ϕ29 development (Meijer *et al*, 2005). Here we show that Spo0A directly prevents *in vitro* ϕ29 DNA replication by preventing the initiation step of DNA replication.

In the studies presented here, we used the Spo0A expression and purification protocol described by Ladds *et al* (2003), who showed that a portion of wild-type Spo0A is functionally phosphorylated when purified from Spo0A-overexpressing *E. coli* cells. Conclusive evidence has been presented that Spo0A forms dimers upon phosphorylation and that dimers constitute the DNA-binding and transcription-regulating active form of Spo0A (Asayama *et al*, 1995; Lewis *et al*, 2002; Ladds *et al*, 2003; Muchová *et al*, 2004). Moreover, contrary to what was observed with the constitutively active Spo0A–Sad67, artificial high-level induction of wild-type Spo0A in logarithmical growing cells, conditions when the Spo0A phosphorelay cascade is not active, did not activate the *spoIIG* promoter (Fujita and Losick, 2005). Similar to the results described by Ladds *et al* (2003), we found that a fraction (15–40%) of our purified Spo0A protein was in its active dimeric form. As the product of the *spo0A–sad67* gene is constitutively active *in vivo* (Ireton *et al*, 1993), it is expected that Spo0A–Sad67 forms dimers in solution and

hence, that it displays its replication-inhibiting effects at lower concentrations than those observed with the wild-type Spo0A protein purified from *E. coli*. We have purified Spo0A–Sad67 and analyzed its effect in ϕ29 DNA amplification assays. Indeed, Spo0A–Sad67 is dimeric in solution and it exerted its inhibiting effects on ϕ29 DNA replication at about 10-fold lower concentrations as compared with wild-type Spo0A (our unpublished results). Altogether, these data show that the DNA-binding and replication initiation-inhibiting effects observed in the experiments described in this and our previous work (Meijer *et al*, 2005) is exerted by the phosphorylated dimers present in the purified Spo0A samples which correspond to the *in vivo* active form of Spo0A generated in wild-type cells starting at the onset of sporulation in response to nutrient limitation.

Here we showed that Spo0A binds specifically to a ~45 bp DNA region at both ϕ29 DNA ends and that either region contains four (im)perfect 0A boxes that are all separated from each other by 3 bp spacers (see Figure 6A). Tandem 0A boxes are present at the *B. subtilis abrB* and *skf* promoters (Strauch *et al*, 1990; Chen *et al*, 2006) and the ϕ29 promoters (Meijer *et al*, 2005). Also, the 0A boxes at the *B. subtilis incB* region have this organization (see Figure 6B). In these cases, the 0A boxes are spaced exactly one helical turn from each other and hence are on the same face of the DNA helix, which would be ideal for the binding of a tandem dimer, which constitutes the active form of Spo0A (see above). Indeed, the DNA used for determination of the crystal structure of the Spo0AC–DNA complex contains dual 0A boxes separated by 3 bp spacers and Spo0AC binds these dual 0A boxes as tandem dimers that are located at the same face of the helix (Zhao *et al*, 2002).

We found that Spo0A can bind to specific sites within the *incA* and *incB* region. Interestingly, except for the ~40 bp region juxtaposed to the three 16-mer AT-rich repeats in *incA*, the Spo0A-binding sites overlap with imperfect DnaA boxes. We have also shown here that Spo0A inhibits open complex formation at *B. subtilis oriC*, which is a crucial step required for the initiation of bacterial chromosome replication.

The mode of DNA replication initiation at the *B. subtilis oriC* is assumed to be similar to that of the R6K plasmid and the Epstein–Barr virus (EBV) (reviewed in Moriya *et al*, 1999). As is the case for the *B. subtilis oriC*, binding of the replication initiator protein to two distant regions at origins in these genomes results in loop formation of the intervening region which is a necessary step for unwinding of the DNA at the actual site of DNA replication initiation (Frappier and O'Donnell, 1991; Su *et al*, 1991; Miron *et al*, 1992). There is a marked difference in the binding affinities of the replication protein for each of the two regions, the low affinity sites being located close to the actual DNA unwinding region in the genome of both R6K and EBV. Moreover, for both systems, it has been shown that binding of the replication initiator protein to the distant high-affinity sites stabilizes its binding to the weak binding sites through loop formation (Miron *et al*, 1992; Frappier *et al*, 1994). Interestingly, in *B. subtilis*, DnaA binds with a clear preference to the *incB* region (Fukuoka *et al*, 1990; Krause *et al*, 1997) and, when present on high multicopy plasmids, the *incB* region confers the highest level of incompatibility (Moriya *et al*, 1988). In analogy with the R6K and EBV origins, these data indicate that the *B. subtilis incB-incA* region functions as a replication enhancer by stabilizing binding of DnaA to the *incC* region permitting subsequent open complex formation at the flanking 27-mer AT cluster. Here we showed that Spo0A binds to sequences in *incA* and *incB* and that Spo0A inhibits open complex formation at the 27-mer AT cluster. It is therefore likely that Spo0A exerts its inhibitory effect on open complex formation by interfering with formation of functional DnaA–nucleoprotein complex at *oriC*. In keeping with the view that Spo0A inhibits initiation of chromosomal DNA replication is the fact that Spo0A binds *in vivo* to the *dnaA* upstream region (Molle *et al*, 2003).

What would be the biological function of Spo0A-mediated inhibition of chromosomal DNA replication *in vivo*? During the early stage of sporulation, the sporangium contains two, and only two, chromosomal copies. One of these is segregated into the forespore and the other to the mother cell (for a review, see Errington, 2001; Piggot and Losick, 2002). Thus, cells that have reached the stage to commit sporulation must not only complete a final round of DNA replication but also prevent a new round of replication from commencing. The following results suggest that Spo0A would be involved in preventing replication initiation at the onset of sporulation (Fujita and Losick, 2005). These authors showed that rapid and efficient induction of sporulation in logarithmical phase cells can be achieved by artificially activating the phosphorelay. As cells growing under these conditions are expected to have multiple replication forks, which would be incompatible with successful entry into sporulation, they suspected that Spo0A prevents new rounds of replication from commencing. Our results presented in this work provide evidence that Spo0A exerts its inhibitory effect on DNA replication initiation by preventing DnaA-mediated open complex formation. Thus, by activating the developmental program of sporulation and preventing new rounds of DNA replication from commencing, Spo0A would accurately couple both processes ensuring their tight coordination required for successful spore formation. A developmental checkpoint has been described that inhibits sporulation in response to conditions that perturb initiation of DNA replication (Burkholder *et al*, 2001). The checkpoint is mediated by Sda that specifically inhibits KinA and KinB, thereby preventing activation of Spo0A. Thus, Sda prevents initiation of sporulation but does not prevent novel rounds of DNA replication from commencing.

It is worth mentioning a remarkable similarity between Spo0A of *B. subtilis* and CtrA of the aquatic bacterium *Caulobacter crescentus*. This latter bacterium divides asymmetrically generating two distinct cell types at each cell division: a stalked cell and a swarmer cell. DNA replication is blocked in the swarmer but not in the stalked cell. Like Spo0A, the CtrA protein belongs to the response regulator family of proteins and its activity is subjected to multiple levels of regulation. The essential CtrA protein is a master transcription factor that controls multiple events in the cell cycle (for a review, see Skerker and Laub, 2004). Interestingly, the *C. crescentus* replication origin contains five functional binding sites for CtrA and binding of CtrA to these sites prevents initiation of DNA replication (Quon *et al*, 1998).

In summary, in this work we present evidence that Spo0A, the master transcriptional regulator for entry in sporulation, prevents initiation of DNA replication of the virulent phage ϕ29 as well as of the *B. subtilis* chromosome by binding to origins of DNA replication of the corresponding genome. For

ϕ29, the Spo0A-mediated inhibition of ϕ29 DNA replication represents a crucial aspect of the alternative ϕ29 infection strategy by exploiting the ability of its host to survive through the formation of endospores. In the case of the *B. subtilis* chromosome, Spo0A-mediated inhibition of DNA replication most likely functions to ensure that an early stage sporangium contains two, and only two, chromosomes.

Materials and methods

Strains, plasmids, and growth conditions

Strains and plasmids, and oligonucleotides (Isogen Bioscience BV, The Netherlands) used are listed in Supplementary Tables SI and SII, respectively. Schaeffer's sporulation medium was prepared as described before (Schaeffer *et al*, 1965). Kanamycin and chloramphenicol were added to *B. subtilis* cultures and plates at final concentrations of 30 and 5 μg/ml, respectively. Ampicillin (50 μg/ml) was used for selection in *E. coli*.

ϕ29 TP-dAMP formation (protein-primed initiation) and ϕ29 TP-DNA amplification

The incubation mixtures of protein-primed initiation reactions, which determines formation of the TP-dAMP initiation product, contained (in 25 μl) 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 20 mM ammonium sulfate, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml BSA, 0.1 μM [α-^{32}P]dATP (1 μCi), 1.6 nM of ϕ29 TP-DNA, 12 nM of ϕ29 DNA polymerase, 12.9 nM TP, 33.3 μM p6, and the indicated amount of Spo0A. After incubation for 5 min at 30°C, the reactions were stopped by adding EDTA to 10 mM and SDS to 0.1%. The samples were filtered through Sephadex G-50 spin columns in the presence of 0.1% SDS. The excluded volumes were subjected to SDS–PAGE and autoradiography was used to detect the level of TP-dAMP initiation product formed. The TP-DNA amplification was carried out as described before (Serna-Rico *et al*, 2000). Spo0A concentrations ranged from 2 to 16 μM and from 1.25 to 80 μM in the initiation and amplification reactions, respectively (two-fold dilution steps).

DNase I footprinting

DNase I footprinting was performed as described (Meijer *et al*, 2005). The origins of replication of ϕ29 and that of *B. subtilis* were amplified by PCR using genomic DNA as template. The left and right ϕ29 origins were amplified using primer sets [Phi_Left-Phi_LL] and [Phi_Right-Phi_LR], respectively. Primer sets [AaU-p_U-AaUp_L2], [Aa_UP_U2-Aa_UP_L], and [AaDOWN_U-Aa_DOWN_L] were used to amplify the *incA*, *incB*, and *incC* regions of *B. subtilis oriC*, respectively. The PCR products were labeled at one of the 5′ ends by treating the appropriate primer with polynucleotide kinase and [γ-^{32}P]ATP before the amplification reaction.

Potassium permanganate footprinting

$KMnO_4$ footprinting was essentially performed as described (Krause *et al*, 1997), with minor modifications. Supercoiled pBsoriC4 plasmid (1 μg) was preincubated with purified *B. subtilis* Spo0A for 10 min at 37°C in 75 μl unwinding buffer (25 mM Hepes–KOH, pH 7.6, 10 mM magnesium acetate, 5 mM ATP, 50 μg/ml BSA, 100 ng HBsu). Protein DnaA, preincubated with 1 mM ATP, was then added and incubated 3 min at 37°C. Next, $KMnO_4$ was added to a final concentration of 1 mM. After 2 min, the reaction was quenched by addition of 6 μl β-mercatoethanol and 6 μl 500 mM EDTA. After phenol/chloroform extraction, the DNA samples were filtered through Sephadex G-50 spin columns and subsequently digested with *Xmn*I. Then, after phenol/chloroform extraction, samples were filtered again through Sephadex G50 columns and the DNA concentration was measured by A_{260}. Next, 250 ng of plasmid DNA was used for each primer extension assay (Meijer *et al*, 2005) using either 1 ng of 5′-labeled oligonucleotide OriC_ATrr_Up (upper strand) or OriC_ATrr_Down (lower strand) and Exo$^-$ Vent DNA polymerase (New England Biolabs, Beverly, MA, USA). After ethanol precipitation, the samples were subjected to electrophoresis on 6% denaturing polyacrylamide sequence gels next to DNA sequencing lanes and visualized by autoradiography on X-ray film.

Real-time PCR

Cells of 1 ml aliquots of *B. subtilis* cultures, withdrawn at different times after infection, were harvested, processed and analyzed by real-time PCR as described before (Meijer *et al*, 2005). Primers used for amplification (Phi_LL and Phi_left) are listed in Supplementary Table SII. The data obtained for the samples were interpolated to a standard curve constructed with known amounts of purified, full-length ϕ29 DNA. The results are expressed as ng of DNA per ml of culture.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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