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Molecular basis for the exploitation of spore formation as survival mechanism by virulent phage ϕ29

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Phage ϕ29 is a virulent phage of *Bacillus subtilis* with no known lysogenic cycle. Indeed, lysis occurs rapidly following infection of vegetative cells. Here, we show that ϕ29 possesses a powerful strategy that enables it to adapt its infection strategy to the physiological conditions of the infected host to optimize its survival and proliferation. Thus, the lytic cycle is suppressed when the infected cell has initiated the process of sporulation and the infecting phage genome is directed into the highly resistant spore to remain dormant until germination of the spore. We have also identified two host-encoded factors that are key players in this adaptive infection strategy. We present evidence that chromosome segregation protein Spo0J is involved in spore entrapment of the infected ϕ29 genome. In addition, we demonstrate that Spo0A, the master regulator for initiation of sporulation, suppresses ϕ29 development by repressing the main early ϕ29 promoters via different and novel mechanisms and also by preventing activation of the single late ϕ29 promoter.

The EMBO Journal (2005) **24,** 3647–3657. doi:10.1038/sj.emboj.7600826; Published online 29 September 2005
Subject Categories: microbiology & pathogens
Keywords: *Bacillus subtilis*; phage ϕ29; Spo0A; Spo0J; transcriptional regulation

Introduction

Bacteriophages have developed a broad range of infection strategies (Calendar, 1988) that may permit them to maintain lysogeny for many generations or to complete their life cycle within a rather short period of time. As viruses are believed to have coevolved with their hosts, it would be surprising if they had not developed the capability to take advantage of special features of their hosts' life style. *Bacillus subtilis* is a member of a large family of bacteria that respond to nutritional stress by forming highly resistant endospores that can remain dormant for long periods of time before germinating to resume growth. The endospore is formed by modified, extremely polar cell division, which generates a tiny prespore compartment near one cell pole and a much larger mother cell. The prespore develops with the cooperation of the mother cell, which eventually lyses to release the completed spore (recently reviewed by Errington, 2003). When certain lytic phages infect *B. subtilis* cells that are in their initial stages of sporulation, they do not cause cell lysis but instead the injected phage genome remains effectively dormant and becomes entrapped in the spore (Sonenshein, 1970). When favorable host growth conditions return, germination of the spore releases the trapped phage genome, which then enters its lytic phase, resulting in lysis of the cell and liberation of phage progeny. Proper execution of this alternative strategy, which allows the phage to take advantage of the remarkable resistance and dormant properties of the spore, presumably requires (i) suppression of phage development and (ii) segregation of the infected phage genome into the small polar prespore compartment. However, the molecular mechanisms responsible for this powerful survival strategy are largely unknown.

One of the *B. subtilis* phages capable of this strategy is phage ϕ29 (Moreno, 1979), whose genome consists of a linear double-stranded DNA (dsDNA) with a terminal protein (TP) covalently linked at each 5′ end (Salas, 1991). This structure precludes integration of the ϕ29 genome into the bacterial chromosome, which would be one obvious way to ensure entrapment in the prespore.

A genetic and transcriptional map of the ϕ29 genome is shown in Figure 1. Phage ϕ29 transcription is divided into early and late stages (for review, see Rojo *et al*, 1998; Meijer *et al*, 2001). All late genes, including those involved in lysis of the infected cell, are clustered in a single, centrally located, operon that is transcribed from the late promoter A3. The early-expressed genes are present in two operons. One, located at the right side of the genome, is under the control of the strong C2 promoter, and the other, located at the left side, is expressed from two strong, tandemly organized promoters named A2b and A2c. The vegetative RNA polymerase containing σ^A (RNAP) recognizes the early promoters and, with the aid of the transcriptional regulator protein p4, the late A3 promoter, which lacks a typical −35 box. The region, comprising promoters A2c, A2b and A3, contains three main protein p4-binding sites. Binding sites 1 and 2 are involved in repression of the early A2c promoter. Binding site 3 is located upstream of the late A3 promoter and partly overlaps the divergently oriented early A2b promoter. Binding of protein p4 to this site simultaneously activates the A3 and represses the A2b promoter.

Segregation of bacterial chromosomes and plasmids is an active process (for review, see Gordon and Wright, 2000; Hiraga, 2000; Wu, 2004). In *B. subtilis*, the Spo0J

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Received: 14 April 2005; accepted: 1 September 2005; published online: 29 September 2005

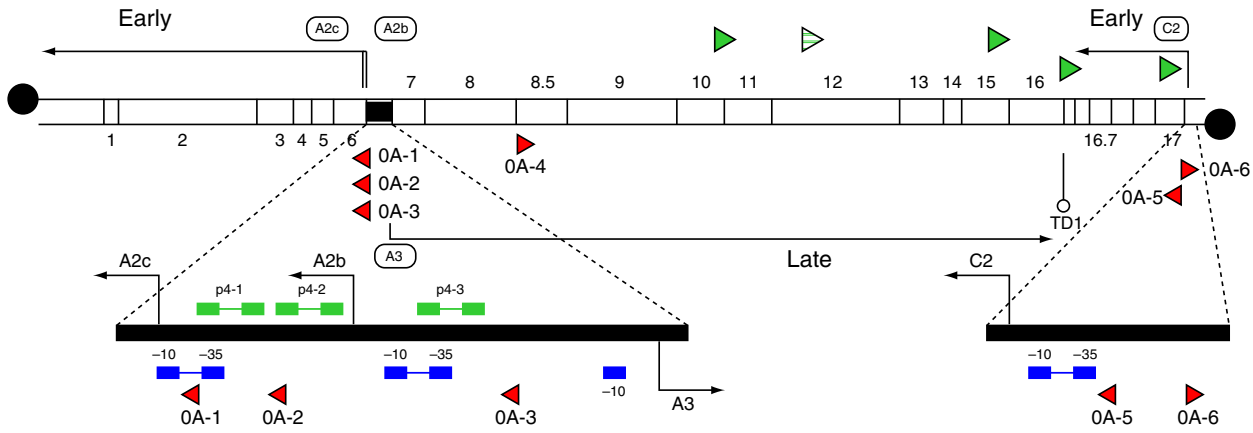


Figure 1 Genetic and transcriptional map of the ϕ29 genome. The direction of transcription and length of the transcripts are indicated by arrows. The positions of the 0A boxes and *parS* sites are indicated with red and green triangles, respectively. The *parS* site having two additional bp with respect to the other ones is hatched. The positions of the various genes are indicated by numbers. The bidirectional transcriptional terminator TD1 is indicated by a hairpin structure. Black circles represent the TP. A black box indicates the region spanning the early promoters A2b and A2c, and the late A3 promoter. Blow-ups of the A2c-A3 and C2 promoter regions are shown in the lower part. Transcription start sites are indicated with bent arrows. The −35 and −10 boxes are indicated with blue filled boxes. Note that the late A3 promoter lacks a consensus −35 sequence. The positions of the main protein p4-binding sites are indicated with green boxes.

protein plays an important role in chromosomal segregation, both in vegetative and sporulating cells (for review, see Draper and Gober, 2002; Wu, 2004). The *spo0J* gene forms an operon with *soj*. Soj and Spo0J are DNA-binding proteins that are related to the ParA/ParB family of proteins, which are required for the stable maintenance of plasmids (reviewed by Gerdes *et al*, 2000). The preferential Spo0J-binding site, *parS*, is the 16 bp imperfectly inverted repeat sequence 5′-TGTTCCACGTGAAACA-3′, which is located within the *spo0J* gene near *oriC*. The *B. subtilis* chromosome contains seven additional Spo0J-binding sites, scattered over the ∼1 Mbp *oriC* region, which have 1 or 2 bp differences with respect to the *parS* site in the *spo0J* gene (Lin and Grossman, 1998). Although Soj does not appear to be required for chromosome segregation in vegetative cells of *B. subtilis*, the combination of *soj, spo0J* and *parS* can stabilize otherwise unstable plasmids in both *B. subtilis* (Lin and Grossman, 1998) and *Escherichia coli* (Yamaichi and Niki, 2000). Furthermore, the combination of *soj* and *spo0J* is involved in segregation of the prespore chromosome during sporulation, together with at least two other proteins, RacA and DivIVA (Ben-Yehuda *et al*, 2003; Wu and Errington, 2003).

Interestingly, the genome of ϕ29 contains five *parS*-like sites, four of which have a sequence identical to the one present in the *spo0J* gene (Murthy *et al*, 1998; see Figure 1). Here, we present evidence indicating that the host-encoded chromosomal segregation machinery is involved in segregation of the ϕ29 genome into the prespore.

The master regulator for initiation of sporulation is the *B. subtilis* Spo0A protein (Hoch, 1993). A multicomponent phosphorelay consisting of five histidine autokinases and two phosphorelay proteins (Spo0F and Spo0B) controls the activity of Spo0A. The activated form, Spo0A∼P, binds to DNA sequences containing a so-called '0A box', where it exerts its role as a transcriptional regulator by activating the expression of certain genes, while repressing others (for review, see Grossman, 1995; Sonenshein, 2000; Perego and Hoch, 2002; Piggot and Losick, 2002).

We present several lines of *in vitro* and *in vivo* evidence that Spo0A (i) directly inhibits transcription from the early ϕ29 promoters by different mechanisms and (ii) prevents activation of the single late A3 promoter. The phage is therefore sensitized to the critical signal transduction system regulating the entry into sporulation. Under appropriate conditions, the phage uses this device to postpone its normal lytic cycle, and arranges to be trapped in the endospore, surviving along with its host until conditions favorable for its lytic cycle prevail.

Results

The ϕ29 parS sites are involved in spore entrapment

It seemed possible that the *parS* sites in ϕ29 DNA might function by exploiting the host-encoded Spo0J chromosome segregation machinery to enhance spore entrapment of the ϕ29 genome. To study whether Spo0J binds *in vivo* to the *parS* sites present on the ϕ29 genome, we performed chromatin immunoprecipitation (CHIP) assays (see Materials and methods). In brief, formaldehyde was added to ϕ29-infected cells to crosslink protein and DNA, cells were lysed and the DNA sheared to an average size of ∼750 bp. The Spo0J–DNA complexes were then immunoprecipitated using polyclonal antibodies against Spo0J, the crosslinks were reversed and the precipitated DNA was analyzed by quantitative real-time PCR. Seven sets of primers were used in the PCR assays; five of these were designed to amplify the ϕ29 *parS* site-containing regions, and two to amplify regions of ϕ29 DNA that do not contain a *parS* site. Binding of Spo0J to the different ϕ29 DNA regions is expressed as immunoprecipitation coefficient (IC) in which the value obtained with antibodies against Spo0J is normalized to the total amount of DNA of that region. Figure 2 shows that the ϕ29 DNA regions containing the *parS* sites were 10- to 23-fold more abundant in immunoprecipitates compared to the non-*parS* site DNA regions. Low ICs (<180) were obtained for all the ϕ29 DNA regions tested when CHIP experiments were performed using an isogenic Δ*soj-spo0J* strain (Figure 2). Together, these results

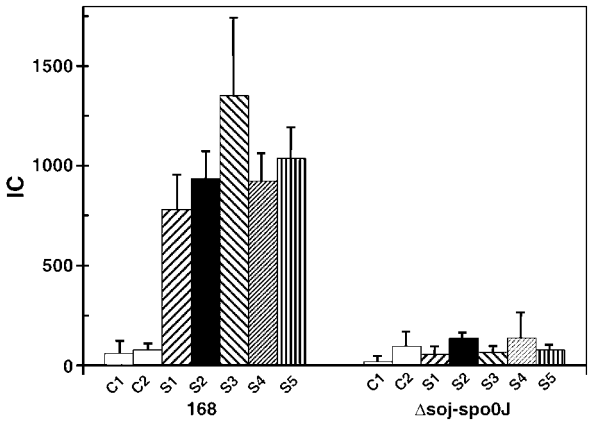


Figure 2 *In vivo* binding of Spo0J to the *parS* sites present in the ϕ29 genome. Wild-type *B. subtilis* 168 (168) or isogenic *soj-spo0J* deletion (Δsoj-spo0J) cultures were infected with phage ϕ29 (m.o.i. 10) and subjected 20 min later to formaldehyde crosslinking. After processing (see Materials and methods), the total and immunoprecipitated DNA samples were analyzed by quantitative real-time PCR using seven sets of primers. Five primer sets were designed to amplify the different ϕ29 *parS* site regions (S1–S5) and two control primer sets to amplify ϕ29 DNA regions not containing a *parS* site (C1 and C2, ϕ29 regions 64–523 and 1022–1548, respectively). The mean values of the ICs ± the standard deviation of two independent experiments are presented.

demonstrate that Spo0J binds preferentially to the ϕ29 regions containing a *parS* site *in vivo*.

Next, experiments were performed to analyze whether the ϕ29 *parS* sites are functionally important for spore entrapment. The linear genome of ϕ29 is not amenable to standard genetic manipulation because of the covalent attachment of TP at both DNA ends, which is essential for ϕ29 DNA replication, excluding the possibility to mutagenize the *parS* sites in the phage genome. As a first approach, we cloned a ϕ29 *parS* site onto a derivative of the low-copy-number *B. subtilis* plasmid pLS20 (Meijer *et al*, 1995) and analyzed the efficiency of spore entrapment of the plasmid with or without the *parS* site in wild-type *B. subtilis* cells. Table I shows that more than 88% of the spores harbored the plasmid when it contained the *parS* site (pParSa/b), versus 28% when it lacked the site (pNeg). When carried out in an isogenic Δ*soj-spo0J* mutant background, ~30% of the spores harbored a plasmid, independent of whether or not it contained the *parS* site (s*po0J* mutants make very few spores because of the negative transcriptional regulator *soj* (Ireton *et al*, 1994), hence the use of a *soj-spo0J* double mutant). Thus, the presence of a ϕ29 *parS* site enhances the segregation efficiency of a heterologous replicon into the prespore in a *soj-spo0J*-dependent manner. As a second approach, we compared the ratio of spore entrapment of the ϕ29 genome between a wild-type and an isogenic Δ*soj-spo0J* strain. Spore entrapment of ϕ29 genome is limited to a narrow window after the onset of sporulation (Moreno, 1979). In our experimental setup, maximum spore entrapment levels were obtained when cells were infected around 40 min after resuspension in sporulation medium (SM). Spore entrapment efficiencies of the ϕ29 genome were consistently higher in the wild-type than in the Δ*soj-spo0J* strain. The ratios between the efficiencies of the 16 values obtained fluctuated, however, between 4- and 13-fold (mean difference 6.5). Probably, these

Table I Spore entrapment efficiencies of plasmids with or without *parS* in wild-type 168 and an isogenic *soj-spo0J* deletion strain

Plasmid	% plasmid containing spores[a]	
	168	Δ*soj-spo0J*
pNeg	28	26
pParSa	89	31
pParSb	93	29

[a]Average of three independent experiments that, among themselves, differed less than 10%.

fluctuations are due to differences in the progress of development between the cultures. The data of both approaches strongly indicate that the *parS* sites on the ϕ29 genome are important for spore entrapment.

Sporulation causes repression of the early ϕ29 promoters and prevents activation of its late promoter

Early reports described that ϕ29 forms large plaques on certain early blocked sporulation mutants but not on the *B. subtilis* wild-type strain 168 (Ito and Spizizen, 1972). To study whether the impairment of phage ϕ29 development in solid medium depends on *spo0A*, we analyzed plaque formation on wild-type *B. subtilis* strain 168, an isogenic *spo0A* deletion strain and a *spo0B* mutant strain (which is blocked for Spo0A activation). Phage ϕ29 formed large plaques on the *spo0A* and *spo0B* mutant strains but not on the wild-type strain (not shown), indicating that activated Spo0A causes impairment of phage ϕ29 development. Spo0A activity is not affected in the absence of both *soj* and *spo0J* (Ireton *et al*, 1994). In accordance with this, ϕ29 did not form large plaques on the Δ*soj-spo0J* strain (not shown).

To study whether suppression of ϕ29 development is because of repression of ϕ29 transcription, we analyzed its expression profile in cells infected at different times after synchronized initiation of sporulation. As shown in Figure 3, relatively high levels of transcripts derived from the main early promoters and of the late A3 promoter were detected when cells were infected immediately after they were resuspended in SM. Much lower or hardly detectable levels of the early A2c and A2b transcripts were observed in samples infected 15, 30 or 45 min after resuspension in SM. A decrease of the early promoter C2 transcription level was also observed when cells were infected 30 or 45 min after resuspension. Moreover, promoter A3-derived transcripts were no longer detected when cells were infected 15 min or later after sporulation induction. The different repression levels suggest that the effects are promoter specific and do not represent a general loss of transcription of nonsporulation promoters. These results together with those of the analyses of transcriptional *lacZ* fusions (see below) indicate that the early ϕ29 promoters become repressed during sporulation.

The ϕ29 genome contains six 0A boxes

Examination of the ϕ29 genome revealed that it contains six 0A boxes, as originally defined by Strauch *et al* (1990). One of them, 0A box 4 (0A-4), is present within gene 8.5 encoding the phage head fiber. Interestingly, the other five 0A boxes are all located in the vicinity of promoters; three of them (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 (see Figure 1). All five promoter-associated 0A boxes, but not the one present within gene 8.5, have a sequence that is identical to the extended 0A box as defined by Liu *et al* (2003) (see Supplementary Table SI).

Early ϕ29 promoters are repressed in vivo in a spo0A-dependent manner

Single-copy chromosomal transcriptional *lacZ* fusions were constructed and used to study the temporal expression of each early ϕ29 promoter with its associated 0A boxes in the wild-type 168 and the isogenic *spo0A* background. The results (Supplementary Table SII) showed that the β-galactosidase activities driven by these ϕ29 promoters started to decline as soon as the cultures entered sporulation (defined as $t=0$) when the *lacZ* fusions were in the wild-type but not in the *spo0A* background. These results, together with the fact that Spo0A becomes overexpressed and activated shortly before $t=0$ (Strauch *et al*, 1992), suggested that Spo0A represses the ϕ29 promoters *in vivo*. The apparent lower repression levels observed by this approach compared to those observed by primer extension (see above) are most likely owing to the higher stability of the β-galactosidase enzyme with respect to the phage mRNA.

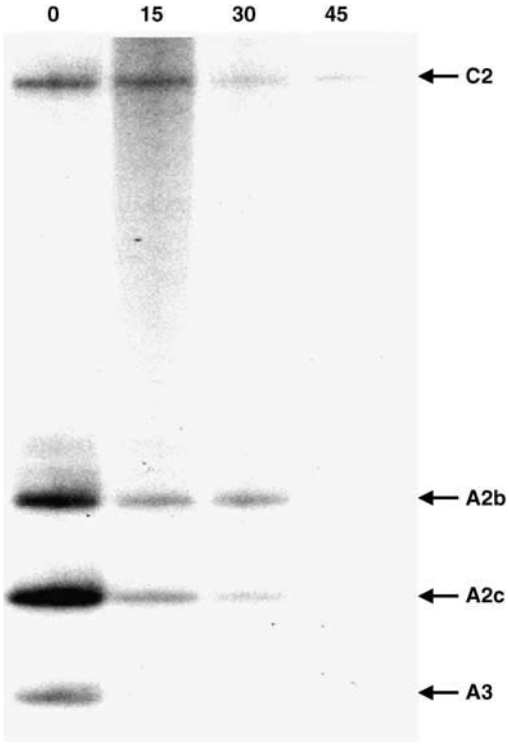


Figure 3 Analysis of *in vivo* phage ϕ29 transcripts produced as a function of cell development. Total RNA was purified 25 min post-infection (m.o.i. 5) from cells that were infected with ϕ29 directly or at different times (15, 30 or 45 min) after resuspension of a mid-logarithmically grown culture in SM. Next, ϕ29 transcripts were analyzed by primer extension and resolved in denaturing polyacrylamide gels. The primers were selected to give a cDNA of different length for each promoter (indicated at the right). Mean values of the relative abundance of the transcripts at the indicated times were as follows: C2, 100 (±5.8), 83.8 (±6.7), 18 (±4.2), 5.2 (±2.8); A2b, 100 (±7.9), 26 (±5.6), 20 (±6.3), <2; A2c, 100 (±2.1), 19 (±2.9), 4 (±2.3), <2; and A3, 100 (±4.5), <2, <2, <2.

The ϕ29 early promoter-associated 0A boxes are bona fide Spo0A-binding sites

DNase I footprinting was used to test whether the ϕ29 promoter-associated 0A boxes are Spo0A-binding sites. The functionality of the Spo0A protein used was inferred from the fact that (i) it produced highly similar footprints on the *B. subtilis spoIIG*-associated 0A boxes to those published (Satola *et al*, 1992) (not shown) and (ii) that it was able to activate the *spoIIG* promoter *in vitro* (see below).

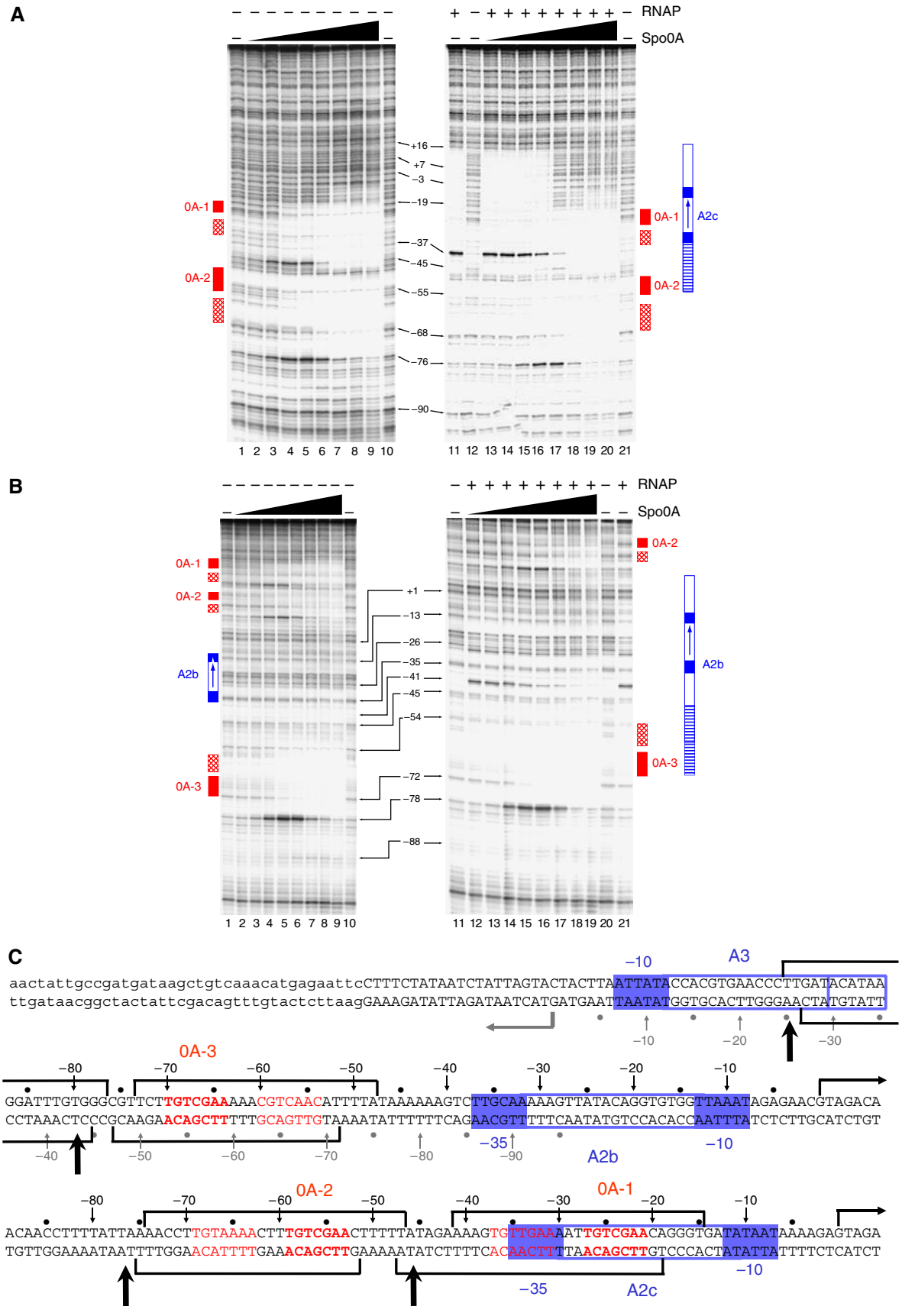
Representative footprints of Spo0A binding to the A2c-A2b and the C2 promoter regions are shown in Figures 4 and 5, respectively. The schematic overviews of the various Spo0A-protected regions on either strand (Figures 4C and 5B) show that binding of Spo0A is not limited to the 0A-box sequence. Inspection of the protected sequences revealed that each 0A box analyzed is flanked by a 3 bp spacer by an imperfect 0A-box sequence that is located either upstream (0A boxes 1 and 2) or downstream (0A boxes 3, 5 and 6) of the consensus heptamer 0A-box sequence. The dual 0A boxes 1 and 5 overlap partly the A2c and C2 promoter, respectively.

Spo0A represses the early ϕ29 promoters by different mechanisms

In vitro transcription assays (Figure 6) were performed to study if Spo0A is directly responsible for repression of the early ϕ29 promoters. The *B. subtilis* σ^A-RNAP-dependent *spoIIG* promoter is activated by Spo0A (Satola *et al*, 1992). Contrary to the Spo0A-induced activation of the *spoIIG* promoter, a Spo0A-dependent decrease of promoter activity was observed for the early ϕ29 promoters, demonstrating that Spo0A represses these promoters directly.

The possibility that Spo0A interferes with binding of RNAP to the early ϕ29 promoters was tested by DNase I footprinting (Figures 4 and 5). RNAP binds efficiently to the A2c promoter, generating a footprint on the template strand that spans

Figure 4 Footprint analyses of the binding of Spo0A with or without RNAP to the A2c (**A**) and A2b (**B**) promoter. DNA fragments labeled at the 3′ end at the template strand for early transcription were incubated with the proteins as indicated above the footprints. The numbering used in panels A and B is according to the transcriptional start sites of promoters A2c and A2b, respectively. The promoters and their directionality are indicated in blue; the filled and striped boxes depict the −35 and −10 hexamers and the UP element, respectively. Filled and hatched red boxes indicate the positions of the originally defined consensus 0A-box sequence and that of the flanking imperfect 0A box (see text), respectively. When indicated, 70 nM of RNAP was added 10 min after Spo0A addition, and Spo0A concentrations ranged from 1.6 nM to 27 μM (four-fold dilution steps). A summary of the Spo0A-protected DNA regions is presented in (**C**). Spo0A-protected regions are bracketed. The consensus heptamer 0A-box sequences and the flanking imperfect 0A-box sequences are given in red and boldface and in red, respectively. Transcription initiation sites are indicated with bent arrows. The core promoter sequences are indicated with blue rectangles; the −35 and −10 hexamers are indicated with blue boxes. Positions relative to the transcription start site are indicated below (promoter A3) or above (promoters A2b and A2c) the DNA sequence. ϕ29 DNA sequences are given in uppercase letters; lowercase letters correspond to vector sequences. Strong hypersensitive sites, observed at low and medium Spo0A concentrations, are indicated with thick arrows. The template used in panels A and B contains ϕ29 sequences up to position −87 relative to the A2b transcription start site; the fragment containing the additional ϕ29 sequences shown here up to position +21 relative to the A3 transcription start site was used in footprint assays presented in Figure 7.



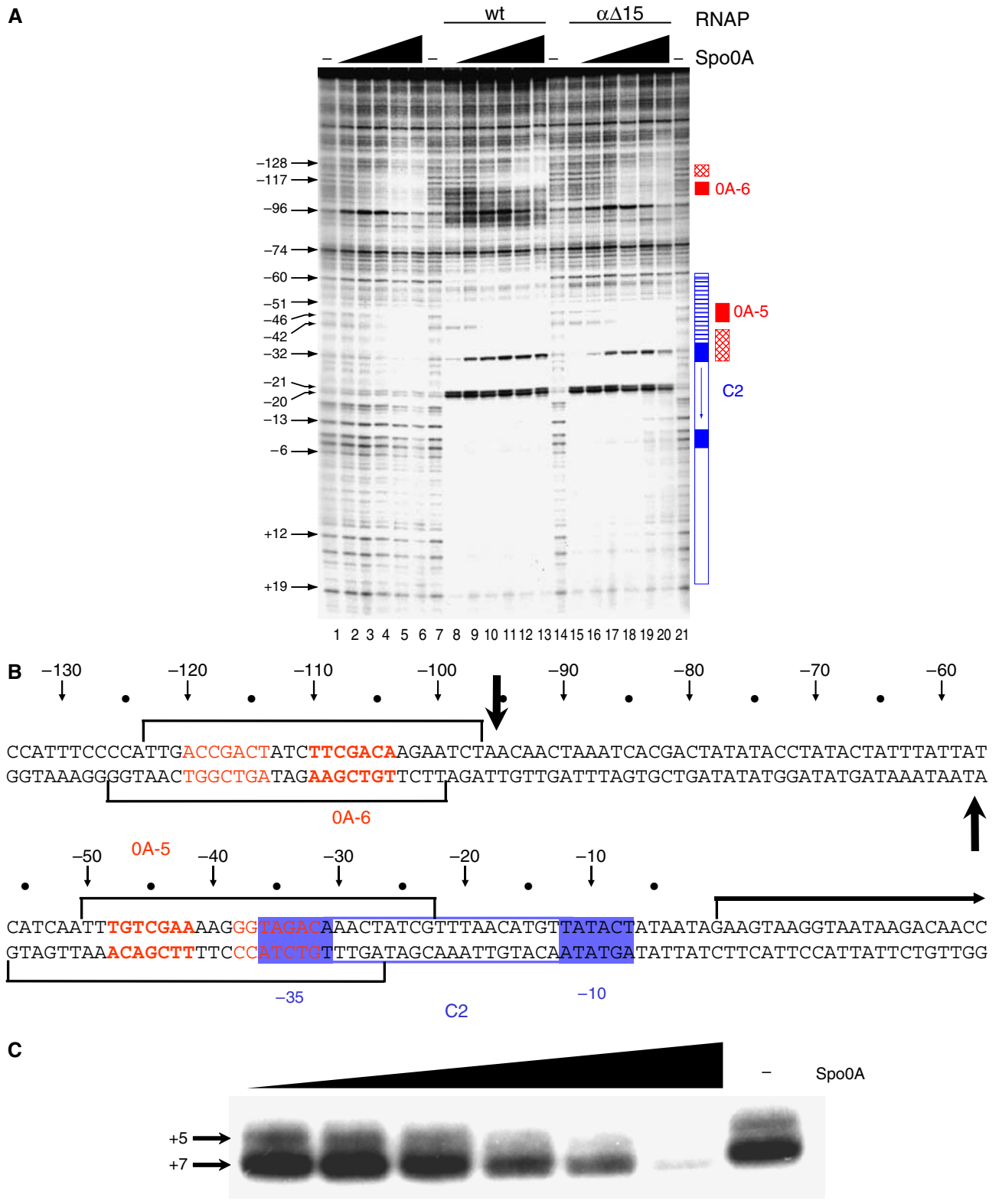


Figure 5 Footprint analyses of the binding of Spo0A in the absence or presence of RNAP at the ϕ29 C2 promoter. (**A**) The C2 promoter-containing DNA fragment, end-labeled at the nontemplate strand, was analyzed for binding of Spo0A (lanes 2–6), σ^A-RNAP containing either the wild type (wt; lanes 8–13) or the αΔ15 mutant (αΔ15; lanes 15–20) without (lanes 8 and 15) or with (lanes 9–13 and 16–20) preincubation of the fragment with Spo0A. The numbering used is according to the transcriptional start site of promoter C2. Color schemes indicating the positions of the 0A boxes and promoter elements are according to those described in Figure 4A and B. Fixed amounts of RNAP (70 nM) were used and Spo0A concentrations (four-fold dilution steps) ranged from 26.4 nM to 6.7 μM. (**B**) Summary of the DNA regions protected by Spo0A from DNase I digestion. Color schemes and symbols are the same as those used in Figure 4C. (**C**) The C2 promoter-encompassing DNA fragment, preincubated without or with increasing amounts of Spo0A, was incubated at 37°C with RNAP (140 nM) (no initiating nucleotides), and DNA melting was probed by $KMnO_4$ footprinting. The indicated positions that became hypersensitive to $KMnO_4$ are relative to the C2 promoter transcription start site. Spo0A concentrations, four-fold dilution steps, ranged from 26.4 nM to 6.7 μM.

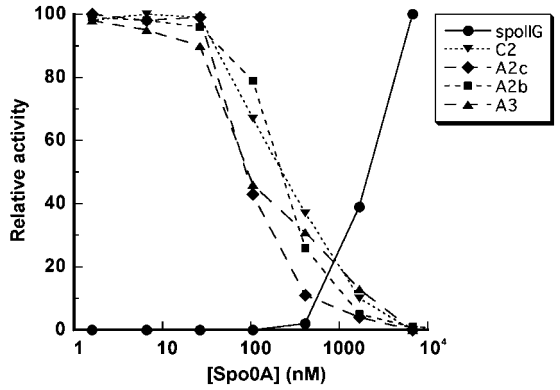


Figure 6 Spo0A represses the ϕ29 early promoters A2c, A2b and C2 and prevents activation of the late A3 promoter *in vitro*. The activities of the ϕ29 early promoters A2b, A2c and C2, the late A3 promoter and the *B. subtilis spoIIG* promoter were measured as a function of Spo0A concentration in *in vitro* run-off assays. Reaction mixtures contained 4 nM of the appropriate template DNA containing the promoter(s) and associated 0A boxes, 40 nM of purified *B. subtilis* RNAP and the indicated amount of Spo0A. Promoter activities are given relative to their maximum activity observed in the absence (ϕ29 promoters) or presence (*spoIIG* promoter) of Spo0A. The fragment containing the *spoIIG* promoter included the 0A-binding sites 1 and 2 (Satola *et al*, 1991). Presented percentages correspond to the mean value of at least three independent experiments that, among themselves, differed less than 10%.

the region from +16 to −57 and inducing a hypersensitivity at position −37 relative to its transcription start site (Monsalve *et al*, 1996; Figure 4A, lane 11). However, RNAP was unable to bind to the A2c promoter when Spo0A was bound to the 0A-box regions 1 and 2 (Figure 4A, lanes 17–20).

Although the A2b promoter is strong, RNAP binds weakly to it. One of the most characteristic changes upon RNAP binding is the generation of a strong hypersensitive site at position −41 of the template strand relative to the promoter A2b transcription start site (Meijer and Salas, 2004; Figure 4B, lane 21). Binding of RNAP to this promoter was inversely related to the binding of Spo0A at the 0A-box 3 region (Figure 4B, lanes 12–19).

The same approach was used for the C2 promoter. RNAP binds efficiently to the C2 promoter generating a footprint at the nontemplate strand spanning positions −50 to +19 relative to its transcription start site. In addition, RNAP binding induces two strong hypersensitivities at positions −20 and −21 and leads to a partial protection of position −60 (Meijer and Salas, 2004; Figure 5A, lane 8). Interestingly, Spo0A bound to 0A-box regions 5 and 6 did not prevent RNAP from binding to the C2 promoter (Figure 5A, lanes 9–13), despite the fact that the upstream part of the C2 core promoter is protected by Spo0A (Figure 5A, lanes 4–6). Simultaneous binding of Spo0A and RNAP caused some alterations in the RNAP binding characteristics, though. Thus, it led to (i) increased hypersensitivity of position −32, (ii) incomplete protection of position −7 and (iii) full protection of position −60. Binding of Spo0A to the 0A-box 5 region is responsible for these alterations, as these were also observed using a shorter DNA fragment that lacked the 0A box 6 (not shown).

The C2 promoter contains a UP element and binding of the C-terminal domain (CTD) of the RNAP α subunit to the UP element is responsible for full protection of the −46 to −50 region and partial protection of position −60 (Meijer and Salas, 2004). Hence, the 0A-box 5 region not only overlaps with part of the C2 core promoter but also with part of its UP element. To test whether binding of Spo0A to the 0A-box 5 region causes the enhanced binding of αCTD to position −60, DNase I footprinting was performed with RNAP holoenzyme containing a mutant α subunit lacking its 15 C-terminal amino acids (αΔ15-RNAP; Mencía *et al*, 1996). Binding of αΔ15-RNAP is limited to the C2 core promoter (Figure 5A, lane 15; Meijer and Salas, 2004). When Spo0A and αΔ15-RNAP are both present, position −46 is protected by Spo0A but position −60 remains unprotected (Figure 5A, lanes 18–20), indicating that αCTD is responsible for full protection of this position by wild-type RNAP when Spo0A is bound to the 0A-box 5 region. Moreover, comparison of the footprints generated by the simultaneous binding of Spo0A with either wild-type RNAP or αΔ15-RNAP to the C2 promoter revealed that (i) in both situations, position −32 became hypersensitive, and (ii) whereas, except for position −7, the C2 core promoter region was fully protected by wild-type RNAP over the entire Spo0A concentration range tested, it was only partially protected by αΔ15-RNAP in the presence of high Spo0A concentrations (compare Figure 5A, lanes 12 and 13 with 19 and 20). These results indicate that the Spo0A-mediated hypersensitivity of position −32 does not require αCTD and that binding of αCTD to position −60 is required for stable binding of RNAP to the C2 promoter in the presence of Spo0A.

As binding of Spo0A to its associated 0A boxes does not prevent RNAP from binding to the C2 promoter, the Spo0A-mediated repression of this promoter should be exerted at a step after formation of the closed complex. Indeed, Spo0A, added either before or after RNAP, affected the amount of open complexes at the C2 promoter as assessed by potassium permanganate footprinting (Figure 5C shows the results obtained when Spo0A was added before RNAP; similar results were obtained when the proteins were added in reverse order (not shown)).

Spo0A prevents activation of the late A3 promoter

The ϕ29 late A3 promoter was not activated when cells were infected 15 min or later after resuspension in SM (Figure 3). Activation of promoter A3, which lacks a typical −35 box, occurs through p4-mediated recruitment of RNAP to the A3 promoter via contacts between protein p4 and the CTD of the RNAP α subunit (Mencía *et al*, 1996). The p4-binding site 3, needed for promoter A3 activation, is located from positions −69 to −95 with respect to its transcription start site. Interestingly, 0A box 3 is positioned in between the A3 promoter and the p4-binding site 3 (see Figure 1). Binding of Spo0A to the 0A-box 3 region might therefore interfere with activation of the late A3 promoter. *In vitro* run-off assays (Figure 6) indeed showed that activation of the late A3 promoter is prevented in a Spo0A-dependent way.

The Spo0A-mediated prevention of promoter A3 activation was studied by DNase I footprinting using a DNA fragment encompassing the late A3 promoter and upstream sequences containing 0A box 3, p4-binding site 3 and the A2b promoter (Figure 7). Surprisingly, using this DNA fragment, it was found that Spo0A not only binds to the 0A-box 3-containing region determined above (Figure 4B), but also to a juxtaposed

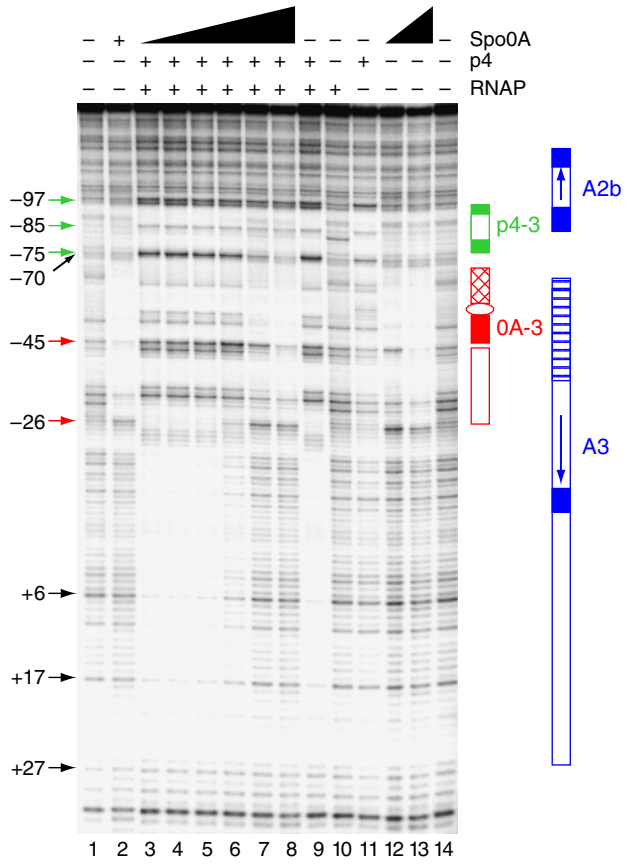


Figure 7 Binding of Spo0A to the 0A-box 3 region prevents recruitment of RNAP to the ϕ29 late A3 promoter. The DNA fragment used, end-labeled at the late strand, includes the A3 promoter and the upstream 0A box 3, p4-binding site 3 as well as the early A2b promoter. The numbering used is relative to the promoter A3 transcription start site. The positions that become hypersensitive to DNase I in the presence of Spo0A or protein p4 are indicated with red and green arrows, respectively, and the regions that become protected upon binding of these proteins are indicated by rectangles using the same color scheme. The inverted repeated sequences of the p4-binding site are boxed. The additional region protected by Spo0A is indicated with an open red box. The upstream A3 promoter region that becomes partially protected upon binding of RNAP to the A3 promoter is striped. Other color schemes are according to those described in Figure 4A and B. Fixed amounts of RNAP (35 nM) and p4 (2.3 μM) were used. Spo0A concentrations, four-fold dilution steps, ranged from 6.6 nM to 6.7 μM. Lanes 2 and 13 contained 6.7 μM and lane 12 contained 1.7 μM Spo0A.

region lacking an obvious 0A-box-like sequence encompassing positions −44 to −26 relative to the A3 promoter transcription start site (Figure 7, lanes 2, 12 and 13). As expected, RNAP bound to the A3 promoter in the presence but not in the absence of protein p4 (compare lanes 9 and 10). In the former situation, the hypersensitive bands that characterize the binding of p4 to its cognate site upstream of the A3 promoter are more pronounced as compared with those generated by protein p4 alone (compare lanes 9 and 11), reflecting cooperative binding of both proteins (Rojo *et al*, 1998). RNAP recruitment was lost, however, under conditions in which binding of Spo0A to the extended 0A-3-containing region became evident (lanes 6–8). Some of the characteristic footprint features of protein p4 were still observed under these latter conditions, indicating that p4 and Spo0A can bind simultaneously to their flanking cognate binding sites. Highly similar footprints were observed when Spo0A was added after the DNA was preincubated with p4 and RNAP (not shown).

Discussion

The mechanisms that (i) suppress *in vivo* ϕ29 phage development and (ii) are involved in segregation of the ϕ29 genome into the *B. subtilis* developing prespore are described in this work. Together, they form the basis for the adaptive ϕ29 infection strategy that (i) permits the phage genome to survive in the highly resistant spore and (ii) restricts phage development to the stage in which conditions are best suited for the production of large numbers of phage progeny.

Spore entrapment

Moreno (1979) showed that a single ϕ29 genome is sufficient for spore entrapment and that viral DNA synthesis is not required for trapping, indicating that spore entrapment of the phage genome involves an active process. Results presented in this work suggest that the *parS* sites present on the ϕ29 genome probably play a role in spore entrapment. Thus, (i) spore entrapment efficiency of ϕ29 genome is lower in a *soj-spo0J* deletion strain as compared to the wild-type strain and (ii) the presence of a ϕ29 *parS* site enhanced the efficiency of spore entrapment of a low-copy plasmid in a *soj-spo0J* dependent way. Moreover, CHIP experiments showed that Spo0J binds specifically to the ϕ29 *parS* site regions *in vivo*. Together, these results indicate that part of the chromosome segregation machinery is employed to partition the ϕ29 genome in the prespore.

Spo0A represses ϕ29 early promoters by different mechanisms

Using *in vivo* and *in vitro* approaches, we have shown here that the main early ϕ29 promoters are directly repressed by Spo0A via different and novel mechanisms. The A2c promoter is repressed by steric hindrance due to binding of Spo0A to the 0A-box 1 region, which overlaps with the core promoter. Binding of RNAP to the A2b promoter is prevented when Spo0A is bound to the 0A-box 3 region. This dual 0A box is located upstream of the core A2b promoter (positions from −54 to −70 relative to the A2b transcription start site). Activity of the A2b promoter depends almost completely on the presence of a UP element (Meijer and Salas, 2004). Interestingly, the position of this UP element coincides with the dual 0A-box 3 region. Thus, binding of Spo0A to the UP element of the A2b promoter prevents docking of the CTD of the RNAP α subunit, which is crucial for its activity. To our knowledge, repression by occupation of a promoter UP element has not been reported previously. In the case of the C2 promoter, Spo0A-mediated repression occurs at a step after RNAP binding.

αCTD is connected to the N-terminal domain by a long flexible linker, permitting a remarkable positional plasticity of αCTD, which can fluctuate in the presence of transcriptional regulators (Blatter *et al*, 1994; Jeon *et al*, 1997). Here, we showed that αCTD completely protects position −60, located upstream of the 0A-box 5 region, when Spo0A and RNAP are bound simultaneously at the C2 promoter. We also showed that binding of αCTD to this position is important for the Spo0A-mediated repression by holding RNAP at the C2

promoter. Binding of αCTD increases the initial equilibrium constant between RNAP and DNA (Rao *et al*, 1994), which probably explains the effect on binding of αΔ15-RNAP at the C2 promoter in the presence of Spo0A. However, as αCTD can also contact various transcriptional regulators (for a recent review, see Browning and Busby, 2004), it is possible that αCTD bound to position −60 interacts with Spo0A, thereby contributing to the binding of RNAP at the C2 promoter.

The *B. subtilis* σ^A-dependent *spoIIE* and *spoIIG* promoters, which have an unusual large spacing of 21 and 22 bp, respectively, between their −35 and −10 promoter boxes, are activated by Spo0A. As for the ϕ29 C2 promoter, a Spo0A-binding site overlaps the upstream part of these *B. subtilis* promoters (Satola *et al*, 1992; York *et al*, 1992). Evidence has been provided that activation of these promoters involves direct contacts between α-helix E of the Spo0AC domain and region 4.2 of the RNAP σ^A subunit (reviewed by Seredick and Spiegelman, 2001). Based on a similar organization, it is possible that Spo0A contacts the RNAP σ^A subunit when bound to the C2 promoter. Thus, the Spo0A-mediated repression of the C2 promoter may result from overstabilization of the closed complex due to interaction of Spo0A with the RNAP σ^A subunit together with binding of αCTD at position −60.

Spo0A prevents activation of late ϕ29 transcription

In vivo and *in vitro* approaches showed that Spo0A also prevents activation of the late A3 promoter. Binding of Spo0A to the 0A-box 3 region prevented p4-mediated recruitment of RNAP to the promoter. RNAP recruitment at the A3 promoter occurs through direct interaction of αCTD with protein p4 bound to its binding site 3 (Mencía *et al*, 1996). This so-called class I type of activation not only involves a surface domain of αCTD that interacts with the transcriptional activator, but also another αCTD surface domain that interacts with the DNA flanking the activator DNA-binding site. Interactions of αCTD with both the activator and the DNA are generally required for class I promoter activation (for reviews, see Busby and Ebright, 1999; Browning and Busby, 2004). The late ϕ29 A3 promoter seems to be no exception, as DNase I footprint analyses of the protein p4–α complex showed that αCTD binds adjacent to the p4-binding site 3 (positions −58 and −65 relative to the A3 promoter transcription start site; Mencía *et al*, 1996). Interestingly, the αCTD binding site of the A3 promoter coincides with the 0A-box 3 region. As described above, the 0A-3 box region also constitutes the docking site of αCTD crucial for binding of RNAP to the divergently oriented early A2b promoter (Meijer and Salas, 2004; Figure 4B). Thus, binding of Spo0A to 0A-box region 3 prevents activation of the late A3 promoter and represses the early A2b promoter by occupying, in both cases, the αCTD-binding site.

In summary, ϕ29 appears to have evolved at least two mechanisms that enable it to adapt and exploit the ability of its host to survive through the formation of endospores. By repressing transcription of genes required for the lytic cycle and providing *cis*-acting sites for segregation into the prespore compartment, the phage encapsulates its genome into one of the most resistant and durable structures in biology. It thereby postpones its replication and the destruction of its host until spore germination occurs, when conditions are likely to be much more favorable for its continued proliferation and spread.

Materials and methods

Strains, plasmids and growth conditions

Strains, plasmids and oligonucleotides (Isogen Bioscience BV, The Netherlands) are listed in Supplementary Tables SIII, SIV and SV, respectively. Standard procedures were used for transformations. Plasmid and strain constructions are described in Supplementary data. Kanamycin and chloramphenicol were added to *B. subtilis* cultures and plates at final concentrations of 30 and 5 μg/ml, respectively. Ampicillin (100 μg/ml) was used for selection in *E. coli*.

ϕ29 genome and plasmid spore entrapment assays

These assays were based on the resuspension method to induce synchronized sporulation (Partridge and Errington, 1993). Thus, for both assays, freshly diluted cultures were grown in casein hydrolysate medium (complemented with kanamycin or 5 mM $MgSO_4$ in the plasmid and ϕ29 genome entrapment assays, respectively) at 37°C until an OD_{600} of 0.7 was reached. Then, cells were centrifuged and resuspended in prewarmed SM. In the case of the plasmid assays, growth was continued for 9 h to allow the sporulation process to be completed. Next, aliquots were incubated for 15 min at 80°C to kill nonsporulated cells after which appropriate dilutions were plated on LB plates without antibiotic. Finally, 200 of the outgrown colonies were transferred to kanamycin-containing plates from which the percentage of plasmid-containing spores was determined. In the case of the ϕ29 genome entrapment assays, the cultures were infected with wild-type phage ϕ29 at different times after infection (20, 30, 40 or 50 min) and further processed as described by Moreno (1979) with the following modifications. After lysozyme and heat treatment (15 min at 80°C), the spores were washed five times with sterile water. Then, the spores were resuspended in PBS containing 50-fold diluted anti-phage ϕ29 serum (determined to neutralize at least 99.9% of the phage particles) and incubated for 30 min at room temperature. Next, the spores were resuspended in sterile water and appropriate dilutions were plated either directly on LB plates or mixed with *B. subtilis* 110NA indicator strain to determine the number of noninfected and infected spores, respectively. Control experiments showed that the supernatants of the centrifuged spore samples gave less than 0.01% plaques with respect to the spore suspension.

In vivo protein–DNA crosslinking, immunoprecipitation and real-time PCR

In vivo crosslinking, immunoprecipitation and real-time PCR were essentially as described (González-Huici *et al*, 2004) with slight modifications. Bacteria, grown at 37°C up to $\sim 10^8$ cells/ml, were infected with ϕ29 at a multiplicity of 10 and subjected to formaldehyde crosslinking 20 min after infection. After crosslinking, cell lysis, sonication and centrifugation, 1/20 of each sample was kept for total DNA analysis and the remaining was used for immunoprecipitation with polyclonal antibodies against Spo0J. Analysis of DNA samples was performed by real-time PCR in a Light-Cycler (primers used are listed in Supplementary Table SV). The data obtained for each region of the total and immunoprecipitated DNA sample were interpolated to a standard curve constructed with known amounts of purified, full-length ϕ29 DNA. The results were expressed as pg of DNA per ml of culture. Binding values are expressed as IC (= [immunoprecipitated DNA/total DNA] $\times 10^6$). Specific amplification of DNA regions by each primer set was checked by PCR reactions using the following purified DNAs as template: full-length ϕ29, ϕ29-infected and noninfected cells. Moreover, a melting analysis was performed at the end of each PCR by continuous fluorescence measurement from 65 to 95°C to ensure that a single product was amplified.

Analysis of in vivo phage transcripts produced as a function of cell development

The resuspension assay (see above) was used to induce synchronized induction of the sporulation process. Thus, after resuspension in SM complemented with 5 mM $MgCl_2$, the culture was divided into four aliquots and incubated at 37°C. One aliquot was infected

with ϕ29 directly after resuspension. The other aliquots were infected 15, 30 or 45 min after resuspension. Infection was allowed to proceed for 25 min after which the cells were harvested and processed to isolate their total RNA and subsequently subjected to primer extension as described before (Monsalve *et al*, 1995). The resulting cDNAs were resolved by electrophoresis in denaturing 6% polyacrylamide gels. Mean values of the relative abundance of the transcripts at the different times were obtained by densitometry of the signals of three independent experiments and were normalized to $t = 0$.

β-*Galactosidase assays*

B. subtilis strains containing *lacZ* fusions were grown in Schaeffer's medium and samples withdrawn at 45 min intervals. Levels of β-galactosidase activity were determined as described (Daniel *et al*, 1996).

Spo0A purification

Spo0A was purified essentially as described (Muchová *et al*, 2004). Similar to published results (Ladds *et al*, 2003), ~40% of the purified Spo0A protein was in its dimeric active form as assessed by gel filtration.

In vitro transcription and footprinting assays

In vitro run-off transcription and DNase I and potassium permanganate footprinting were performed as described (Rojo and Salas, 1995). Plasmid pDM1_A2bc DNA containing the 205 bp ϕ29 region spanning promoters A2c and A2b was used in PCR reactions with primer sets seq2–seq4 or seq5–seq1 to obtain DNA templates used in Figure 4A and B, respectively. The PCR products were labeled by filling-in the protruding ends, generated by *Eco*RI (seq2–seq4) or *Bam*HI (seq1–seq5) digestion, using Klenow enzyme. Similar strategies were used for templates used in Figures 5 and 7. Thus, the PCR products obtained with plasmid pDM1_C2A and primer set seq3–seq4 (Figure 5), and with plasmid pDM_A3 and primer set seq5–A2bL2 (Figure 7), were labeled by filling-in the *Hin*dIII and *Bam*HI restriction site, respectively. The C2 promoter fragment used in the $KMnO_4$ footprint assay was obtained by PCR with primer set seq3–seq8 and plasmid pDM1_C2A as template DNA. The PCR product was labeled by filling-in the *Bam*HI-digested PCR product.

Supplementary data

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

Acknowledgements

We thank Imrich Barák for sending the *E. coli* Spo0A overproducing strain and helpful comments on protein purification, and Martín Alcorlo for help with real-time PCR. We also gratefully acknowledge helpful discussions with Fernando Rojo and Richard Daniel. This investigation was supported by grants 2RO1 GM27242-24 from the National Institutes of Health and BMC2002-03818 from the Spanish Ministry of Science and Technology to MS and an Institutional grant from Fundación Ramón Areces to the Centro de Biología Molecular 'Severo Ochoa'. VC is holder of a predoctoral fellowship from the Spanish Ministry of Science and Technology, which also supported WJJM by means of the 'Ramón y Cajal' program.

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