Differential Spo0A-mediated effects on transcription and replication of the related *Bacillus subtilis* phages Nf and φ29 explain their different behaviours *in vivo*

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**ABSTRACT**

Members of groups 1 (e.g. φ29) and 2 (e.g. Nf) of the φ29 family of phages infect the spore forming bacterium *Bacillus subtilis*. Although classified as lytic phages, the lytic cycle of φ29 can be suppressed and its genome can become entrapped into the *B. subtilis* spore. This constitutes an alternative infection strategy that depends on the presence of binding sites for the host-encoded protein Spo0A in the φ29 genome. Binding of Spo0A to these sites represses φ29 transcription and prevents initiation of DNA replication. Although the Nf genome can also become trapped into *B. subtilis* spores, *in vivo* studies showed that its lytic cycle is less susceptible to spo0A-mediated suppression than that of φ29. Here we have analysed the molecular mechanism underlying this difference showing that Spo0A differentially affects transcription and replication initiation of the genomes of these phages. Thus, whereas Spo0A represses all three main early promoters of φ29, it only represses one out of the three equivalent early promoters of Nf. In addition, contrary to φ29, Spo0A does not prevent the *in vitro* initiation of Nf DNA replication. Altogether, the differences in Spo0A-mediated regulation of transcription and replication between φ29 and Nf explain their different behaviours *in vivo*.

**INTRODUCTION**

The Gram-positive soil bacterium *Bacillus subtilis* responds to environmental changes by inducing an appropriate differentiation process and, as a last resort mechanism, forms highly resistant endospores that can remain dormant for long periods of time. Upon sensing nutrient limitation, a signal-transduction pathway is stimulated leading to the activation by phosphorylation of the key regulator for entry into sporulation, Spo0A [for reviews on sporulation see (1–3)]. Once activated, Spo0A~P forms dimers (4,5) that can bind to DNA sequences containing (im)perfect ‘OA-boxes’ (5’-TGTCGAA-3’), where it exerts its role as a transcriptional activator or repressor. In addition, Spo0A~P can block the initiation of DNA replication by binding to OA boxes located at the origin of DNA replication (6).

Besides being essential for the activation of early sporulation genes, Spo0A is also involved in the regulation of other *B. subtilis* developmental pathways that act before sporulation. In fact, the expression of more than 500 genes (>10% of the total genes of *B. subtilis*) is affected by Spo0A (7). Fujita et al. (8) showed that genes of the Spo0A regulon can be categorized into groups that respond differently to high and low Spo0A concentrations depending on their binding affinity for Spo0A. Moreover, the cellular level of activated Spo0A is subjected to several auto-stimulatory loops that involve both transcription of spo0A and phosphorylation of the Spo0A protein (9,10). These complex regulatory systems lead to heterogeneous levels of Spo0A~P in individual cells with their maximum levels during vegetative growth being lower than those reached during sporulation (11–13).

*B. subtilis* cells do not lyse when they are infected during the initial stages of sporulation with phage φ29 (14). Instead, the injected φ29 genome becomes trapped in the spore. Upon germination of the spore, the phage genome enters its lytic phase resulting in lysis of the cell and liberation of phage progeny [for review see (15)]. The phage φ29 genome consists of a linear double-stranded DNA (dsDNA) with a terminal protein (TP) covalently linked at each 5’ end [for review see (16)]. A genetic and transcriptional map of the φ29 genome is shown in
Figure 1. Phage φ29 DNA transcription is divided into early and late stages [for review see (17,18)]. All late genes, encoding phage structural, morphogenetic and lysis proteins, are clustered in a single, centrally located operon that is transcribed from the late promoter A3. The late operon is flanked at either side by an early operon. The one on the left, which contains, among others, genes coding essential DNA replication proteins, is under the control of the tandemly-organized promoters A2b and A2c. The right-side early operon, which encodes proteins that are involved in internalization of phage DNA during the genome injection step and in DNA replication, is transcribed from the C2 promoter. Initiation of φ29 DNA replication occurs via a so-called protein-primed mechanism [for review see (16)]. The essential φ29 protein p6 functions as initiator of DNA replication by forming a nucleoprotein complex near the origins of DNA replication constituted by the TP-containing DNA ends (19,20).

The genome of φ29 contains binding sites for the host-encoded proteins Spo0J (parS sites) and Spo0A (0A boxes), which play crucial roles in the alternative infection strategy (6,21). On the one hand, evidence has been provided that the Spo0J partitioning protein is involved in the segregation of the φ29 genome into the prespore (21). On the other hand, Spo0A is directly responsible for suppression of the lytic cycle of φ29 acting at the level of transcription and replication. Thus, Spo0A-binding sites are located near the late A3 and early A2c, A2b and C2 promoters (Figure 1). Binding of Spo0A to these sites inhibits transcription from the early promoters and prevents activation of the late promoter (21). In addition, Spo0A-binding sites are located near the left and right φ29 origins of replication. Binding of Spo0A to the origin regions interferes with protein p6 binding preventing the initiation step of φ29 DNA replication (6). In vivo studies indeed showed that φ29 development is suppressed in sporulating cells. Interestingly, these studies also revealed that φ29 development is suppressed in a subpopulation of exponentially growing cells expressing low levels of spo0A. Together, these results showed that φ29 development is suppressed by low levels of Spo0A (11).

φ29 belongs to a family of related phages that is divided into three groups [for review see (18)]. The phages belonging to groups 1 and 2 infect B. subtilis. We were interested to know if the alternative infection strategy of φ29, which belongs to group 1, is conserved in phages belonging to group 2, such as Nf. Previous studies showed that, like φ29, the genome of Nf becomes trapped into spores when cells are infected during the initial stages of sporulation (22). However, Nf has a clearly distinct behaviour in vivo. Thus, although development of Nf is affected in a spo0A-mediated way, its lytic cycle is not or hardly suppressed in cells infected during exponential growth and substantial levels of Nf DNA replication are still observed in cells infected under sporulating conditions (22). Determination and analysis of the Nf genome sequence revealed that it has a genetic organization similar to that of φ29 (Figure 1). However, the genome of Nf contains only one consensus 0A box, versus the six present on the
\( \phi 29 \) genome. As Spo0A can bind to DNA sequences that deviate from the consensus one, it remained unclear however if and how the differences in 0A boxes are related to the dissimilar \textit{in vivo} behaviour of \( \Phi f \) and \( \phi 29 \). In this work we have analyzed the effect of Spo0A on the \( \Phi f \) \textit{in vitro} transcription and DNA replication initiation step. The results obtained reveal differences in the way Spo0A regulates \( \Phi f \) and \( \phi 29 \) development. As in \( \phi 29 \), Spo0A prevents activation of the late \( \Phi f \) promoter, but only represses one out of its three main early promoters. In addition, unlike \( \phi 29 \), Spo0A does not prevent the \textit{in vitro} initiation of \( \Phi f \) DNA replication. Together, our findings strongly indicate that differences in the presence and location of binding sites for the host-encoded Spo0A protein form the molecular basis for the different \textit{in vivo} behavioural strategies of \( \Phi f \) and \( \phi 29 \).

**MATERIALS AND METHODS**

**Strains, plasmids and growth conditions**

Strains and plasmids, and oligonucleotides (Isogen Life Sciences BV, The Netherlands) used are listed in Supplementary Tables SI and SII, respectively. Kanamycin (30 \( \mu \)g/ml) was used for selection in \textit{Escherichia coli}.

**Spo0A purification**

Spo0A was purified essentially as described (5) with the indicated modifications (21). Protein Spo0A dimers, produced upon phosphorylation, constitute the Spo0A active form (4,5,23,24). Similar to published results (24), \~40% of the purified Spo0A protein was in its dimeric active form as assayed by gel filtration.

**DNase I footprinting and \textit{in vitro} transcription assays**

DNase I footprinting and \textit{in vitro} transcription assays were performed as described (25). DNA fragments were amplified with the appropriate oligonucleotides using either the \( \phi 29 \) or \( \Phi f \) genome as template. The PCR products used as templates in the DNase I footprint assays were labelled at one of the 5' ends by treating the appropriate oligonucleotide with polynucleotide kinase and \([\gamma^{32}\text{P}]\text{ATP} \) before the amplification reaction.

**\( \Phi f \) TP-dAMP formation (protein-primed initiation of replication)**

The incubation mixture of the protein-primed initiation reactions contained, in 25 \( \mu \)l, 50 mM Tris–HCl, pH 7.5, 1 mM MnCl\( _2 \), 40 mM ammonium sulphate, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml BSA, 0.1 mM MnCl\( _2 \), 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml BSA, 0.1 mM [\( \gamma^{32}\text{P}]\text{ATP} \) (1 \( \mu \)Ci), 1.8 nM of \( \Phi f \) TP-DNA, 6 nM of \( \Phi f \) DNA polymerase (DNAP), 13 nM of \( \Phi f \) TP, 33 \( \mu \)M of \( \Phi f \) p6 and the indicated amounts of Spo0A. After incubation for 10 min at 30\( ^\circ \)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 formed. Spo0A concentration used ranged from 2 to 16\( \mu \)M (2-fold dilution steps).

**RESULTS**

**Effect of Spo0A on transcription of the main \( \Phi f \) promoters**

As a first approach to study the role of Spo0A in transcriptional regulation of the \( \Phi f \) genome, we performed \textit{in vitro} transcription assays of the three main \( \Phi f \) early promoters A2b, A2c and C2, and of the late A3 promoter in the absence or presence of Spo0A. In \( \phi 29 \), Spo0A is directly responsible for repression of the early A2c, A2b and C2 promoters and prevents protein p4-mediated activation of the late A3 promoter (21). The functionality of the Spo0A protein used was inferred from DNase I footprinting assays and from \textit{in vitro} transcription assays using the \textit{B. subtilis} spoIIG promoter that is activated by Spo0A (26) (data not shown). The results of the \( \Phi f \) transcription assays revealed that Spo0A represses the A2c promoter (Figure 2A), does not affect transcription of the C2 promoter (Figure 2B), and slightly affects transcription of the A2b promoter at the highest Spo0A concentration used (Figure 2C). In addition, the results show that Spo0A inhibits the p4-mediated activation of the late A3 promoter (Figure 2D).

**Footprint analysis of Spo0A binding to the \( \Phi f \) promoters**

The genome of \( \Phi f \) contains one consensus 0A box 5'-TGT CGAA-3' (22), as originally defined by Strauch et al. (27), which is located in between the divergently oriented early A2b and late A3 promoters (Figure 1). The results presented above show that the A2c promoter of \( \Phi f \) is repressed by Spo0A, although it has no consensus 0A box near it. Since Spo0A can also bind to DNA sequences that deviate from the consensus 0A box, we studied possible binding of Spo0A to the A2c and other \( \Phi f \) promoters. Spo0A did not protect any region at or near the C2 promoter, nor did it affect binding of RNA polymerase (RNAP) to the C2 promoter (not shown). These results are in line with the findings presented above showing that Spo0A does not affect transcription from the C2 promoter. Representative footprints of Spo0A binding to the A2c and A2b-A3-promoter regions are shown in Figure 3A and B, respectively. Binding of Spo0A at the A2c-promoter region results in protection of 25–30 bp that includes sequences upstream from and part of the core promoter. Inspection of the protected region revealed that it contains two imperfect 0A boxes separated by 3 bp (Supplementary Figure S1). Each of these imperfect 0A boxes contains the same single deviation (5'-TGTGAA-3') with respect to the consensus 0A box sequence (5'-TGTGAA-3'). The position of these 0A boxes relative to the A2c promoter is identical to the 0A box region, 0A-1, at the \( \phi 29 \) A2c promoter. Accordingly, we named this Spo0A-binding site in \( \Phi f \) 0A-1'. In \( \phi 29 \), another Spo0A-binding site, 0A-2', is located in between the A2c and A2b-promoter region. The footprint analysis showed that an equivalent Spo0A-binding site is absent in \( \Phi f \).
As expected, Spo0A also binds to the Nf region encompassing the single perfect 0A box located between the divergently oriented early A2b and late A3 promoter (Figure 3B). As in the case of the A2c promoter, binding of Spo0A to this region results in protection of about 25–30 bp. Based on the comparative location of the 0A-3 box region in the genome of φ29, this Spo0A-binding site in Nf was named 0A-3'. Phage φ29 0A box 3 region constitutes a relative high Spo0A-binding site (11) according to the classification in high and low-affinity-binding sites described by Fujita et al. (8).

The amount of Spo0A needed to observe retardation of a DNA fragment containing the 0A-3' region (Supplementary Figure S2) is similar to that needed with φ29 Spo0A-binding site 3 (11), suggesting that this Spo0A-binding site of the Nf genome also constitutes a high-affinity binding site. A more extensive analysis of Spo0A binding to this site is presented below.

Spo0A prevents binding of RNAP to the A2c promoter but hardly interferes with binding of RNAP to the A2b promoter

The possibility that Spo0A interferes with binding of RNAP to the Nf early A2c and/or A2b promoters was tested by DNase I footprinting (Figure 4 and Supplementary Figure S3, respectively). RNAP binds efficiently to the A2c promoter, generating a footprint on the template strand that spans the region from +18 to –51 and inducing hypersensitivities at positions /C0 36 and /C0 37 relative to its transcription start site (Figure 4, lanes 2 and 10). However, RNAP was unable to bind to the A2c promoter when Spo0A was bound to the 0A-1' region (Figure 4, lanes 7–8).

RNAP binds weakly to the A2b promoter of Nf (Supplementary Figure S3), a feature that is also observed for the φ29 A2b promoter (21,28,29). One of the most characteristic changes upon RNAP binding to Nf A2b promoter is the generation of a hypersensitive site at position –37 on the template strand relative to its transcription start site (Supplementary Figure S3, lane 3). This hypersensitivity is maintained in the presence of increasing amounts of Spo0A, except in the presence of the highest Spo0A concentration tested at which the hypersensitivity is diminished (Supplementary Figure S3, lane 8). These results show that binding of Spo0A to the Nf 0A-3'
region hardly interferes with binding of RNAP to the early A2c promoter, in agreement with the observation that Spo0A hardly affects transcription from the A2b promoter (see above).

Altogether, various in vitro approaches show that Spo0A represses the Nf A2c promoter but hardly affects the activity of its A2b promoter. These in vitro results were corroborated by in vivo primer extension analysis. Thus, cultures of wild-type and isogenic spo0A null mutant cells were infected with Nf during the early stationary phase. Quantification of the primer extension experiments revealed a ratio of 19.2 for the A2c/A2b-promoter activity in the spo0A-deletion strain. This ratio decreased to 9.1 in the case of the wild-type strain (Supplementary Figure S4).

Spo0A prevents activation of late Nf transcription by precluding p4-mediated recruitment of RNAP to the late A3 promoter

The results of the in vitro transcription assays presented in Figure 2D show that Spo0A prevents activation of the

region hardly interferes with binding of RNAP to the early A2c promoter, in agreement with the observation that Spo0A hardly affects transcription from the A2b promoter (see above).

Altogether, various in vitro approaches show that Spo0A represses the Nf A2c promoter but hardly affects the activity of its A2b promoter. These in vitro results were corroborated by in vivo primer extension analysis. Thus, cultures of wild-type and isogenic spo0A null mutant cells were infected with Nf during the early stationary phase. Quantification of the primer extension experiments revealed a ratio of 19.2 for the A2c/A2b-promoter activity in the spo0A-deletion strain. This ratio decreased to 9.1 in the case of the wild-type strain (Supplementary Figure S4).

Spo0A prevents activation of late Nf transcription by precluding p4-mediated recruitment of RNAP to the late A3 promoter

The results of the in vitro transcription assays presented in Figure 2D show that Spo0A prevents activation of the

NF late A3 promoter. This promoter, which lacks a typical −35 box, is activated by p4-mediated recruitment of RNAP. The single consensus 0A box of NF is positioned in between the A3 promoter and the upstream p4-binding site 3, as well as the early A2b promoter. Binding of spo0A to this 0A box 3 region might therefore interfere with activation of the late A3 promoter. This possibility was examined by DNase I footprinting using a DNA fragment encompassing the late A3 promoter and upstream sequences that include 0A box 3 and the p4-binding site 3. Figure 5 shows the footprint generated by binding of spo0A to NF box region 3 (lanes 3–5). Binding of p4 to its binding site 3 generates a typical pattern consisting of hypersensitive bands and intermittent protected regions (lane 7). Lanes 8–12 show that protein p4 and spo0A can bind simultaneously to their flanking cognate-binding sites. As expected, RNAP binds to the A3 promoter only in the presence of protein p4 (compare lanes 14 and 15). The fact that the hypersensitive bands and protected intermittent regions, characteristic of the binding of p4 to its cognate site 3, are more pronounced in the presence of RNAP, as compared with those generated by protein p4 alone (compare lanes 7 and 15), strongly indicate cooperative binding of p4 and RNAP to the A3-promoter region. Importantly, RNAP recruitment was lost under
conditions in which binding of Spo0A to its cognate 0A box 3' region became evident (lanes 18–21). These results show that binding of Spo0A to NF 0A-3' region prevents p4-mediated recruitment of RNAP to the late A3 promoter.

Differences in Spo0A-mediated regulation of the A2b-A3 promoters of NF and \( \phi 29 \) are due to a different location of the imperfect 0A box relative to the perfect 0A box 3

The results presented above show that the single consensus 0A box, 0A-3', at the NF genome forms part of a bona fide Spo0A-binding site. This consensus 0A box is located at a similar position to that of 0A box 3 at the \( \phi 29 \) genome. In both genomes the consensus 0A box is located 40-bp upstream the -10 sequence of the late A3 promoter and 27 and 26-bp upstream the -35 sequence of the early A2b promoter of NF and \( \phi 29 \), respectively. In \( \phi 29 \), it has been demonstrated that binding of Spo0A to this region has a dual effect. On the one hand, it represses the A2b promoter, and on the other hand it prevents activation of the divergently oriented late A3 promoter (21). In contrast, although binding of Spo0A to the 0A-3' region at the NF genome prevents activation of the late NF promoter A3, it hardly affects the activity of the early A2b promoter. DNase I footprinting was used to examine whether this different effect of Spo0A on the regulation of the early A2b promoters of NF and \( \phi 29 \) is due to differences in binding of Spo0A to their 0A-3 regions (Figure 6). As expected, for both NF and \( \phi 29 \), sequences corresponding to the consensus 0A box are protected upon Spo0A binding. Interestingly, whereas in \( \phi 29 \) additional Spo0A-protected sequences extend in the direction of the A2b promoter, additional sequences are protected in the opposite direction in the case of NF; i.e. in the direction of the A3 promoter. Inspection of the Spo0A-protected regions of \( \phi 29 \) and NF revealed that in both cases the additional Spo0A-protected sequences contain an imperfect 0A box with 3 mismatches that is separated from the consensus 0A box sequence by 3 bp. However, these imperfect 0A boxes are located at opposite sides of the consensus one in NF and \( \phi 29 \) (Figure 8A). The different location of the imperfect 0A box with respect to the consensus 0A box is most likely responsible for the observed different Spo0A-mediated effects on A2b-promoter activity of NF and \( \phi 29 \) (see also ‘Discussion’ section).

Spo0A does not block in vitro p6-stimulated initiation of NF DNA replication

In \( \phi 29 \), binding of Spo0A to the origin regions inhibits initiation of DNA replication in vitro by interfering with protein p6 binding (6). To examine whether Spo0A inhibits also the p6-stimulated replication initiation step of NF, in vitro NF TP-DNA replication initiation assays were performed in the absence or presence of Spo0A. Supplementary Figure S5 shows that the initiation of TP-DNA replication is not inhibited in the wide range of Spo0A concentrations tested.

Spo0A binds to NF origins of DNA replication but hardly interferes with binding of the replication initiator protein p6

For \( \phi 29 \) it has been shown that Spo0A binds to an approximate 40-bp region located 30 bp from the extreme left and right DNA ends, and that this binding interferes with formation of the replication initiation p6-nucleoprotein complex, blocking the initiation step of DNA replication (6). DNase I footprinting analyses were used to examine whether Spo0A has a similar effect in NF. The results obtained show that Spo0A binds to an ~20-bp region (Figure 7, lanes 7–10) which is located more closely to the right DNA end than in the case of \( \phi 29 \) (6). Similar results were obtained for the left NF origin region (not shown). Inspection of the Spo0A protected regions revealed that both origin regions contain two imperfect 0A boxes, located more proximal to the DNA ends than the four consecutive 0A boxes at the left and right origins of \( \phi 29 \) (see discussion and Figure 8B and C).
Binding of protein p6 to the origin regions of Nf generates a typical footprint characterized by protected regions alternating with hypersensitive sites (30). Interestingly, the p6-induced footprint remained intact in the presence of a wide range of Spo0A concentrations (Figure 7, lanes 12–16). Only in the presence of the highest concentration of Spo0A tested the p6-induced footprint became partially lost (Figure 7, lane 17). Similar results were obtained for the left Nf origin region (data not shown).

DISCUSSION

Early reports described that various phages that infect B. subtilis exploit the ability of this bacterium to form spores in response to conditions of prolonged stress by trapping the infecting phage genome into the spore. Presumably this alternative infection strategy enhances the fitness of the phage under natural conditions. B. subtilis spores are among the most resistant biological entities known and hence the phage genome present within the spore is optimally protected from environmental insults.

So far, the molecular mechanisms underlying this alternative infection strategy have been studied in detail only for φ29. These studies revealed that the adaptation involves at least two host-encoded DNA-binding proteins, Spo0J and Spo0A, and the presence of binding sites for these proteins on the phage genome (parS sites and 0A boxes, respectively). The φ29 genome contains five parS sites and evidence has been provided that φ29 exploits part of the host-encoded chromosomal segregation machinery for spore-entrapment of the infecting φ29 genome (21). φ29 DNA also contains various binding sites for Spo0A. 0A boxes are located near the φ29 late A3 and early A2c, A2b and C2 promoters. Binding of Spo0A to these sites results in repression of the three early promoters and in inhibition of activation of the late promoter (21). In addition, bona fide Spo0A-binding sites are located near the left and right DNA ends of φ29 (6). Initiation of DNA replication at the TP-containing DNA ends (replication origins) requires binding of the replication initiator protein p6 to an extended region at the DNA ends and this protein is essential for in vivo φ29 DNA replication (19,31). Binding of Spo0A to the φ29 right and left DNA end regions interferes with formation of the p6-nucleoprotein complex blocking initiation of φ29 DNA replication (6). Thus, Spo0A suppresses phage φ29 development at the level of transcription and DNA replication ensuring a strict regulation by Spo0A, as demonstrated by subsequent in vivo studies. These latter studies showed that φ29 development was not only suppressed in cells that were in the initial stages of sporulation, when Spo0A is overexpressed, but also in a subpopulation of exponentially growing cells where low-levels of active Spo0A are produced (11).

In previous work we showed that phage Nf development is less stringently suppressed by Spo0A than that of φ29. To gain insight into these differences, the entire sequence of the Nf genome was determined. Analysis of the sequence revealed that, whereas the φ29 genome contains six consensus 0A box sequences, the genome of Nf only contains one which corresponds to φ29 0A box 3 located between the divergently oriented early A2b and late A3 promoters (22). Here we studied in detail the molecular mechanisms underlying the different behaviours of Nf and φ29.

Fujita et al. (8) analyzed various B. subtilis Spo0A-regulated genes and showed that there is a good correlation between the affinity for Spo0A in vitro and their responsiveness to high or low doses of Spo0A in vivo. Based on this analysis, retardation of DNA fragments encompassing high-affinity-binding sites required 20–60-fold less Spo0A than fragments containing low-affinity-binding sites. Here we showed that the Nf Spo0A-binding site 0A-3′ (Supplementary Figure S2) constitutes, like φ29 Spo0A-binding site 3 (11), a high-affinity-binding site (retardation requires 2- to 4-fold higher amounts of
Figure 8. Sequence alignments of the A2b-A3 promoter region (A), of the left origins (B) and of the right origins (C) of the genomes of some group 1 (φ29, φ15, PZA and BS32) and group 2 (Nf, B103 and M2Y) members of the φ29-family of phages. The perfect and imperfect oA boxes are indicated with dark and light grey rectangles, respectively. The directionality of the oA boxes is indicated with arrows. The nucleotides that deviate from the consensus SpolA-binding site are indicated with lower case letters. The striped boxes depict the /C010 and /C035 promoter elements. The different accession numbers of the sequences used are the same as in Pecenkova and Paces (33). The Clustal W program (www.ebi.ac.uk/Tools/clustalw2/index.html) was used for aligning the DNA sequences.
Spo0A than the classified high-affinity-binding sites present in the *B. subtilis* genome), making it unlikely that this would be the cause of the different *in vivo* behaviour between Nf and φ29. Rather, as outlined below, it is most likely that the different behaviours are due to differences in the number and location of the Spo0A-binding sites.

Results presented in this work show that Spo0A represses the early Nf A2c promoter and prevents activation of the late Nf A3 promoter in a similar way as the equivalent promoters of φ29. Importantly however, we show here that, in contrast to the situation in φ29, Spo0A does not bind near the C2 promoter and in line with this does not affect the activity of this promoter that drives expression of the right-side early operon. In addition, in φ29, binding of Spo0A to the 0A box region 3 does not only prevent activation of the late A3 promoter but also causes repression of the divergently oriented early A2b promoter. Based on the almost identical position of the single consensus 0A box, 0A-3', present in the Nf genome to the consensus 0A box 3 in the φ29 genome, it was reasonable to expect that binding of Spo0A to the 0A-3' region of Nf would exert the same effects as in φ29. However, we found that binding of Spo0A to the Nf 0A box 3' region hardly affects binding of RNAP to the Nf A2b promoter and consistent with this, hardly affects its activity. Moreover, the A2c promoter was more strongly repressed than the A2b promoter *in vivo* in a spo0A-dependent manner when cells were infected during the early stationary phase. An explanation for this was obtained by analyzing in detail the Spo0A-binding boundaries to this region in Nf. Thus, like the φ29 0A-3-binding site, also the 0A-3'-binding site of Nf is composed of a consensus and a flanking imperfect 0A box. However, the imperfect 0A box in Nf is located in the direction of the late A3 promoter, in contrast with the situation in φ29, where the flanking imperfect 0A box is located in the direction of the early A2b promoter. In φ29, Spo0A binding represses its A2b promoter by preventing the C-terminal domain of the RNAP α subunit from binding to the UP element, which is required for its activity (21,28). Spo0A does not bind to the corresponding sequences of the Nf A2b promoter and most likely this explains why Spo0A does not repress this promoter. To our knowledge, this is the first time in which the position of an imperfect 0A box with respect to a perfect one is crucial for differential transcriptional regulation.

Thus, contrary to φ29, Spo0A does not or hardly represses the early Nf promoters C2 and A2b that drive expression of the right and left-side early operons, respectively (Figure 1) encoding all essential DNA replication proteins. Nf DNA replication may therefore occur in Spo0A expressing cells if Spo0A will not block Nf DNA replication initiation. The results presented here show that, unlike the situation in φ29, Spo0A does not affect the *in vitro* Nf DNA replication initiation step. These results explain the *in vivo* data in which it was found that substantial levels of Nf DNA replication occurs when cells were infected during the early stages of sporulation when Spo0A is over expressed. Hence, the different *in vivo* behaviours between the related phages Nf and φ29 are most likely due to differential effects exerted by Spo0A on the level of transcription and replication initiation of these phages.

Results presented here and those published before (6) show that Spo0A is able to bind near the left and right DNA ends of the genomes of both φ29 and Nf. However, in φ29, Spo0A binds to a ~40-bp region located 30 bp from the DNA ends (6). These binding sites overlap with the nucleation site of protein p6 (32). Consequently, binding of Spo0A to these sites efficiently prevents binding of p6 to this and flanking origin regions (6). Footprinting analyses presented here show that the situation is different for Nf. In this case Spo0A binds a ~20-bp region which is located more closely to the DNA ends than in φ29. In addition, except for the highest concentration tested, Spo0A binding to these regions does not affect protein p6 binding. The fact that the Nf genome contains bona fide Spo0A-binding sites near its DNA ends suggests that Spo0A may influence DNA replication initiation under certain *in vivo* conditions. Support for this possibility is the observation that Nf is able to infect wild-type *B. subtilis* cells in late stationary phase cultures but that under these conditions DNA replication is suppressed in a spo0A-dependent way (22).

Phages belonging to the φ29 family infecting *B. subtilis* have been classified into two groups [for review see (18)]. The first group includes phages φ29, PZA, φ15 and BS32 and the second group includes B103, Nf, and M2Y. This classification was based on serological properties, DNA physical maps, peptide maps and partial or complete DNA sequences. It is interesting to note that it is likely that this classification can be extended to differences in stringency of Spo0A-mediated suppression of phage development. The entire genome of two members of group 1 (φ29 and PZA) and two members of group 2 (Nf and B103) are known. In addition, sequences corresponding to the left and right DNA end regions of φ15 (group 1) and M2Y (group 2) are known, as well as the sequence of the right DNA end region of group 1 phage BS32. Multiple alignments of the sequences corresponding to the central promoter region, and the left and right DNA end regions of these phages are shown in Figure 8. Analysis of the centrally located A2c-A3-promoter region shows that the number, location and sequences of the perfect and imperfect 0A boxes are conserved within members of group 1 (φ29 and PZA) and within members of group 2 (Nf and B103) (Figure 8A).

Similar conservation of the 0A boxes is observed for the left and right side DNA ends (Figure 8B and C, respectively). Most interestingly, in these latter cases it is clear that members of group 2 contain a deletion of 25 and 50 bp at their left and right DNA end regions, respectively, corresponding to various imperfect 0A boxes in the group 1 phage genomes. Most probably this deletion is related to the different Spo0A-mediated effects on DNA replication initiation between φ29 and Nf and strongly argues that these differences can be extended to all members of group 1 and group 2.

*In toto*, the combination of these and previous results shows that differences in the number and position of 0A-boxes on the genomes of phages belonging to the
\(\phi 29\) family lead to a different adaptation of their life cycle to the physiological state of the infected cell.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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