A functional Spo0A is required for maximal *aprE* expression in *Bacillus subtilis*

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Abstract The initiation of sporulation in *Bacillus subtilis* is under control of the transcriptional factor Spo0A. Most Spo0A mutants fail to initiate the sporulation process and all the sporulation initiated processes such as the synthesis of subtilisin. However, the product of *spo0A9V*, one of the several spo0A mutants characterized, distinguishes itself in the fact that, while it appears to effectively repress abrB, it fails to activate the *spoIIA* operon. The aim of this study was to examine the effect of the *spo0A9V* mutation on *aprE* expression and we found that in different genetic backgrounds, the *spo0A9V* mutation has a negative effect on *aprE::lacZ* expression.

Key words: Sporulation; *abrB* expression; β-Galactosidase fusion; Phosphorelay; Transition state regulator

1. Introduction

The initiation of sporulation in *Bacillus subtilis* is under control of the Spo0A transcriptional factor. Spo0A belongs to a large family of phosphorylation-activated signal transduction proteins that regulate adaptative responses in bacteria [1]. It is phosphorylated by a complex phosphorelay that includes several components needed to integrate multiple signals [2]. The *spo0A* gene product regulates negatively the *abrB* gene [3,4], a repressor of several genes which transcription is initiated during the transition between exponential growth and stationary phase [5]. As a positive effector, Spo0A is indispensable for activating the expression of at least three important stage II genes or operons [6]. Different mutants of *spo0A* gene have been isolated. Among them, the *spo0A9V* gene product (Spo0A9V) represses *abrB* but fails to activate the *spoII*A operon. Also, as deduced from qualitative assays on milk plates, when a strain carries the *spo0A9V* allele, the production of extracellular proteases appears normal [7]. These data support the suggestion that in a strain carrying the *spo0A9V* mutation, it is possible to dissociate the positive and negative regulatory properties of Spo0A [7].

The *aprE* gene of *Bacillus subtilis* codes for the extracellular protease subtilisin and its expression is controlled by Spo0A, AbrB and several other regulators [8]. In the present study we examined the effect of the *spo0A9V* mutation on *aprE* expression. We have found that in different genetic backgrounds, the *spo0A9V* mutation has a negative effect on *aprE* expression.

2. Materials and methods

2.1. Bacterial strains and plasmids

The genotypes of bacterial strains used in this work are listed in Table 1. All strains are derivatives of BG125 [9]. Strain JH13541 was kindly provided by J. Hoch. The rest of the strains were constructed for this study using the method described by Anagnostopoulos and Spizizen in [10]. To measure *aprE* gene expression we used an *aprE::lacZ* fusion integrated in the *amy* locus, as previously described [9].

2.2. β-Galactosidase assays

B. subtilis cells carrying pSG35.1 and pSub-204 (Fig. 1) were grown at 37°C in Schaeffer's sporulation media [11]. At regular time intervals, samples of 1 ml were taken and assayed for ß-galactosidase specific activity as described in [9].

2.3. Plasmid construction

Plasmid pSub-204 was constructed using standard recombinant DNA techniques. A DNA fragment that contains part of the *aprE* regulatory region was generated using the polymerase chain reaction (PCR) and the synthetic primers Sub1 and Sub2. The sequence of the primers is the following: 5'GGAATTCGGACTCAGGAGCATTTAACC3' (Sub1); 5'CGGGATCCCGTTAACGCAAACAACAAGC3' (Sub2). These primers hybridize with the −204 to −185 region of the *aprE* regulatory region and from the +83 to +100 of the *aprE* structural gene respectively [12]. The recognition sequence for the *Eco*RI or *Bam*HI restriction enzymes were introduced in the primers to facilitate the cloning of the DNA fragment into pSG35.1 (see Fig. 1).

3. Results and discussion

Since the mutation *spo0A9V* generates a SpoOA protein that can still repress *abrB* efficiently, but cannot activate the transcription of the *spoIIA* operon [7], we were interested in studying the effect of such mutation on *aprE* expression in different genetic backgrounds. For such a purpose we constructed the *B. subtilis* strains described in Table 1. It is important to mention that *hpr2* [13] and *degU32* [14–16] mutations cause *aprE* overexpression and that their effects are synergistic [9–17]. We measured *aprE* expression using an *aprE::lacZ* fusion in all these strains. In all the analyzed strains, β-galactosidase peaked between 2 and 3 h after ending exponential growth (data not shown). To facilitate comparisons between strains, the β-galactosidase specific activities reached at T_2 were compared. As can be seen in Table 2, the *spo0A9V* mutation had a negative effect, even in the strains carrying the *degU32* or *hpr2* mutations that cause *aprE* over-expression. The level of β-galactosidase activity was approximately 5-fold lower in the *spoOA9V* background in all the analyzed strains. We believe that such difference is small for a qualitative assay, like the measurement of halos on skim milk plates, and could explain the discrepancy between

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our data and the ones reported in [7]. This suggestion is also supported by the fact that milk plates reflect more accurately the levels of the neutral protease NprE.

These data suggest that a fully functional Spo0A is needed for an adequate *aprE* expression. Since *abrB* is regulated normally in a *spo0A9V* background [7], Spo0A should have another role on *aprE* expression (see below). The fact that the negative effect of *spo0A9V* was observed even in *a hpr2* mutant, suggests that this effect is not due to a secondary effect on *hpr*. It is also clear that the *degU32* mutation does not overcome the repressive effect of *spo0A9V*.

A possible explanation for these results could be that in the *spo0A9V* mutant, SinR activity is not adequately regulated. SinR is a regulator that prevents premature expression of stage II sporulation genes [18–20]. Its overproduction has a negative effect on *aprE* expression [21]. Furthermore, purified SinR binds to a specific region on the *aprE* regulatory region [22]. It is known that SinR is regulated by the action of SinI by protein–protein interaction, and that in a *spo0A* mutant, *sinI* is repressed [23].

To test this hypothesis, we analyzed again the effect of *spo0A9V* mutation in *wt*, *hpr2* and *degU32* strains. However, in this set of experiments, we used an *aprE* regulatory region devoided of the SinR binding site as shown in Fig. 1; it is known that such regulatory region is insensitive to SinR repression [12,18]. Essentially, this construction is the same as the one reported previously [12] in plasmid pSG35.8 and integrated into the *amy* locus. However, because we have found that some of the original construct carry an uncharacterized deletion on the *aprE* regulatory region, we built a new plasmid called pSub-204 carrying the upstream region of the *aprE* promoter up to the −204 position ($PaprE_{-204}$) (Fig. 1). The correct integration and size of this fusion was corroborated by PCR (data not shown).

After measuring ß-galactosidase levels in these strains (Table 3) it was found that in a *wt* background, after deleting the SinR binding site of the *aprE* regulatory region, the negative effect of the *spo0A9V* mutation disappeared. The same results was obtained in an *hpr2* background. Here is important to mention that as previously reported, and by unknown reasons, the effect of the *hpr2* mutation on this shorter version of the *aprE* regulatory region, is lost [12]. Furthermore, $PaprE_{-204}$ lost partially its capacity to respond to the *degU32* mutation. However, in this genetic background, the repression factor due to the *spo0A9V* mutation was similar (see Tables 2 and 3).

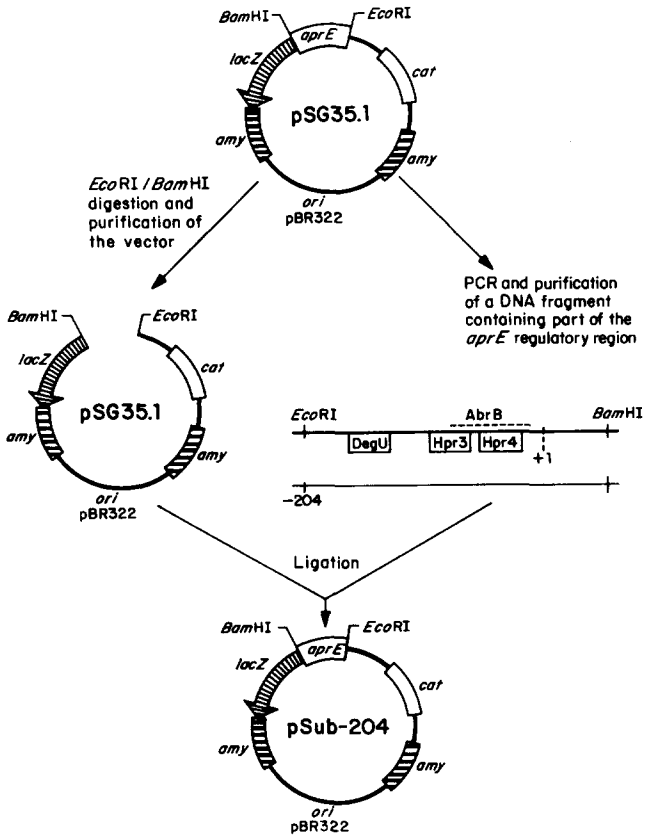

Fig. 1. Construction of plasmid pSub-204. Using PCR and synthetic oligonucleotides Sub1 and Sub2 (see section 2), a DNA fragment comprising part of the *aprE* regulatory region was obtained. It contains the putative DegU recognition site, two of the Hpr binding sites (Hpr3 and Hpr4) and the AbrB binding site previously located (17,3). These sites as well as other relevant features of this DNA fragment are also indicated.

These data indicate that part of the negative effect of the *spo0A9V* mutation is due to the binding of SinR to the *aprE* regulatory region. However in a *degU32* background, a fully functional Spo0A is required for maximal expression.

Recently it has been reported that a mutation in *spo0A* gene codon 97, suppressed the sporulation defect caused by the

Table 1
Bacterial strains

Strain	Genotype or description	Source and/or Ref.
BB17	BB80 *degU32*	E. Ferrari
BB80	Δ*npr hisA glyB*	E. Ferrari
BB82	Δ*npr hisA hpr2*	E. Ferrari
BB809	BB80 *amy(aprE::lacZ) cat*	This work
BB810	BB82 *amy(aprE::lacZ) cat*	This work
BB811	BB809 *degU32*	This work
BB812	BB80 *amy*($paprE_{-204}$::lacZ) cat	This work
BB813	BB82 *amy*($paprE_{-204}$::lacZ) cat	This work
BB814	BB812 *degU32*	This work
JH13541	*spo0A9V phe*	J. Hoch
BB815	BB809 *spo0A9V*	This work
BB816	BB810 *spo0A9V*	This work
BB817	BB811 *spo0A9V*	This work
BB818	BB812 *spo0A9V*	This work
BB819	BB813 *spo0A9V*	This work
BB820	BB814 *spo0A9V*	This work

Table 2
β-Galactosidase specific activity of the P*aprE::lacZ* fusion at T_2

	wt	*spo0A9V*	Ratio *wt/spo0A9V*
wt	2,262	394	5.7
hpr2	10,500	2446	4.3
degU32	55,600	8835	6.3

Table 3
β-Galactosidase specific activity of the P$aprE_{-204}$*::lacZ* fusion at T_2

	wt	*spo0A9V*	Ratio *wt/spo0A9V*
wt	1194	1024	1.1
hpr2	1273	972	1.3
degU32	23813	4353	5.5

spo0A9V mutation. This suppression was evident only in the presence of a mutation in the *spo0H* locus [24]. This result support the hypothesis that Spo0A interacts directly with the transcription machinery [7].

The fact that *spo0A9V* revertants required a mutation on *spo0H* is important because apparently δ^H, transcribes a diverse group of genes that play important roles in the bacterium responses to nutrient depletion, and it is required to amplify the response initiated by Spo0A-P [25]. In this sense, it is known that a deletion of *spo0H* lowers *aprE::lacZ* expression approximately five-fold [26], the same value observed in the *spo0A9V* mutants.

Based on these, it is possible to speculate that in a *spo0A9V* mutant, some genes of the δ^Hregulon that require a direct interaction between Spo0H and Spo0A-P, are not properly activated or repressed.

Finally, the data presented in this paper support the notion that in *B. subtilis*, the *aprE* gene can be expressed at different levels; one is obtained in *wt* strains under normal laboratory conditions, and is controlled by Spo0A mainly through the action of SinR. Another level is only obtained through the action of *degU32* mutation, and requires a fully functional Spo0A. This level of expression depends on an unidentified signal.

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