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Effects of the *sinR* and *degU32* (Hy) mutations on the regulation of the *aprE* gene in *Bacillus subtilis*

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Abstract The *aprE* gene of *Bacillus subtilis* codes for the serine alkaline protease known as subtilisin. Its expression is regulated by a complex network of activators and repressors that includes the products of *hpr*, *degU* and *sinR*. In order to understand the effect of these gene products on subtilisin expression, strains carrying combinations of the *degU*32(Hy), *hpr*2 and *sinR* null mutations, were constructed. We found that in all the genetic backgrounds tested, the *sinR* null mutation decreased *aprE* expression. Also, by measuring alkaline phosphatase synthesis and the formation of heat-resistant spores, as indicators of sporulation, we found that some of the mutant strains showed alterations in the sporulation process. These results suggest that these alterations are partially responsible for some of the observed changes in *aprE* expression.

Key words Sporulation process · Proteases · Gene regulation · Transition state regulators

Introduction

The *aprE* gene codes for the extracellular protease subtilisin and has been used as a model to understand the complex regulatory network that controls gene expression during the transition stage and the stationary phase in *Bacillus subtilis* (Kunst et al. 1974; Ferrari et al. 1985, 1986, 1988). AprE production is controlled by several regulators, such as DegU/DegS (Henner et al. 1988b; Kunst et al. 1988; Msadek et al. 1990), AbrB (Strauch et al. 1989), Hpr (also known as ScoC) (Kallio et al. 1991), and SinR (Gaur et al. 1991; Mandic-Mulec et al. 1992; Bai et al. 1992). Furthermore, direct binding of AbrB, Hpr and SinR to the *aprE* regulatory region has been demonstrated by in vitro gel retardation assays and/or footprinting analysis (Strauch et al. 1989, Gaur et al. 1991; Kallio et al. 1991).

A *B. subtilis* strain carrying the *degU*32(Hy) mutation overproduces several degradative enzymes, forms filaments and is deficient in competence and motility. On the other hand, SinR is a regulatory protein with dual function exerting both positive and negative effects on gene expression. Inactivation of *sinR* results in an increase in extracellular protease activities, filamentous growth, loss of competence and motility, and a deficiency in autolysin production (Gaur et al. 1986; Bai et al.1992). SinR binds to the *aprE* promoter at a region between −217 and −263 bp with respect to the transcription start point (Gaur et al. 1991). Furthermore, it is known that high levels of SinR repress production of extracellular proteases and the expression of an *aprE::lacZ* fusion (Gaur et al. 1986, 1991).

Based on all these results, it is generally assumed that SinR is a repressor directly acting on *aprE*. A prediction of this model is that a *sinR* null mutation might elevate *aprE* expression. However, to our knowledge this experiment has never been reported. Furthermore, the effect of such a mutation in strains that overproduce extracellular enzymes has not been described.

The analysis of the interactions between different regulators is necessary to comprehend the mechanism of *aprE* regulation, and especially because of the similarities between some of the *sinR* and *degU*32(Hy) phenotypes. Therefore, we decided to study in more detail the combined effects of these two mutations on the expression of *aprE*. In addition, the effect of the *hpr*2 mutation was also analyzed.

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Materials and methods

Strains and plasmids

The bacterial strains utilized in this work are listed in Table 1. The JM101 strain of *Escherichia coli* was used for plasmid amplification and was transformed by standard procedures (Sambrook et al. 1989). Strains of *B. subtilis* were derived from strain BG125 (Ferrari et al. 1986). The transformation procedures used for strain construction were as described by Anagnostopoulos and Spizizen (1961). The integrative plasmids pSG35.1 and pJF751 have been described previously (Ferrari et al. 1985, 1988). Plasmid *papr::lacZ* was constructed during this work, using standard gene cloning techniques (Sambrook et al. 1989). This plasmid carries a 634 bp DNA fragment containing the *aprE* regulatory region, derived from plasmid pSG35.1, and cloned into pJF751, as an *Eco*RI-*Bam*HI DNA fragment, creating an *aprE::lacZ* reporter gene. This recombinant vector allows integration into the chromosomal *aprE* locus by a single crossover event. Plasmid *papr::lacZ* also carries the chloramphenicol (Cm) transacetylase and the β-lactamase structural genes for selection.

Growth media and antibiotics

LB medium (Sambrook et al. 1989) was used for routine liquid growth, and maintenance of *E. coli* and *B. subtilis* strains. To study *aprE* expression and the sporulation process in *B. subtilis*, Schaeffer's sporulation medium was utilized (Schaeffer et al. 1965). Depending on the *B. subtilis* strain used (see Table 1), Cm and/or phleomycin were used at 5 μg/ml in LB plates.

Enzymatic assays

The β-galactosidase activity of the *B. subtilis* strains was measured as previously reported (Ferrari et al. 1985). Extracellular subtilisin activity was quantified using the synthetic substrate N-succinyl-l-Ala-l-Ala-l-Pro-l-Phe-*p*-nitroanalide and optimal conditions for alkaline protease activity (T. Graycar, pers. commun.). Briefly, culture supernatants were assayed in a 1 ml final volume containing 10 μl of a 20 mg/ml stock solution of N-succinyl-l-Ala-l-Ala-l-Pro-l-Phe-*p*-nitroanalide dissolved in dimethyl sulfoxide, 890 μl of a solution of 0.1 M TRIS, pH 8.6 at 25°C and 100 μl of supernatant. The assays measured the increase in absorbance at 410 nm/min due to hydrolysis and release of *p*-nitroanaline ($\in_{410}$ = 8480 M/cm, Estell et al. 1985).

Measurements of sporulation process markers

To measure alkaline phosphatase (APase) activity and heat-resistant spores by the method described by Nicholson and Setlow (1990), 1.5 ml samples were taken at the same time, from the same cultures in which β-galactosidase was assayed.

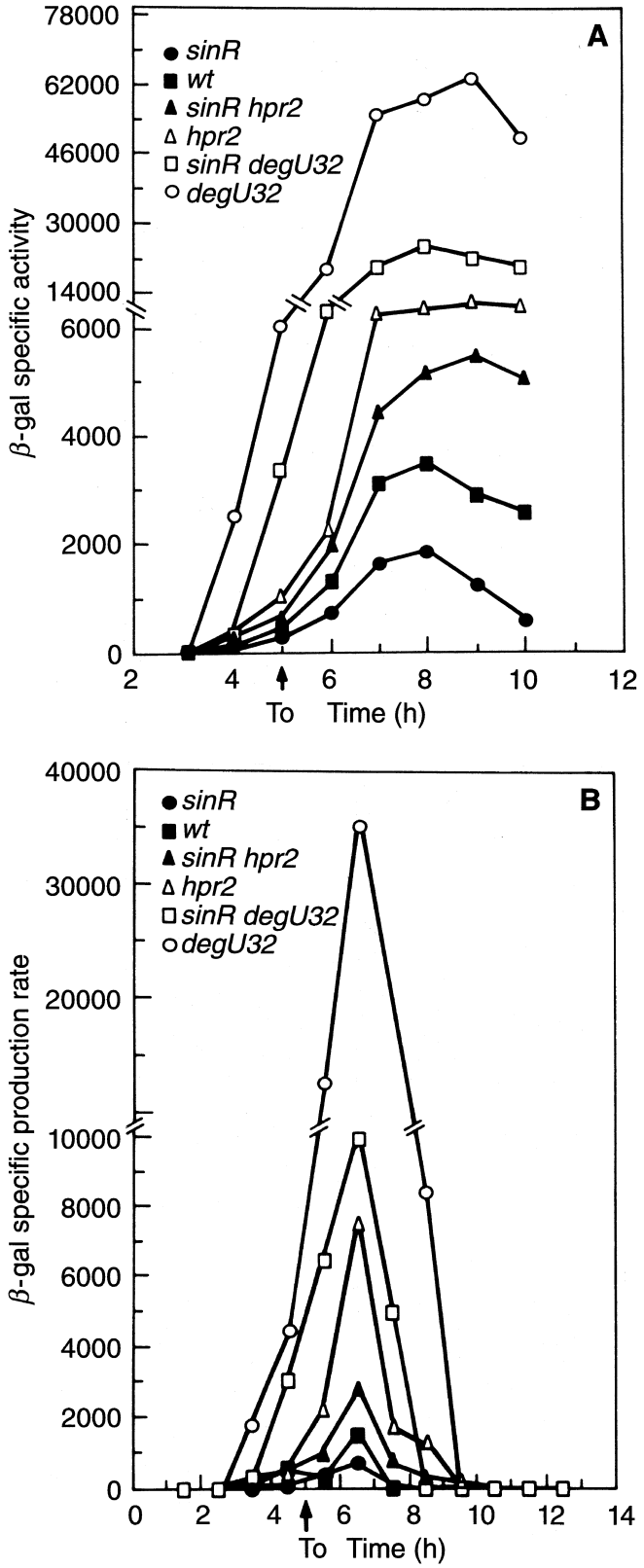
Results

Expression of the *aprE::lacZ* fusion

Studies on *aprE* expression using an *aprE::lacZ* transcriptional/translational fusion, in wild type, *hpr*2 and *degU*32 (Hy) strains have been previously reported (Ferrari et al. 1985, 1986; Henner et al. 1988a). These studies have shown that the highest specific subtilisin production rate is obtained between stages 0 and II of the sporulation process. In addition, it is known that *hpr2* and *degU*32 have a synergistic effect on the expression of the *aprE* gene (Ferrari et al. 1995). Because of this synergy, we wanted to investigate the effect of a *sinR* null mutation, which has been described as a subtilisin repressor, on the expression of *aprE*, in strains carrying *hpr*2 and/or *degU*32. For this purpose, we constructed a set of isogenic strains carrying one or more of these mutations (Table 1) and measured the level of expression of *aprE* using the *aprE::lacZ* fusion present in plasmid *papr::lacZ* (see Materials and methods). The β-galactosidase levels obtained in shake flask cultures in wild type, and in the *hpr*2 and *degU*32 strains (Fig. 1) were similar to those reported by others (Ferrari et al. 1986). Interestingly, in all genetic backgrounds tested, the interruption of *sinR* caused an approximately 50% decrease in β-galactosidase expression without changing the timing and profile of expression (Fig. 1B). These results were unexpected because, as mentioned above, the SinR phenotype has been associated with higher levels of extracellular proteases (Gaur et al. 1986) and several lines of evidence

Table 1 Bacterial strains. (*pm* phleomycin gene, *cat* chloramphenicol acetyltransferase gene)

Strain	Description	Source
Bacillus subtilis		
IS432	*sinR::cat metB5 hisA*1 *leuA*8	M. Strauch
IS720	*sinR::pm metB*5 *hisA*1 *leuA*8	E. Ferrari
BB11	BB80 *sinR::cat*	This work
BB17	BB80 *degU*32(Hy)	E. Ferrari
BB18	BB80 *sinR::cat degU*32(Hy)	This work
BB80	Δ*npr hisA glyB*	E. Ferrari
BB82	Δ*npr hisA hpr*2	E. Ferrari
BB83	BB80 *aprE::lacZ cat*	This work
BB84	BB82 *aprE::lacZ cat*	This work
BB85	BB83 *sinR::pm*	This work
BB86	BB84 *sinR::pm*	This work
BB87	BB83 *degU*32(Hy)	This work
BB88	BB83 *sinR::pm degU*32(Hy)	This work
BB89	BB80 *nprE*18 Δ*aprE*3	This work
BB90	BB89 *sinR::cat*	This work
Escherichia coli		
JM101	*supE thi* (*lac-proAB*) [F′*traD*36 *proAB lacI*^{q}*Z* M15]	Laboratory stock



indicate that SinR could be a directly acting repressor of *aprE*. This discrepancy could be explained if one assumes that a *sinR* null mutant has a positive effect on the level of one or more proteases other than subtilisin.

To test this hypothesis, we decided to investigate the production of extracellular proteases in more detail. Since the main goal of this study was to analyze the possible interaction between *degU*32 and *sinR* on *aprE* expression, only combinations of mutations in these two genes were investigated. The first parameter studied was the total extracellular protease activity as described in Materials and methods. Because the strains used carry a deletion of the *npr* gene and the assays were performed at pH 8.6, measurements should detect primarily subtilisin and other protease(s) active in similar alkaline conditions. Figure 2A shows that the *sinR* null mutant produced about twofold more extracellular protease activity than the parental strain. This result is in good agreement with previously reported data (Gaur et al. 1986). However, in a *degU*32 background, *sinR* appears to have a negative effect by decreasing the level of extracellular protease activity to 50% of the *degU*32 parental strain (Fig. 2A). As mentioned before, one way to reconcile these data with previous reports is to assume that the *sinR* null mutation, while decreasing *aprE* expression, increases the expression level of some other unidentified protease(s). To confirm this hypothesis, we compared the total extracellular protease production in two *B. subtilis* strains in which both the neutral and alkaline protease structural genes have been deleted. As shown in Fig. 2B, it is evident that, compared with the control strain, the *sinR* mutant produced a higher level of total extracellular proteases. These results confirm that the *sinR* null mutation decreases *aprE* expression, while increasing the level of one or more unidentified proteases.

Alterations in the sporulation process

In an attempt to understand how the *sinR* null mutation affects *aprE* expression, we decided to analyze some other events of the sporulation process like expression of APase, timing of appearance of heat-resistant spores and sporulation efficiency of the cultures. APase expression is an indicator of stage II in the sporulation process as defined by the appearance of a completed asymmetric septum (Gaur et al. 1986). Appearance of heat-resistant spores indicates the completion of the sporulation stage. Sporulation efficiency is a measure of the fraction of the culture that is able to differentiate into heat-resistant spores. This fraction varies depending on the composi-

Fig. 1A, B Expression of the *aprE::lacZ* fusion carried in plasmid *papr::lac*, in different genetic backgrounds. **A** β-galactosidase levels in the wild-type (BB83), *hpr*2 (BB84), *sinR* (BB85), *sinR hpr*2 (BB86), *degU*32 (Hy) (BB87) and *sinR degU*32 (Hy) (BB88) strains. T_o in the x-axis indicates the beginning of the sporulation process. Due to the high level of β-galactosidase produced by the BB87 and BB88 strains and to fit the data in the plot, a break in the scale in the y-axis was introduced indicated by the symbol //. B To determine the time at which maximal *aprE* expression was achieved, the specific production rate of β-galactosidase was calculated and plotted

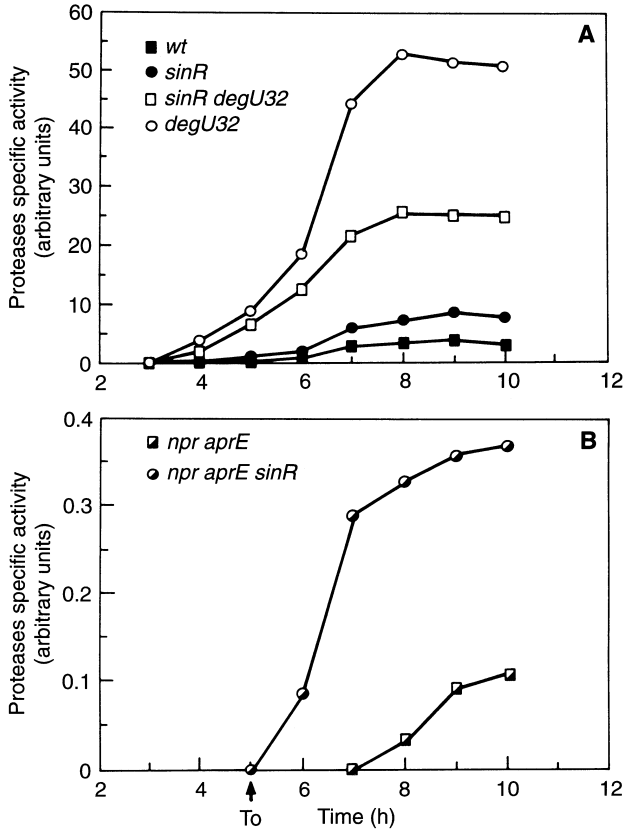


Fig. 2A, B Total extracellular alkaline protease levels. **A** Measurements on the wild type (BB80), *sinR* (BB11), *degU*32 (Hy) (BB17) and *sinR degU*32 (Hy) (BB18) strains. **B** Extracellular proteases detected in the *npr aprE* (BB89) and *npr aprE sinR* (BB90) strains. T_o indicates the initiation of the sporulation process

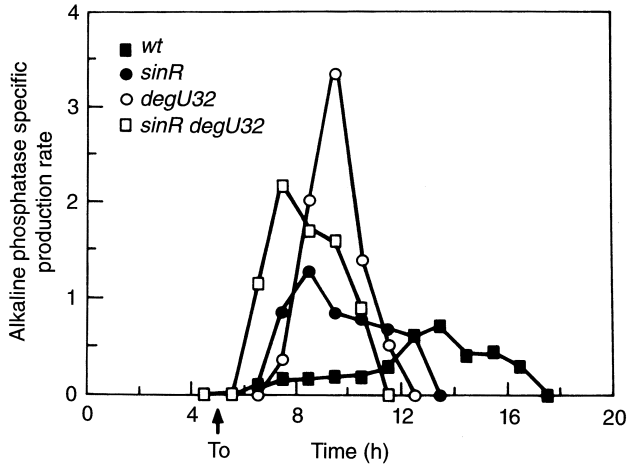


Fig. 3 Specific production rate of alkaline phosphatase in the wild-type (BB83), *sinR* (BB85), *degU*32 (Hy) (BB87) and *sinR degU*32 (Hy) (BB88) strains. T_o indicates the initiation of the sporulation process

tion of the medium and the genetic background (Schaeffer et al. 1965; Chung et al. 1994).

In the wild-type strain, as shown in Fig. 3, total APase specific activity increased first at T_2 (7.5 h), and synthesis resumed later on, around T_8, as described by others (Bookstein et al. 1990). However, in strains carrying *sinR* and/or *degU*32 mutations, the maximal APase productivity was reached very early in the sporulation process, between T_2 and T_3, and their APase levels were two- and five-fold higher, respectively, than the wild-type strain. Our results confirm a previous report that demonstrated that the *sinR* null mutation resulted in the expression of stage-II genes 1 h earlier (Mandic-Mulec et al. 1992). However, to our knowledge, the observation that the *degU*32(Hy) mutation affects APase has not been reported.

The changes in the level and timing of APase expression were also reflected in the appearance of heat-resistant spores. In all tested mutants, mature spores appear much earlier than in the wild-type strain (Fig. 4). Furthermore, strains carrying the *sinR* null mutation sporulate at a frequency twice that of the wild-type strain. This difference was only observed by measuring the sporulation percentage 12 h after T_0 (Fig. 4). We want to stress the point that strains lacking SinR sporulate with higher efficiency than the wild type, as has been reported previously (Mandic-Mulec et al. 1992). However, the earlier detection of heat-resistant spores has not been reported for strains with these two genetic backgrounds.

Discussion

It has been reported that in *B. subtilis* SinR is a repressor of certain stage-II events, thus preventing a premature commitment to a dormant stage (Louie et al. 1992,

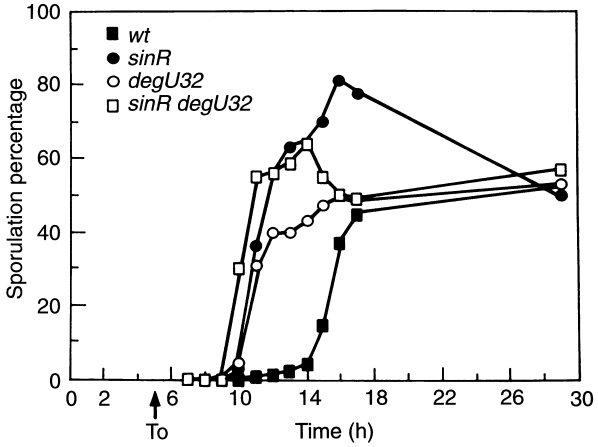


Fig. 4 Formation of heat-resistant spores in strains with wild-type (BB83), *sinR* (BB85), *degU*32 (Hy) (BB87) and sinR degU32 (Hy) (BB88) genetic backgrounds. Samples were taken at different time points and diluted serially in phosphate buffer; 0.1 ml samples were plated on LB agar plates to determine the total number of colony forming units. The rest of the sample was heated at 80°C for 10 min and plated on LB agar plates. The percentage of the population that survived heat treatment was calculated and plotted. T_o indicates the initiation of the sporulation process

Mandic-Mulec et al. 1992). Therefore, the absence of this regulatory element in a *sinR* null mutant could increase the size of the subpopulation completing stage II, i.e., the number of cells committed to sporulate. As a consequence, some of the events that follow septum formation, such as APase activity and the appearance of heat-resistant spores, could occur earlier and/or in a larger fraction of the cell population. Furthermore, by changing the time frame in which certain genes are turned on or off, the absence of SinR could also affect some of the events preceding septum formation. For instance, if in the wild-type strain the expression of a gene lasts 2 h, the time needed to overcome the repressive effect of SinR and proceed to stage II, in the absence of the repressor, this period could be shorter. This is an important factor to consider since, in the wild-type strain, the expression of *aprE* ceases around T_3 (Fig. 1A). Hence shortening the expression period of the subtilisin structural gene could explain the observed negative effect of *sinR* null mutations on *aprE*.

Another aspect to consider is the fact that in a sporulating culture of *B. subtilis*, only a subpopulation of cells actually produces mature heat-resistant spores (Schaeffer et al. 1965). Furthermore, recently it has been shown that cells that do not produce spores are unable to express some of the earliest induced developmental genes that are directly activated by Spo0A~P (Chung et al. 1994). It is conceivable that mutations in some of the transition state regulators could also affect the behavior of those subpopulations.

Considering that *aprE* expression is coupled to a developmental process in which the magnitude and the timing of a cascade of responses is monitored and controlled constantly, it is reasonable to expect that some of the effects caused by mutations in certain transition state regulators alter subtilisin production by altering the sporulation process. It should be mentioned that, to date, no mutations of the transition state regulators that affect solely *aprE* expression have been described.

Finally, it should be emphasized that some of the mutations studied affected the sporulation program by increasing the percentage of cells capable of forming heat-resistant spores. These results suggest that in *B. subtilis* there is a mechanism to ensure that only a percentage of the population forms spores, while other members of the population perform other activities. Such a mechanism would assure that, after cell growth, part of the population forms spores to ensure survival in adverse conditions, while other cells can reassume exponential growth as soon as the appropriate conditions arise. It appears that *B. subtilis* cultures behave more like a well-programmed microbial society where there is a division in the roles that certain subpopulations perform, in order to ensure the continuous permanence of this microorganism in its ecological niches.

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