Research Article

Transcriptional Regulation of the rsbV Promoter Controlling Stress Responses to Ethanol, Carbon Limitation, and Phosphorous Limitation in Bacillus subtilis

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The σB-dependent promoter in front of the rsbV gene of Bacillus subtilis is induced ∼5-fold in response to (1) the addition of 4% ethanol, (2) carbon starvation, and (3) phosphorous starvation. Binding sites for the global carbon and nitrogen regulators, CcpA and TnrA, were mutated, and the consequences of their loss and that of CcpA or TnrA were studied using rsbV-lacZ fusions. These responses proved to be dependent on CcpA, TnrA, and their putative binding sites upstream of the promoter. Induction in response to glucose limitation was largely abolished by loss of CcpA or the upstream region, while induction in response to phosphorous limitation was largely abolished only by the upstream mutations. The results suggest that CcpA directly influences the carbon starvation response and that both proteins exert indirect effects on all three stress responses. The integrity of the DNA sequence is important for all three responses.

1. Introduction

In Gram-positive bacteria such as species of Bacillus, the complex rsb operon encodes the stress sigma factor, σB, and many of its regulatory proteins [1, 2]. This operon includes two promoters, a σA-dependent promoter in front of the rsbR gene at the beginning of the operon, and a σB-dependent promoter in front of the rsbV gene, the fifth gene in this 8-cistron operon [3]. Much is known about the biochemical mechanism of sigma B control which involves an anti-σB, an anti-anti-σB, and protein phosphorylation [4–6]. Additionally, induction of the σB promoter in part mediates adaptation to heat shock, (40–54°C), cold shock (4–12°C), NaCl (10%) shock, ethanol (4–10%) shock, and various starvation (carbon, phosphorous, and nitrogen) stresses [5, 7]. It has been argued that a drop in ATP levels may trigger σB activation [8]. Elements of this regulatory system are found in a wide range of bacteria which in addition to low and high G+C, Gram-positive bacteria include bacteroidetes, cyanobacteria, proteobacteria, and deinococci [2].

We have investigated the dependencies of various stress responses (ethanol stress and limitation for carbon or phosphorous), showing that these responses are dependent on the principal carbon-limitation transcription factor in B. subtilis, CcpA [9–11], as well as the principal nitrogen regulator TnrA [12]. However, we show that point mutations and deletions in the region upstream of the rsbV promoter, within the rsbU gene, largely abolish all of these stress responses. Because these studies were conducted with an rsbV-lacZ fusion reporter system integrated at the amyE locus, we can conclude that RsbU function is not involved in these effects. Our preliminary results suggest that CcpA and TnrA exert their effects (with the possible exception of carbon starvation) through secondary effects. They further suggest that the DNA conformation upstream of the rsbV promoter plays a critical role in responses of the rsbV promoter to multiple stresses.
2. Materials and Methods

2.1. Strains and Plasmids. Bacterial strains used in this study are listed in Table 1. E. coli DH5α was used as a general cloning host. Transformation of B. subtilis was carried out by a method described elsewhere [13]. Plasmid pDG1661 was obtained from the Bacillus Genetics Stock Center in Ohio. The promoter of rsbV was amplified by PCR using Platinum Pfx DNA polymerase (Invitrogen Corp.) with primers rsbV1 (5′-ttaaatagctttctctctgacagc-3′) and rsbV2 (5′-aaaggatcctgactgccaaca-3′), digested with HindIII and BamHI and cloned into the corresponding sites of plasmid pDG1661 to construct pDG-rsbV1. The fragment rsbV3 amplified by PCR with primers rsbV7 (5′-ttagatcctctgacagcaggaagc-3′) and rsbV2 was digested with HindIII and BamHI and cloned into the corresponding sites of plasmid pDG1661 to construct pDG-rsbV3. The fragment rsbV5 amplified by PCR with primers rsbV11 (5′-ttaatatgccgtcctgctgtc-3′) and rsbV2 was digested with HindIII and BamHI and cloned into the corresponding sites of plasmid pDG1661 to construct pDG-rsbV5. The promoter of ctc was amplified by PCR with primers ctc1 (5′-ttagatctctggtgctttcttctc-3′) and ctc2 (5′-ttagatctctggtgctttcttctc-3′), digested with EcoRI and BamHI, and cloned into the corresponding sites of plasmid pDG1661 to construct pDG-ctc.

2.2. Site-Directed Mutagenesis. The Sall fragment containing the rsbV promoter, TRE (the TnrA binding site), and CRE (the CcpA binding site) from pDG-rsbV1 were cloned into pUC19 to construct pUC-rsbV1. pUC-rsbV1m1 containing the mutated CRE site in which the central C was changed to A was constructed by site-directed mutagenesis of pUC-rsbV1 using a QuikChange Site-Directed Mutagenesis kit (Stratagene Corp.) with primers rsbV5 (5′-gaatgcagaacggaaaaagctttctgattc-3′) and rsbV6 (5′-caggagctctggagaaatcttctgattc-3′). The mutation was confirmed by sequencing. The EcoRI and BamHI fragment of pUC-rsbV1m1 containing the rsbV promoter and the mutated CRE was cloned into pDG1661 to construct pDG-rsbV1m1.

2.3. Growth Conditions and Enzyme Assays. Bacillus cells were grown in LB, glucose limitation medium (GLM), and phosphate limitation medium (PLM) [14]. GLM contains 0.05% glucose and the 0.15 mM of KH₂PO₄ was used for PLM. But the similar results was obtained from 0.18 mM of KH₂PO₄. Environmental stress was imposed by adding ethanol to a final concentration of 4% (vol/vol) to cells growing exponentially in LB medium. For the β-galactosidase assay, overnight cultures grown in 1 mL of corresponding medium were diluted 200-fold into fresh medium and grown at 37°C with shaking at 200 rpm. Samples were collected at hourly intervals for the determination of the optical density at 600 nm. β-Galactosidase activity was determined as previously described [15].

3. Results

3.1. Strain Construction. Figure 1 shows the constructs made and the mutations introduced in order to investigate the ethanol stress, carbon limitation, and phosphorous limitation responses of the σ⁸-dependent promoter in the sigB (rsb) operon. The rsb operon has a stress-insensitive σ⁸ promoter in front of the rsbR gene, indicated by the first arrow above the operon, and a stress-sensitive σ⁸ promoter in front of the rsbV gene, indicated by the second arrow above the operon [3]. Upstreams of the σ⁸ promoter and in the rsbU structural gene are two putative transcription factor-binding sites, one specific for TnrA (open arrow) and one specific for CcpA (a so-called catabolite responsive element (CRE)) (closed arrow) see Figure 1 [16]. A lacZ fusion was constructed at position +282, well after the σ⁸ promoter. The different strains have (1) the wild-type regulatory region (rsbV1), (2) the same with a C → A point mutation in the CcpA binding site (rsbV1m1), (3) a deletion lacking the
3.2. The Ethanol Stress Response. The results of studies on the ethanol stress response with these constructs are presented in Figures 2(a)–2(d). Upon addition of 4% ethanol, \( \sigma^B \)-promoter activity increased about five fold in the wild-type genetic background in agreement with earlier studies [5, 18]. As shown in Figures 2(a) and 2(b), the loss of either CcpA or HprK, both required for catabolite repression in B. subtilis, resulted in a moderate loss of the ethanol activation response. The loss of TnrA or of both TnrA and CcpA resulted in slightly less response than when just the ccpA gene was deleted.

Figure 2(a) shows that the loss of the upstream regulatory region including the TnrA site (rsbV3), the loss of both the TnrA and CcpA binding sites (rsbV5), or the point mutation in the CcpA binding site (rsbV1m1) resulted in a much more substantial loss of activation. The three mutant constructs showed comparable responses. These results show that while CcpA and TnrA may play regulatory roles, a point mutation in the CcpA binding site or modification of the upstream region with loss of the TnrA binding site either alone or together with the CRE has a much more dramatic effect.

We then examined the effect of glucose on the ethanol response (Figure 2(c)), both in the wild-type genetic background (squares) and in the ccpA mutant background (circles). With glucose (closed symbols), the ethanol response was identical to that without glucose (open symbols) within experimental error. We therefore conclude (1) that the DNA sequence in front of the \( \sigma^B \) promoter is more important to regulation than the presence or absence of CcpA and/or TnrA, and (2) that glucose does not influence the ethanol stress response either with or without CcpA [19]. Since the action of CcpA usually depends on the presence of glucose [20], we suggest that CcpA and TnrA exert indirect effects on the activation of the \( \sigma^B \) promoter in response to ethanol addition (see Section 4). The fact that even a single nucleotide substitution in the CRE has as dramatic an effect as deletion of the entire region suggests (but does not prove) that the CRE is important for the ethanol stress response even though CcpA and glucose are not. If this suggestion is correct, then a requirement for the presence of the TnrA binding site can also be inferred (see Section 4).

Figure 2(d) shows the effects of various mutations on the ethanol stress response of the \( \sigma^B \)-dependent ctc gene in B. subtilis. Constructs were made and inserted at the amyE locus as described in “Section 2.” The experiment was conducted in LB medium with the addition of 4% ethanol at \( t = 0 \). The responses observed followed those for the rsb operon. Thus, the cpaA mutation moderately diminished the activity of the ctc promoter, relative to that of the wild type, but the rsbV1m1 and rsbV5 mutations exerted much more drastic effects. On the basis of these results, we suggest that the effects observed on the stress response of rsbV operon expression are transmitted to the ctc gene, and that \( \sigma^B \) is therefore rate limiting for expression of the ctc gene. These results also confirm the validity of the data presented in Figure 2(a).

3.3. The Carbon Limitation Response. As observed upon the addition of ethanol, glucose limitation causes a substantial
increase in expression of the rsbV promoter in a wild-type genetic background (Figure 3). The response to a point mutation or loss of part or most of the upstream region in the rsbU gene was dramatic. The basal activity was diminished only slightly by the point mutation, but starvation induction was largely abolished. The deletion mutations reduced the basal activity more, but in all cases, an inductive response, although much reduced, was still observed (Figure 3(a)).

Figure 3(b) shows the effects of loss of TnrA, CcpA, or both factors. Loss of TnrA was essentially without effect, but the loss of CcpA + TnrA resulted in premature induction followed by dramatic repression. When the consequences of the loss of CcpA were determined for the σB-controlled ctc gene (Figure 3(c)), the dramatic induction observed upon glucose starvation was essentially abolished. The relative responses of the rsbV and ctc promoters were comparable.

3.4. The Phosphorous Limitation Response. Phosphorous limitation similarly caused substantial induction of the rsbV promoter (Figure 4(a)). Mutation of the CRE or deletion of part or most of the upstream region greatly reduced this response. The effects of loss of CcpA and/or TnrA proved to be less dramatic with the loss of CcpA having a greater effect than the loss of TnrA (Figure 4(b)). Loss of
both reduced expression even further. Correlating with the minimal response to the loss of CcpA on rsbV promoter expression, induction of expression of the ctc gene was not appreciably altered by deletion of the ccpA gene (Figure 4(c)).

4. Discussion

At the onset of this project, we discovered binding sites for two transcription factors in front of the stress-responsive rsbV promoter within the rsbU structural gene. The first was a typical binding site for the global nitrogen regulator of B. subtilis, TnrA, while the second one was a typical CRE site which normally binds the global carbon regulator of B. subtilis, CcpA. We introduced a debilitating point mutation in the CRE and deleted either the TnrA binding site or both the TnrA and CcpA binding sites (see Figure 1). Surprisingly, regardless of the type of mutation introduced, changing this upstream region largely abolished the stress responses to (1) ethanol addition, (2) carbon limitation, and (3) phosphorous limitation. In all cases, the responses were reduced to similar degrees, and all three types of mutations had similar, but not identical, effects.

Multiple interpretations are possible for these observations. For example, the CRE and adjacent regions might bind a protein or RNA, and binding might depend on the secondary structure of the upstream DNA. Even subtle changes introduced by a point mutation in the CRE might abolish the activating interactions. Alternatively, the upstream region might inherently control promoter activity in response to stress signals by transmitting a signal through the DNA helix to the promoter. This could affect binding of RNA polymerase or another factor that contributes to the stress responses. Still, another possibility is that the CRE and the TnrA binding sites are important to the
stress induction, but they do not function primarily in this regard to respectively bind CcpA and TnrA. Regardless, our experiments clearly indicate that the generalized effects of these upstream changes in the DNA are not mediated by either CcpA and/or TnrA alone.

We believe that these transcription factors, CcpA and TnrA, exert their effects which in general are less dramatic than those of the upstream mutations, through indirect means. For example, the responses to ethanol and phosphorous limitation stresses were only moderately dependent on CcpA and TnrA. Further, although CcpA binding to the DNA is usually dependent on the presence of metabolites generated in the presence of exogenous glucose or another glycolytically metabolized sugar, the presence of glucose exerted no effect on the ethanol stress response (Figure 2(c)). By contrast, carbon starvation induction of the rsbV promoter was dramatically affected by the loss of CcpA although deletion of tnrA was without effect. Since the ccpA mutation affects growth rate and glucose utilization, it is possible that even this dramatic effect of CcpA could be indirect. Regardless, its effect does not appear to be mediated by the CRE identified in the rsbU gene.

The studies reported here clearly indicate the importance of the rsbU region upstream of the rsbV promoter on both basal and induced promoter activities. It is clear, also, that both TnrA and CcpA influence these stress responses. The phenomenology is therefore defined. The detailed molecular mechanisms, providing explanations for the observations reported here, have yet to be determined. The functions of the putative binding sites for TnrA and CcpA in the rsbU gene similarly must be investigated further.

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