Analysis of the expression and regulation of the gerB spore germination operon of *Bacillus subtilis* 168

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The gerB spore germination operon of *Bacillus subtilis* 168 is a homologue of the gerA spore germination operon. The expression and regulation of the gerB operon has been examined using a lacZ transcriptional fusion and the transcriptional start defined. The gerB operon is expressed during sporulation under the control of RNA polymerase containing the forespore-specific sigma factor, σ^G_. This is a further homology to the gerA operon, which is similarly regulated. It is predicted from the localization of expression and the encoded primary sequences that the GerB proteins are located at the inner spore membrane.

**Keywords**: *Bacillus subtilis*, sporulation, germination, gerB expression and regulation, sigma factor

**INTRODUCTION**

The production of endospores by *Bacillus subtilis* is achieved by expression of many sporulation-specific genes in a strict temporal and spatial order. A regulatory cascade operates whereby genes involved in successive stages in the developmental process are regulated by a series of RNA polymerase sigma factors with promoter specificity for those genes. This, and additional levels of control, were reviewed recently by Errington (1993). The sigma factor σ^G_ regulates a number of genes that are expressed solely in the forespore compartment of the sporangium after engulfment by the mother cell. These genes include *spoIIIA* (sigG), the structural gene for σ^G_, the *spg* genes, encoding small acid-soluble proteins which stabilize spore core macromolecules, and *gpr*, encoding the protease which degrades them during germination (Moir, 1992). Several spore germination genes, i.e. the gerA operon and gerD, have been shown to be expressed in the forespore compartment of the sporangium, and are exclusively regulated by σ^G_ (Feavers et al., 1990; Kemp et al., 1991). The gerA operon is concerned with alanine-triggered spore germination and may encode an alanine receptor; the gerD gene is involved in both the known germination pathways, but its function is unknown. We recently reported the nucleotide sequence of the gerB operon of *B. subtilis* and showed that it was a homologue of the gerA operon in terms of both polypeptides encoded and gene organization, despite the two operons being associated with different spore germination pathways (Corfe et al., 1994). The gerB operon has a potential σ^G_-binding site upstream of the first ORF (Corfe et al., 1994; Fajardo-Cavazos et al., 1991), which may suggest a further degree of homology between the two operons. All three polypeptides encoded by gerB are predicted to be membrane-linked to some extent, so the location of gerB expression in spores may indicate whether the proteins are likely to be associated with the inner or outer spore membrane, assuming that they would become associated with the membrane surrounding the compartment in which they are expressed.

**METHODS**

**Bacterial strains and media.** All strains used are listed in Table 1. *B. subtilis* strains were grown and stored on nutrient agar (Oxoid). Plasmids were constructed in, and prepared from, *Escherichia coli* strain DH5α. Transformation of *B. subtilis* was by the method of Anagnostopoulos & Spizizen (1961) and of *E. coli* by the method of Mandel & Higa (1970). Synchronous sporulation experiments were done as described by Sterlini & Mandelstam (1969) and IPTG-induction of *PepA* was as described by Sun et al. (1989).

**Plasmid construction.** Plasmid pAZ106-gerB was made by cloning the 1.5 kb *HindIII* fragment internal to the gerB operon (Corfe, 1992) into the *HindIII* site upstream of the promoterless *lacZ* gene of pAZ106 in the correct orientation to give a transcriptional fusion to *lacZ* (Corfe, 1992).
**Table 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. subtilis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS432</td>
<td>Prototroph</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>SG64</td>
<td>lacA1 lacR1</td>
<td>Errington &amp; Vogt (1990)</td>
</tr>
<tr>
<td>BC100</td>
<td>lacA1 lacR1 gerBB::pAZ106</td>
<td>SG64 × pAZ109-gerB</td>
</tr>
<tr>
<td>BC101</td>
<td>lacA1 lacR1 gerBB::pA106 Pspac-sigE</td>
<td>BC100 × pDG180</td>
</tr>
<tr>
<td>BC102</td>
<td>lacA1 lacR1 gerBB::pA106 Pspac-sigF</td>
<td>BC100 × pRS11</td>
</tr>
<tr>
<td>BC103</td>
<td>lacA1 lacR1 gerBB::pA106 Pspac-sigG</td>
<td>BC100 × pDG298</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5s</td>
<td>supE44 lacU169 (q80 lacZΔM15) bidR17 * recA1 relA1</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td><em>Plasmids</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAZ106</td>
<td>MLSR</td>
<td>Zuberi et al. (1990)</td>
</tr>
<tr>
<td>pAZ106-gerB</td>
<td>MLSR gerB-lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>pDG180</td>
<td>Ap R Km R Pspac-sigE</td>
<td>Schmidt et al. (1990)</td>
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<tr>
<td>pRS11</td>
<td>Ap R Km R Pspac-sigF</td>
<td>Sun et al. (1989)</td>
</tr>
<tr>
<td>pDG298</td>
<td>Ap R Km R Pspac-sigG</td>
<td>Corfe et al. (1994)</td>
</tr>
<tr>
<td>p5113</td>
<td>Ap R Cm R gerBA</td>
<td></td>
</tr>
</tbody>
</table>

* Ap R, ampicillin resistance; Cm R, chloramphenicol resistance; Km R, kanamycin resistance; MLS R, macrolide-lincosamide-streptogramin B resistance.

Enzyme assays. β-Galactosidase and glucose dehydrogenase (GDH) were assayed as described by Kemp et al. (1991). One unit of β-galactosidase activity was defined as the amount of enzyme which releases 1 pmol methyl umbelliferone (MU) unit of P-galactosidase activity was defined as the amount of macrolide-lincosamide-streptogramin B resistance.

Isolation and analysis of gerB mRNA. RNA was extracted from *B. subtilis* 168 at 0, 2, 4 and 6 h after initiation of sporulation in 2x SG medium (Goldrick & Setlow, 1983), and the 5' end of the gerB mRNA was determined by primer extension analysis (Feavers et al., 1990; Mason et al., 1988). The primers used were (1) a synthetic oligonucleotide 3'-GGTCTTGTTAAA'C-5', which is complementary to residues 54-72 in the gerBA coding sequence (Corfe et al., 1994), or (2) a second synthetic oligonucleotide 3'-CCCTGTTTTGCTGCTATA-5', which is complementary to residues 21-42 in the gerB coding sequence (Corfe et al., 1994). The synthetic oligonucleotides were labelled with polynucleotide kinase and [γ^32P]ATP. Samples of primer extended with reverse transcriptase in the presence of RNA (40 μg) from sporulating cells were run on 6% (w/v) polyacrylamide/8 M urea sequencing gels alongside the four lanes of DNA sequence determinations by the chain termination method, which was carried out using these same primers with plasmid p5113 (Corfe et al., 1994).

**RESULTS AND DISCUSSION**

**Time of expression of gerB during sporulation**

Synchronous sporulation of strain BC100 (see Table 1) was induced by the resuspension method (Sterini & Mandelstam, 1969) and the results are shown in Fig. 1. The time of induction of gerB, as indicated by β-galactosidase activity from the gerB-lacZ transcriptional fusion, was between 2.5 and 3 h after resuspension (T2.5 - T3). The measured activity reached a peak at T3 and subsided thereafter, probably due to the progressive increase in resistance of the forespore to the lysozyme-dependent extraction procedure used. The samples were

![Fig. 1. Time of induction of gerB-lacZ expression during sporulation. β-Galactosidase levels were measured following induction of synchronous sporulation in strains BC100 (○) and SG64 (■). Enzyme units were calculated per ml of culture and expressed as a percentage of the maximum value obtained with strain BC100 [2 pmol MUG hydrolysed min^-1 (ml culture)]. Results are the means of ten experiments.](image-url)
also assayed for GDH activity (data not shown), which showed the same kinetics of induction as gerB-lacZ, and peaked at the same time. Direct comparison of the level of expression observed with that of gerA-lacZ fusions is not strictly possible as the two fusions were in different genetic backgrounds, but we estimate that the level of gerB expression is about one-fifteenth that of gerA. The mean maximum level of β-galactosidase activity [2 pmol methylumbelliferyl β-D-galactoside (MUG) hydrolysed min⁻¹ ml⁻¹] was extremely low, but was consistently above the level of the control strain, SG64, lacking the fusion. This control strain of B. subtilis has a very low level of endogenous β-galactosidase activity and is generally considered as having a Lac⁻ phenotype. Nevertheless, the background in the Lac⁻ strain becomes significant at the very low levels of lacZ expression observed in these experiments—presumably the lacA1 allele is slightly leaky. The data presented in Fig. 1 are the mean of ten experiments; although differences in the absolute level of β-galactosidase of up to twofold were obtained in different sporulation experiments, the profile of time of expression was similar in each case. GDH is regulated by σG, and the time of induction of both gerB and gbd correspond well with the published data for genes regulated by σG (Setlow, 1989).

Spores of the gerB-lacZ fusion strain were shown to possess β-galactosidase activity, indicating that the activity was present in spores after mother-cell lysis (data not shown).

### Induction of gerB expression

In order to ascertain the elements responsible for regulation of a sporulation gene, most workers have introduced lacZ fusions into a number of different Spo⁺ genetic backgrounds. Owing to the very low level of expression of gerB this was not considered feasible. Instead plasmids carrying different inducible sigma factor (sig) genes were introduced into BC100 (see Table 1). It should be noted that, whilst the Pspac-sigG and sigE plasmids were autonomously replicating in B. subtilis, the Pspac-sigF was integrated into the chromosome. In order...
to establish in which background the gerB-lacZ fusion would be switched on, the sigma factors were induced in vegetative cultures. The results are shown in Fig. 2. The gerB-lacZ fusion is expressed after induction of sigG in vegetative cells, but shows virtually no activity after induction of sigE or sigF. These results were obtained in three independent experiments. GDH activity was also measured after induction of sigG, and showed a similar profile to gerB-directed β-galactosidase activity (data not shown). It might be expected that a σ^G-dependent promoter, such as gerB, would also show some delayed induction following the expression of sigF, as that would lead to σ^G-dependent expression of sigG (Partridge et al., 1991). There is a slight suggestion of some eventual expression of gerB-lacZ after the induction of Pspac-sigF. The very low level observed in this case may be a result of the lower gene dosage of sigF and the observed weakness of the gerB promoter.

Analysis of gerB mRNA

Further evidence pertinent to the timing of gerB expression and the promoter sequences involved was obtained by primer extension analysis using RNA obtained from cells 0, 2, 4 and 6 h into sporulation (Fig. 3). This analysis indicated that significant levels of gerB mRNA were only present at the fourth hour of sporulation. While a number of closely spaced extension products were obtained from gerB mRNA at T_n, taking the darkest band as the major start point localized the transcription to a T residue 36 nt upstream of the initiating ATG codon (Fig. 3). This same transcription start point was found when the second oligonucleotide was used for primer extension analysis (data not shown). Comparison of sequences upstream of the gerB transcription start point with the consensus for σ^G-dependent genes (Fig. 4) shows that gerB exhibits 3/6 matches in the −35 region, and 5/7 matches in the −10 region. In addition, at several of the positions where the gerB sequence differs from the σ^G-consensus sequence, the residues found in gerB are present in at least one other σ^G-dependent promoter (Fig. 4, underlined residues). While the good match between the σ^G-consensus and gerB promoter sequences is consistent with gerB being a σ^G-dependent gene, there are four very highly conserved positions in the σ^G-consensus at which gerB differs—a two in the −35 region and two in the −10 region. Although the differences between the gerB sequence and the σ^G-consensus in the −35 region have been shown to have only a small effect on σ^G-dependent promoter expression, the differences in the −10 region may reduce transcription significantly (Fajardo-Cavazos et al., 1991). These latter changes may be the reason for the relatively weak transcription of gerB during sporulation. We also note that there are apparently much better matches to the σ^G −10 and −35 promoter consensus sequences located 4 and 5 bp, respectively, closer to the ATG initiation codon (Corfe et al., 1994). Why this potential promoter sequence is not used is unclear.

We conclude that gerB is transcribed by EoG exclusively during sporulation, similarly to the gerA spore germination operon. There was an apparent background level of gerB expression in BC100 during vegetative growth and early sporulation in the synchronous sporulation experiment (see Fig. 1). This residual level is close to the limits of detection and may be explained by some asynchrony of the sporulating cultures, both within and between experiments. The conclusions about the time of expression are reinforced by data which showed that the only significant expression came from induction of sigG, and from the primer extension analysis, which showed that the gerB transcript was only found in late sporulation and not in vegetative or early sporulation cultures.

Both the gerA and gerB operons encode three proteins which are membrane-associated to some extent, and pairs of which have very similar hydropathy profiles (Feavers et al., 1985; Zuberi et al., 1987; Corfe et al., 1994). It seems likely that, if the GerB proteins are expressed in the forespore and are membrane-associated, then the membrane in which they are located is the inner forespore membrane, which may be the only intact membrane in spores. This model has already been proposed for the GerA proteins (Feavers et al., 1990) and is in accord with models that suggest that the initial events of spore germination occur at the inner membrane (Keynan, 1978).

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REFERENCES


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