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 *spoIIIG* (*sigG*), the structural gene for σ^G , the *ssp* 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 DH5 α . 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 *Pspac* was as described by Sun *et al.* (1989).

Plasmid construction. Plasmid pAZ106-*gerB* was made by cloning the 1.5 kb *Hind*III fragment internal to the *gerB* operon (Corfe, 1992) into the *Hind*III site upstream of the promoterless *lacZ* gene of pAZ106 in the correct orientation to give a transcriptional fusion to *lacZ* (Corfe, 1992).

Abbreviations: GDH, glucose dehydrogenase; MU, methylumbelliferone; MUG, methylumbelliferyl β -D-galactoside.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype*	Source or reference
<i>Bacillus subtilis</i>		
PS832	Prototroph	Laboratory stock
SG64	<i>lacA1 lacR1</i>	Errington & Vogt (1990)
BC100	<i>lacA1 lacR1 gerBB::pAZ106</i>	SG64 × pAZ109- <i>gerB</i>
BC101	<i>lacA1 lacR1 gerBB::pAZ106 Pspac-sigE</i>	BC100 × pDG180
BC102	<i>lacA1 lacR1 gerBB::pAZ106 Pspac-sigF</i>	BC100 × pRS11
BC103	<i>lacA1 lacR1 gerBB::pAZ106 Pspac-sigG</i>	BC100 × pDG298
<i>Escherichia coli</i>		
DH5 α	<i>supE44 ΔlacU169 (ϕ80 <i>lacZ</i>ΔM15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i></i>	Laboratory stock
Plasmids		
pAZ106	MLS ^R	Zuberi <i>et al.</i> (1990)
pAZ106- <i>gerB</i>	MLS ^R <i>gerB-lacZ</i>	This study
pDG180	Ap ^R Km ^R <i>Pspac-sigE</i>	Popham & Stragier (1991)
pRS11	Ap ^R Km ^R <i>Pspac-sigF</i>	Schmidt <i>et al.</i> (1990)
pDG298	Ap ^R Km ^R <i>Pspac-sigG</i>	Sun <i>et al.</i> (1989)
p5113	Ap ^R Cm ^R <i>gerBA</i>	Corfe <i>et al.</i> (1994)

* 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) min⁻¹, after Zuberi *et al.* (1987).

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 2× 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'-GGTCTTGTTAAATC-TGTGTGAC-5', which is complementary to residues 21–42 in the *gerB* coding sequence (Corfe *et al.*, 1994), or (2) a second synthetic oligonucleotide 3'-CCCTGTTTGCTGCTATA-5', which is complementary to residues 54–72 in the *gerBA* 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 μ g) 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 (Sterlini & 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 (T_{2.5}–T₃). The measured activity reached a peak at T₄ 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)⁻¹]. Results are the means of ten experiments.

Fig. 2. Induction of *gerB-lacZ* expression in *Pspac-sig* genetic backgrounds. β -Galactosidase was measured at intervals after induction of various sporulation-specific sigma factors in strains BC103 (□, *Pspac-sigG*), BC101 (■, *Pspac-sigE*), BC102 (▲, *Pspac-sigF*). The data are expressed as a percentage of the maximum activity normalized for the OD_{600} of the culture, observed in strain BC103 (950 pmol min⁻¹ per ml culture of OD_{600} 1.0). Data are the means of three independent experiments. Cultures were grown in L-broth containing 5 μ g kanamycin ml⁻¹ and were induced by the addition of IPTG to a final concentration of 1 mM.

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 σ^G , and the time of induction of both *gerB* and *gdb* correspond well with the published data for genes regulated by σ^G (Setlow, 1989).

Fig. 3. Primer extension analysis of the 5' end of *gerB* mRNA *in vivo*. RNA was isolated and purified from cells of strain PS832 at 0, 2, 4 and 6 h of sporulation as described in Methods. Samples of these RNAs were hybridized with end-labelled oligonucleotide-1 and extended with reverse transcriptase. A sample of the same oligonucleotide was also used to prime DNA synthesis with the appropriate dideoxynucleotides on plasmid p5113. Samples were resolved by electrophoresis as described in Methods, and labelled DNA located by autoradiography. The lanes are: C, T, A and G, DNA synthesis reactions with ddGTP, ddATP, ddTTP and ddCTP, respectively; 0, 2, 4 and 6, extended primer with RNA isolated from sporulating cells after 0, 2, 4 or 6 h of sporulation, respectively. The sequence on the left shows the *gerB* sequence in the region of the extended primer (Corfe *et al.*, 1994). The arrow at the T residue denotes the 5'-end of *gerB* mRNA.

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

-35		-10	
TGAATA	17-18nt	CATACTA	σ^G consensus
CGATAA	18nt	GATACAA	<i>gerB</i>
AGTATA	17nt	AAAGATA	<i>gerA</i>

Fig. 4. Comparison of the σ^G -consensus promoter sequence with the *gerB* and *gerA* promoter sequences. The consensus σ^G promoter sequence in the -10 and -35 regions is from Nicholson *et al.* (1989) and Sun *et al.* (1991); the *gerB* sequence is from Corfe *et al.* (1994) as determined in Fig. 3; the *gerA* sequence from Feavers *et al.* (1990). Bold face bases are conserved in > 80% of all *B. subtilis* promoters transcribed by $E\sigma^G$. Underlined bases in the *gerB* promoter which differ from the consensus sequence are found at this position in at least one other *B. subtilis* σ^G -dependent promoter.

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^F -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_4 , 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 – 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 $E\sigma^G$ 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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