Complete spore-cortex hydrolysis during germination of *Bacillus subtilis* 168 requires SleB and YpeB

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The role of the *sleB* gene of *Bacillus subtilis*, which encodes a putative spore-cortex-lytic enzyme, and the downstream *ypeB* gene were investigated. Both SleB and YpeB were required for normal germination to occur. The corresponding mutants formed phase-bright, heat-resistant spores with no apparent defects in dormancy. However, mutant spore suspensions lost optical density slower than the wild-type and spores were phase-grey even 12 h after the triggering of germination. Since the loss of heat resistance and release of dipicolinic acid was similar to the wild-type, these mutants were blocked in the later stages of germination. The mutants were nevertheless capable of outgrowth on rich agar to form colonies, indicating that other spore components can compensate for their function sufficiently to allow outgrowth. The expression and regulation of the operon was examined using a *lacZ* transcriptional fusion. Expression of the operon began 2 h after the onset of sporulation and was under the control of RNA polymerase containing the forespore-specific sigma factor, σ^G . The application of reverse phase HPLC revealed that the mutants do not have any structural defect in the dormant spore cortex and therefore these genes are not required for normal spore-cortex synthesis. The analysis of peptidoglycan dynamics during germination showed, however, that the cortex was only partially hydrolysed in both mutants. This analysis also revealed that the likely hydrolytic bond specificity of SleB is likely to be that of a lytic transglycosylase.

Keywords: *Bacillus subtilis*, endospores, germination, peptidoglycan, cortex hydrolysis

INTRODUCTION

The formation of resistant dormant endospores is a survival strategy used by members of the genera *Bacillus* and *Clostridium* during nutrient deprivation. The spore cortex maintains heat resistance and dormancy (Ellar, 1978). The cortex consists of a thick layer of peptidoglycan with a unique spore-specific structure (Atrih *et al.*, 1996; Popham *et al.*, 1996a). Hydrolysis of the cortex peptidoglycan is essential for later germination events and outgrowth (Atrih & Foster, 1999; Atrih *et al.*, 1998; Popham *et al.*, 1996b).

A number of spore-cortex-lytic enzymes have been

isolated from spores of different organisms. Two lytic enzymes have been isolated from *Clostridium perfringens* S40 spores – a 31 kDa enzyme encoded by the gene *sleC* (Miyata *et al.*, 1995) and a 38 kDa enzyme encoded by the gene *sleM* (Chen *et al.*, 1997). The *Bacillus cereus* IFO 13597 gene *sleB* encodes a 24 kDa enzyme (Makino *et al.*, 1994; Moriyama *et al.*, 1996b), and its homologue has been identified and inactivated in *Bacillus subtilis*. The resulting mutant germinates slower than the wild-type (Moriyama *et al.*, 1996a).

Foster & Johnstone (1987) isolated a germination-specific lytic enzyme (GSLE) that was capable of cortex hydrolysis from the spores of *Bacillus megaterium* KM. The enzyme was activated *in vivo* during germination and has a high specificity for intact spore cortex. Western blot analysis revealed cross-reactivity with proteins from spore fractions of other species and that the enzyme was

Abbreviations: GSLE, germination-specific lytic enzyme; MUG, methyl-umbelliferyl β -D-galactoside; RP-HPLC, reverse phase HPLC.

capable of germinating permeabilized spores of other species (Foster & Johnstone, 1988).

The recent application of reverse phase HPLC (RP-HPLC) to monitor peptidoglycan structural dynamics during the germination of *B. subtilis* revealed that the mechanism of cortex hydrolysis is complex and involves several hydrolases of differing specificity (Atrih *et al.*, 1998).

In *B. subtilis*, the *sleB* and adjacent downstream *ypeB* gene form a bicistronic operon (Moriyama *et al.*, 1999). In this study, we have shown that a defect in either the *sleB* or the *ypeB* genes results in incomplete germination. Analysis of cortex dynamics during germination revealed the likely hydrolytic bond specificity of SleB.

METHODS

Bacterial strains, growth and preparation of spores. All *B. subtilis* strains used in this study are shown in Table 1. Mutations were transferred into the laboratory wild-type

background (HR) used for mucopeptide analysis (Atrih *et al.*, 1996). Vegetative cells of *B. subtilis* were grown in nutrient broth (Oxoid) or on nutrient agar plates. Plasmids were constructed in, and prepared from, *Escherichia coli* strain TG1 grown in Luria–Bertani (LB) broth or on LB agar. When appropriate, chromosomal drug resistance markers in *B. subtilis* were selected with chloramphenicol (5 µg ml⁻¹), kanamycin (10 µg ml⁻¹), or erythromycin (1 µg ml⁻¹) and lincomycin (25 µg ml⁻¹). All bacterial cultures were grown at 37 °C.

Construction of mutants insertionally inactivated in *sleB* or *ypeB*

(i) **Plasmid construction.** Plasmids pFB101 and pFB102 were made by cloning internal fragments of the *sleB* and *ypeB* genes, respectively, into pMUTin2 (Vagner *et al.*, 1998) using nucleotide sequence from the *B. subtilis* complete genome sequence (Kunst *et al.*, 1997). Genomic DNA was used as template in the PCR amplification of the 250 bp internal fragment of *sleB* using the forward primer (5'-GCCG-AAGCTTA₆₄TTAATACTGAAACGATCTC₈₃) and the reverse primer (5'-CGCGGATCCT₂₉₄ATTAAGGTTTGCTT-

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype*	Source or reference†
Strains		
168 HR	<i>trpC2</i>	Laboratory stock
FB101	<i>trpC2 sleB-lacZ</i> , Em ^r	(pFB101) → 168 HR
FB102	<i>trpC2 ypeB-lacZ</i> , Em ^r	(pFB102) → 168 HR
FB103	<i>trpC2 spoIIIGB::kan sleB-lacZ</i> , Em ^r Km ^r	(1295) → FB101
FB104	<i>trpC2 spoIIAABC::cat sleB-lacZ</i> , Em ^r Cm ^r	(JE650) → FB101
FB105	<i>trpC2 spoIIIG::kan sleB-lacZ</i> , Em ^r Km ^r	(1296) → FB101
FB106	<i>trpC2 spoIIIC::cat sleB-lacZ</i> , Em ^r Km ^r	(618) → FB101
FB107	<i>trpC2 Pspac-sigE sleB-lacZ</i> , Em ^r Km ^r	(pDG180) → FB101
FB108	<i>trpC2 Pspac-sigF sleB-lacZ</i> , Em ^r Cm ^r	(pRS11) → FB101
FB109	<i>trpC2 Pspac-sigG sleB-lacZ</i> , Em ^r Km ^r	(pDG298) → FB101
FB110	<i>trpC2 Pspac-sigK sleB-lacZ</i> , Em ^r Cm ^r	(SH132) → FB101
HC145	<i>trpC2 sleBA</i>	(pGSB21) → 168 HR
SH132	<i>sigKΔ19::pVO12 (Pspac-sigK)</i> , Cm ^r	Oke & Losick (1993)
1295	<i>trpC2 spoIIIGB::kan</i> , Km ^r	J. Errington, Oxford University, UK
650	<i>trpC2 ilvB2 leuB16 spoIIAABC::cat</i> , Cm ^r	J. Errington, Oxford University, UK
618	<i>trpC2 spoIIIC::cat</i> , Cm ^r	Turner <i>et al.</i> (1986)
1296	<i>trpC2 spoIIIG::kan</i> , Km ^r	J. Errington, Oxford University, UK
Plasmids		
pFB101	<i>sleB-lacZ</i>	This study
pFB102	<i>ypeB-lacZ</i>	This study
pGSB21	<i>sleBA</i>	This study
pRS11	<i>Pspac-sigF</i>	R. Schmidt, Harvard University, USA
pDG180	<i>Pspac-sigE</i>	Popham & Stragier (1991)
pDG298	<i>Pspac-sigG</i>	Sun <i>et al.</i> (1989)

* Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Em^r, erythromycin resistance.

† DNA transformation crosses are indicated; DNA source is in brackets and the arrow points to the recipient strain.

TG₂₇₈) where the internal sequence of the gene is italicized (numbering is with respect to the A of the translational start codon of the gene) and the *Hind*III site of the forward primer and the *Bam*HI site of the reverse primer are underlined. The primers (5'-GCCGAAGCTTC₁₅₇TTCATGATAAAATCGG₁₇₃) and (5'-CGCGGATCCA₄₀₈TATAGCTTATTTAGT₃₉₃) were used to amplify a 271 bp segment of the *ypeB* gene. pMUTin2 and the PCR fragments were digested with *Hind*III and *Bam*HI and then ligated by the method of Sambrook *et al.* (1989).

(ii) Transformation of *E. coli* and *B. subtilis*. Transformation of *E. coli* was performed as described by Hanahan (1983). Transformation of *B. subtilis* 168 with pFB101 and pFB102 was performed by the competent cell method (Anagnostopoulos & Spizizen, 1961). Disruption of the *sleB* and *ypeB* genes by means of Campbell-type recombination was confirmed by Southern blot analysis, using the appropriate plasmid as probe. Hybridization, probe labelling and detection were done with the Boehringer Mannheim nonradioactive DNA labelling and detection kit.

Construction of *sleB* in-frame deletion mutant. To construct an in-frame deletion of *sleB*, a 5.6 kb DNA fragment spanning the *sleB* and *ypeB* coding sequence was synthesized by PCR using *B. subtilis* HR chromosomal DNA as template. Primers 5'-ATGCGAGCTC₂₆₄₀CGGCTAAGAAGAGAGGCATC₂₆₂₁ and 5'-CTGAGTCGACC₂₉₉₅GCCTGATGCAGTATTGAAG₃₀₁₄ were used as forward and reverse primers, respectively (the chromosomal sequences are italicized and the *Sac*I and *Sal*I restriction sites added are underlined; the numbering is with respect to the A of the translational start codon of *sleB*). The PCR product was cloned in *E. coli* DH5 α using plasmid pGEM3Z, after appropriate restriction digestion and ligation. A recombinant plasmid, pGSB1 containing the cloned DNA was isolated and this was used as template DNA for inverse-PCR using primers 5'-ATGCGGATCCG₈₄₃ATACGGCTACAAGTCCGTG₈₂₄ and 5'-ATGAGGATCCC₄₄₄TGCCGTGCTTCTGCTTGTG₄₆₄ as forward and reverse primers, respectively (the chromosomal sequences are italicized and the restriction site for *Bam*HI is underlined). The PCR product from this experiment resulted in a deletion of 399 bases in the coding region of the *sleB* gene, removing amino acid residues 148–281 of SleB. The PCR product was then restricted with *Bam*HI and religated before transformation into *E. coli* DH5 α . A plasmid, pGSB21, carrying the *sleB* deletion was isolated and verified by restriction analysis. The *sleB* deletion was then transferred into *B. subtilis* HR by congression using pGSB21 linearized with *Sca*I and *trp*⁺ DNA. Congressant colonies (*trp*⁺) were selected on SS minimal agar and then screened by PCR for the *sleB* deletion. One congressant, which showed the correct deletion, was verified by Southern blot and named HC145.

Spore preparation and germination. Sporulation was initiated in CCY medium and spores of *B. subtilis* were prepared as described by Stewart *et al.* (1981). Spores were stored at a concentration of 10 mg dry weight ml⁻¹ in distilled water at -20 °C. Purified spores were heat-activated at 70 °C for 30 min and cooled in ice. Germination was initiated by the addition of L-alanine to a final concentration of 1 mM, or by the addition of asparagine to 30 mM with glucose and fructose each to a concentration of 5.6 mM (AGF). Germinant was added to a 5 mg dry weight ml⁻¹ spore suspension in 10 mM Tris/HCl pH 7 containing KCl (10 mg ml⁻¹). Spores were germinated at 37 °C and the extent of germination was monitored by recording the decrease in OD₆₀₀ (Foster &

Johnstone, 1987) over a 2 h period. Phase-darkening of spores was determined by phase-contrast microscopy.

(i) Determination of loss of heat resistance during germination. Germinating spore samples were diluted serially in 10 mM D-alanine and incubated at 70 °C for 30 min. After cooling in ice, viability was measured by plate counting on nutrient agar.

(ii) Measurement of loss of dipicolinic acid during germination. Samples of germination spore suspension (3 ml) were filtered through a 0.45 μ m membrane and the dipicolinic acid content was measured as described by Scott & Ellar (1978).

RP-HPLC analysis of spore peptidoglycan. Cortex extraction from dormant and germinated spores, mucopeptide separation by RP-HPLC, and amino acid and mass spectrometry analyses were performed as previously described (Atrih *et al.*, 1996, 1998).

Analysis of gene expression

(i) Expression under the control of the *Pspac* promoter. Induced expression of sigma factor genes under the control of *Pspac* was carried out by adding IPTG (400 μ M final concentration) to cells growing in LB at an OD₆₀₀ of 0.25 (Sun *et al.*, 1989).

(ii) Expression during sporulation. Synchronous sporulation was performed by the resuspension method of Sterlini & Mandelstam (1969). Samples were harvested every hour after the initiation of sporulation (*t*₀) for 8 h and sporulation morphology was monitored by microscopy.

(iii) Measurement of β -galactosidase activity. β -Galactosidase assays, using MUG (methylumbelliferyl β -D-galactoside) as the substrate, were performed as described by Youngman (1990), except that cells were permeabilized by incubation with lysozyme on ice for 20 min, MUG was used at a final concentration of 600 μ g ml⁻¹ in DMSO and the assay was incubated at 28 °C. Fluorescence was measured on a fluorometer (Hoefer). One unit of β -galactosidase activity was defined as the amount of enzyme which releases 1 pmol methylumbelliferone min⁻¹ ml⁻¹ (Zuberi *et al.*, 1987), normalized to a culture OD₆₀₀ of 1.0.

RESULTS

Characterization of the germination response of *sleB* and *ypeB* mutants

B. subtilis strains FB101 and FB102 contain an *sleB-lacZ* and a *ypeB-lacZ* transcriptional fusion, respectively, which resulted in insertional inactivation of the *sleB* and *ypeB* genes. An in-frame deletion in *sleB* was constructed to give strain HC145. In HC145, YpeB could still be produced in the absence of active SleB. All strains showed comparable sporulation efficiency to the parent. Spores prepared from these mutant strains were phase-bright and heat-resistant (70 °C, 30 min). During L-alanine-triggered germination, wild-type spores lost 55–60% of their initial optical density and became phase-dark, whereas mutant strains FB101 (*sleB*), FB102 (*ypeB*) and HC145 (*sleB* Δ) lost optical density at a slower rate, and became phase-grey (Fig. 1 and data not shown). Thus both SleB and YpeB are necessary for the germination process to occur normally. Similar results were obtained when germination was triggered in AGF (data not shown), suggesting that both *sleB* and *ypeB* are also required for the AGF germination pathway.

Fig. 1. Spore germination of *B. subtilis* HR (wild-type, ○), FB101 (*sleB*, ●), FB102 (*ypeB*, □). Germination was monitored by measurement of OD₆₀₀ at the times indicated after addition of 1 mM L-alanine and is expressed as the percentage loss of initial optical density.

The loss of heat resistance and the release of DPA during germination of strains FB101 (*sleB*), FB102 (*ypeB*) and HC145 (*sleBA*) were comparable to the wild-type (data not shown) in two independent experiments, indicating that these mutants are blocked in the later stages of germination.

The germinated spores from the mutant strains, in germination buffer, remained phase-grey even 12 h after germination was triggered. Strains FB101 (*sleB*), FB102 (*ypeB*) and HC145 (*sleBA*) were, however, capable of outgrowth to form colonies when plated on nutrient agar overnight at 37 °C, indicating that *B. subtilis* can overcome the loss of SleB and/or YpeB to outgrow to form a new vegetative cell.

Peptidoglycan structural analysis of dormant spores

To determine whether the germination defect in the *sleB* and *ypeB* mutants is caused by a structural modification of spore peptidoglycan, the RP-HPLC profiles of Cellosyl-digested peptidoglycan from these mutants were compared to that of the wild-type. No peptidoglycan structural defect could be detected in strains FB101 (*sleB*), FB102 (*ypeB*) and HC145 (*sleBA*) compared to the wild-type (data not shown). The *sleB* and *ypeB* gene products are both likely therefore to be involved in the processes associated with cortex hydrolysis during germination.

(i) Peptidoglycan dynamics during germination. RP-HPLC profiles of germinated-spore-associated peptidoglycan in the wild-type and FB101 (*sleB*) are shown in Fig. 2(a) and 2(b), respectively. After 2 h germination, the ratio of dormant spore mucopeptides and germination-associated mucopeptides was altered between the wild-type (HR; Fig. 2a) and strain FB101 (*sleB*; Fig. 2b). In strain FB101 (*sleB*) there were less germination mu-

Fig. 2. Analysis of mucopeptides by RP-HPLC during germination of *B. subtilis* 168 HR spores. Mucopeptide-containing samples were taken after 2 h germination and separated by RP-HPLC and the A₂₀₂ of the eluates monitored. (a) *B. subtilis* HR germinated-spore-associated material; (b) FB101 (*sleB*) germinated-spore-associated material; (c) germination exudate from HR; and (d) germination exudate from FB101 (*sleB*).

peptides (G1–G7) and correspondingly more dormant spore mucopeptides (10, 11, 20, 21). Strains FB102 (*ypeB*) and HC145 (*sleBA*) showed identical profiles to strain FB101 (*sleB*) (data not shown). This result indicates a partial hydrolysis of the cortex in the *sleB* mutant. The amount of peptidoglycan released, calculated as the ratio of retained material to that of primordial cell wall (mucopeptides 1 and 8) at time 0 and after 2 h germination, for strains FB101 (*sleB*), and FB102 (*ypeB*) was 44% and 38%, respectively. This amount is approximately 20% lower than that released from the wild-type, where 64% of peptidoglycan fragments were released in the germination exudate after 2 h.

Table 2. Calculated and observed m/z values for protonated sodiated and deprotonated molecular ions of new mucopeptides from germination exudate of FB101 (*sleB*)

Mucopeptide*	Ion	m/z		Δm (Da)†	Identity
		Observed	Calculated		
G8A	$[M+H]^+$	785.9	989.0	−203.1	Trisaccharide alanine
	$[M-H]^-$	783.3	987.0	−203.7	
G8B	$[M+Na]^+$	1600.0	1801.7	−201.7	Pentasaccharide tetrapeptide
	$[M-H]^-$	1575.9	1777.7	−202.8	
G8C	$[M+Na]^+$	1227.7	1429.4	−201.7	Pentasaccharide alanine
	$[M-H]^-$	1203.2	1405.4	−202.2	

* Mucopeptides as numbered in Fig. 2(c) and 2(d).

† Difference between observed and calculated protonated, sodiated or deprotonated molecular mass values. Boldface characters denote deviations where the calculated values are the most likely combinations of the substituent components.

(ii) **Germination exudate.** The RP-HPLC profiles of germination exudate of the wild-type and the *sleB* mutant are shown in Fig. 2(c) and 2(d), respectively. The first impression from the RP-HPLC mucopeptide profile of strain FB101 (*sleB*) is the relative abundance of mucopeptides G8, G8A, G8B and G8C compared to the wild-type. Mucopeptide G8 has been previously identified as a trisaccharide tetrapeptide (Atrih *et al.*, 1998). The mucopeptides G8A, G8B and G8C are present in the wild-type, but they are produced in substantial amounts in the *sleB* mutant only. Amino acid analysis and mass spectrometry revealed that mucopeptides G8A, G8B and G8C are trisaccharide alanine, pentasaccharide tetrapeptide and pentasaccharide alanine, respectively (Table 2). These mucopeptides are therefore the products of an *N*-acetylglucosaminidase already suggested to be involved in cortex hydrolysis during germination of *B. subtilis* endospores (Atrih *et al.*, 1998). The most striking difference in the peptidoglycan RP-HPLC profile of strain FB101 (*sleB*) compared to the wild-type is the apparent absence of mucopeptides G9, G10, G12 and G13 (all are reduced by >90% compared to background levels). These mucopeptides have previously been identified as anhydromucopeptides generated by a lytic transglycosylase (Atrih *et al.*, 1998). The RP-HPLC profile of peptidoglycan fragments from germination exudates of strains FB101 (*sleB*), FB102 (*ypeB*) and HC145 (*sleBA*) mutants are similar, suggesting the important role of the gene products in cortex hydrolysis during germination.

Expression of *sleB* during sporulation

Synchronous sporulation of the mutant strains was induced by the resuspension method and β -galactosidase activity from the *sleB-lacZ* and *ypeB-lacZ* transcriptional fusions was measured. In both cases, activity was first detected between t_2 and t_3 (2–3 h after the onset of sporulation, after asymmetric septation) and was maximal at around t_5 , in two separate experiments,

Fig. 3. Timing and the effect of sigma factor mutations on *sleB-lacZ* expression during sporulation. β -Galactosidase levels were measured following induction of synchronous sporulation in strains FB101 (*sleB*, ●) and FB102 (*ypeB*, ■) and various sigma factor mutant background strains [FB103 (*sigE*, △); FB104 (*sigF*, ○); FB105 (*sigG*, □) and FB106 (*sigK*, ▲)].

suggesting that the operon is regulated by a sporulation-specific sigma factor (Fig. 3). The time of induction of both *sleB* and *ypeB* correspond well with the published data for genes regulated by σ^G (Setlow, 1989). An apparent decrease in LacZ activity after t_5 of sporulation has been observed in experiments using fusions to forespore-specific genes such as *gdh* and *gerA* (Mason *et al.*, 1988; Feavers *et al.*, 1990) and is due to the progressive increase in resistance of the developing forespore to lysozyme used in the assay.

Chromosomal DNA carrying the *sleB-lacZ* fusion was transferred by transformation into strains carrying various *spo* mutations and β -galactosidase was measured during sporulation. The absence of expression of *sleB-lacZ* in strain FB105 carrying a mutation in the

Fig. 4. Expression of *sleB-lacZ* in strains carrying various sporulation-specific sigma factors under *Pspac* promoter control during vegetative growth. Strain FB103 (*Pspac-sigF*, ○), FB104 (*Pspac-sigE*, △), FB105 (*Pspac-sigG*, ▲) and FB106 (*Pspac-sigK*, ■) were grown in L-broth and induced by the addition of IPTG to a final concentration of 1 mM. At various times, samples (1 ml) were harvested, treated with lysozyme and assayed for β -galactosidase activity.

spoIIIG gene (σ^G) but still containing an intact *spoIIGB* gene (σ^E) indicates that σ^G is required for the *in vivo* expression of *sleB-lacZ*. Expression was blocked in strain FB104 by an inactivation of the *spoIIABC* (σ^F) operon, as expected, since this sigma factor is required for subsequent expression of other sigma factors during sporulation. As found previously with a number of forespore-specific genes (Setlow, 1989; Mason *et al.*, 1988), *lacZ* expression was not blocked by a mutation in the *spoIIIC* gene (strain FB106) required for σ^K expression which regulates mother-cell-specific gene expression (Fig. 3).

To test the sigma-factor specificity of RNA polymerase which could transcribe the *sleB* gene, the *sleB-lacZ* fusion was introduced by transformation into strains FB107, FB108 and FB109 (Table 1), which contain plasmids having *Pspac*-inducible genes encoding *spoIIGB* (σ^E), *spoIIAC* (σ^F) and *spoIIIG* (σ^G), respectively (Popham & Stragier, 1991; Oke & Losick, 1993; Sun *et al.*, 1989). The *sleB-lacZ* fusion was also introduced into strain FB110, which contains a chromosomally located σ^K coding sequence under *Pspac* control.

Expression of β -galactosidase activity was detected on induction of σ^G , showing that production of σ^G is sufficient to direct expression from the *sleB* promoter (Fig. 4). A putative consensus sequence for σ^G binding is detectable upstream of the transcriptional start site of *sleB*. Expression of β -galactosidase did not occur following induction of σ^K or σ^F , but expression was detected after induction of σ^E in vegetative cells, in three separate experiments (Fig. 4). No expression of *sleB-lacZ* was observed *in vivo* in a *spoIIIG* mutant, which would contain functional σ^E . The expression in veg-

etative cells in response to σ^E induction may be artefactual, or may reflect further levels of regulation.

DISCUSSION

Recent analysis of *B. subtilis* and *B. megaterium* cortex hydrolysis during germination has revealed a complex common process involving at least three hydrolytic enzymes; an *N*-acetylglucosaminidase, a lytic transglycosylase and a possible amidase (Atrih & Foster, 1999; Atrih *et al.*, 1998, 1999). Another activity, suggested to be an epimerase, produces muropeptides with longer retention times than their counterparts in dormant spores. This activity has been extracted from germinated spores of *B. megaterium* along with an *N*-acetylglucosaminidase (Atrih *et al.*, 1999). In this study, we analysed the role of *sleB* and *ypeB*, which form a bicistronic operon (Moriyama *et al.*, 1999) involved in cortex hydrolysis. *sleB* has been previously insertionally inactivated in *B. subtilis* and was shown to be a crucial gene in germination (Moriyama *et al.*, 1996a). Peptidoglycan analysis of the spore-associated material of strain FB101 after 2 h germination revealed a partial hydrolysis of peptidoglycan. More interestingly, the analysis of the muropeptides in the germination exudate showed a major difference compared to that of the wild-type. The anhydromuropeptides generated by the lytic transglycosylase (Atrih *et al.*, 1998) were absent in the exudate, indicating that the gene may encode the lytic transglycosylase. Mutant spores release approximately 20% less peptidoglycan material than the wild-type and the spores remain phase-grey 12 h after addition of L-alanine. These observations indicate the crucial role of the enzyme and demonstrate that it is probably acting on spore-associated material as well as peptidoglycan fragments as previously suggested (Atrih *et al.*, 1998). Another interesting feature of the germination exudate of FB101 (*sleB*) is the increase of *N*-acetylglucosaminidase products. The *N*-acetylglucosaminidase was previously thought to have only a minor role in cortex hydrolysis during germination of *B. subtilis* (Atrih *et al.*, 1998). In *B. megaterium* KM, an extract from germinating spores contains primarily *N*-acetylglucosaminidase activity and is able to cause germination-like changes in permeabilized spores of *B. subtilis* and *B. megaterium* (Atrih *et al.*, 1999). The increase in *N*-acetylglucosaminidase activity may compensate for the absence of lytic transglycosylase, since both enzymes cleave glycan strands. Spore cortex is loosely cross-linked, links occurring at only 2.9% of muramic acid residues (Atrih *et al.*, 1996). This conserved spore peptidoglycan structural feature suggests that cleavage of relatively few bonds in the cortex may result in the mechanical constraint being removed (Atrih & Foster, 1999). The fact that *N*-acetylglucosaminidase is only able to achieve partial hydrolysis of peptidoglycan suggests a co-operative action of the different GSLEs. It is possible that the glucosaminidase may have specific requirements and only cleaves at specific locations. The *sleB* gene was suggested to code an amidase by analogy to the gene in

B. cereus (Moriyama *et al.*, 1996a, 1999). However, our present result indicates that it is likely to be a lytic transglycosylase. Recent findings did not show an amidase activity in the form of amidase products during cortex hydrolysis of *B. subtilis* (Atrih *et al.*, 1998).

The *sleB* gene is the first in the operon and its inactivation is likely to have an effect on the downstream gene, *ypeB*. Insertion inactivation of *ypeB* or in-frame deletion of *sleB* produced mutants with similar germination defects. This indicates that both genes are necessary for cortex hydrolysis during germination and therefore are essential for the activity of the lytic transglycosylase encoded by *sleB*. SleB has recently been shown to be located just inside the spore coat layer in the dormant spore and to exist in mature form but lacking a signal sequence (Moriyama *et al.*, 1999). The enzyme is translocated across the forespore's inner membrane by a secretion signal peptide and is deposited in cortex layer synthesized between the forespore inner and outer membrane (Moriyama *et al.*, 1999).

The role of YpeB is still unclear. It appears to be required for either expression, localization, activation or function of SleB. The N-terminal region of YpeB could represent a hydrophobic anchor for the localization of the protein in the membrane or a signal peptide sequence involved in the translocation of the protein across the membrane. The *ypeB* gene has homologues in *B. cereus* and *B. megaterium* KM, all in the same operon organization, downstream of a *sleB* homologue. In *B. cereus*, the gene corresponding to *ypeB* encodes a protein that has 75 % identity to YpeB from *B. subtilis* (Moriyama *et al.*, 1996b) and the equivalent gene in *B. megaterium* KM encodes a protein having 65 % identity (Pettigrew, 1996). These data suggest that *ypeB* may have the same role in *B. cereus* and *B. megaterium* as in *B. subtilis*.

There are two more homologues of SleB in *B. subtilis*, CwlJ and YkvT, which exhibit 28 % and 30 % identity, respectively, with the putative catalytic C-terminal domain of SleB. CwlJ, like SleB, is involved in the later stages of germination (Ishikawa *et al.*, 1998), although its effects are less pronounced. The double mutant *sleB cwlJ* is blocked completely in later germination so that colony formation is not possible. Interestingly, the lack of cortex hydrolysis does not affect the loss of optical density or dipicolinic acid release, indicating that cortex hydrolysis and release of small solutes during germination are probably two separate events (Sekiguchi *et al.*, 1995; Atrih *et al.*, 1996, 1998; Popham *et al.*, 1996b). However, unlike *sleB*, *cwlJ* and *ykvT* do not occur in an operon with a homologue of *ypeB*. The observation that the phenotype of the *sleB* or *ypeB* mutants are identical suggests that *ypeB* is not involved in the expression or the function of *cwlJ*. The genes *sleB* and *cwlJ* differ in their compartment-specific regulation during sporulation; *cwlJ* is transcribed by $E\sigma^E$ RNA polymerase in the mother cell (Ishikawa *et al.*, 1998), and this work demonstrates that *sleB* expression is dependent on σ^G , a forespore-specific sigma factor. Expression of *sleB* has

also recently been shown to be controlled by σ^G using primer extension analysis (Moriyama *et al.*, 1999).

The mechanism of SleB and CwlJ activation and their molecular interplay, as an integral part of the germination triggering response, is currently under investigation.

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