***In vivo* roles of the germination-specific lytic enzymes of *Bacillus subtilis* 168**

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Germination of endospores of *Bacillus subtilis* involves the activities of several germination-specific lytic enzymes, including glucosaminidase and lytic transglycosylase. Another non-hydrolytic activity, likely to be due to an epimerase, also occurs. The effect of pH on enzyme activities and the overall germination rate was measured. Optimal germination occurred between pH 7–9; however, optimum glucosaminidase and epimerase activities were noted at pH 5. Conversely, the lytic transglycosylase activity was greatest at pH 8. Treatment of spores (15 min) with heat (90 °C) or NaOH (0.25 M) led to impaired cortex hydrolysis/modification, but with <20% loss in viability. Analysis of mucopeptides in the germination exudate revealed a reduction of >85% in glucosaminidase and epimerase products, when compared to untreated spores. Conversely, lytic transglycosylase activity was increased by alkali or heat treatment, which was possibly due to increased substrate availability. FB101 (*sleB*) spores, which lack lytic transglycosylase activity, showed 90-fold greater loss in viability than the wild-type after 1 h at 90 °C. Similarly, 97% of FB101 (*sleB*) spores were unable to form a colony on nutrient agar after 130 min exposure to 0.25 M NaOH at 4 °C, whereas the wild-type was unaffected. This demonstrates the crucial role of the lytic transglycosylase in cortex hydrolysis of damaged spores. The respective targets of heat and alkali in spores and their role during germination are discussed.

Keywords: spores, peptidoglycan, cortex, resistance, lytic transglycosylase

INTRODUCTION

Sporulation occurs in spore-forming bacteria in response to nutrient deprivation, resulting in the formation of spores with high levels of heat and chemical resistance (Nicholson *et al.*, 2000; Warth, 1978). The thick spore-specific peptidoglycan layer, known as the spore cortex, is responsible for the maintenance of spore dormancy (Ellar, 1978; Gerhardt & Marquis, 1989). The success of this survival mechanism lies in the retention of an efficient mechanism for returning the organism to the vegetative state, allowing outgrowth and multiplication when nutrients become available. Dormant spores of *Bacillus subtilis* respond to nutrient germinants, represented by L-alanine or a mixture of L-asparagine, glucose, fructose and KCl (AGFK) (Wax & Freese, 1968), or to non-nutrient germinants (as recently demonstrated by Paidhungat & Setlow, 2000). The trigger

reaction commits spores to undergo a series of degradative events, which result in the loss of spore dormancy and spore-resistance properties (Foster & Johnstone, 1990). Hydrolysis of the spore cortex by germination-specific lytic enzymes (GSLEs) is considered a late event in germination (Atrih *et al.*, 1998; Atrih & Foster, 1999). However, it is essential for the removal of the physical constraint on the core so that outgrowth can occur (Atrih *et al.*, 1998; Atrih & Foster, 1999; Popham *et al.*, 1996).

Recent findings have shown that cortex hydrolysis during germination is a complex process with at least three GSLEs involved: a glucosaminidase, a lytic transglycosylase and a possible amidase (Atrih *et al.*, 1998, 1999; Atrih & Foster, 1999; Boland *et al.*, 2000). Another non-hydrolytic activity, suggested to be an epimerase, has also been noted (Atrih *et al.*, 1998). Two components involved in cortex hydrolysis during *B. subtilis* germination have been identified. Spores of a *sleB* mutant germinate more slowly than the wild-type and analysis of peptidoglycan dynamics during germination has

Abbreviations: AGFK, a mixture of L-asparagine, glucose, fructose and KCl; A₂pm, diaminopimelic acid; GSLE, germination-specific lytic enzyme.

Fig. 1. Hydrolytic activity of GSLEs during germination of *B. subtilis* 168 spores at different pH values. (a) Strain HR (wild-type). (b) Strain FB101 (*sleB*); lytic transglycosylase activity is not shown as FB101 is deficient for this enzyme. □, Germination of *B. subtilis* spores (percentage decrease in OD₆₀₀); ○, glucosaminidase products [nmol mucopeptide (mg dry wt spores)⁻¹]; ▲, epimerase products [nmol mucopeptide (mg dry wt spores)⁻¹]; ●, lytic transglycosylase products [nmol anhydromuropeptide (mg dry wt spores)⁻¹]; ■, hydrolytic activity of GSLEs in terms of A_{2pm} released into the exudate. Muropeptides and A_{2pm} were quantified in germination exudates.

revealed that the gene is likely to encode a lytic transglycosylase (Boland *et al.*, 2000). The *cwlJ* gene product is homologous to SleB (Ishikawa *et al.*, 1998). A *cwlJ* mutation results in slower germination and a *sleB/cwlJ* double mutant cannot hydrolyse the cortex during germination (Ishikawa *et al.*, 1998).

A number of GSLEs have been purified from germinated or broken spores of *Bacillus megaterium*, *Bacillus cereus* and *Clostridium perfringens* (Chen *et al.*, 1997, 2000; Foster & Johnstone, 1987; Makino *et al.*, 1994; Miyata *et al.*, 1995; Moriyama *et al.*, 1996). The common property shared by these GSLEs is the requirement for the presence of the muramic δ -lactam moiety on the spore cortex for activity. Purified GSLEs extracted from spores are not unusually heat resistant (Chen *et al.*, 1997, 2000; Foster & Johnstone, 1987; Moriyama *et al.*, 1996; Warth, 1978); therefore they must be protected against heat within the internal environment of spores. Unfortunately in *B. subtilis*, attempts to isolate active

GSLEs from germinated or physically broken spores have been unsuccessful (T. J. Smith & S. J. Foster, unpublished). In *C. perfringens* heat and NaOH inactivate GSLEs involved in cortex hydrolysis, since treated spores can be partially rescued by lysozyme (Duncan *et al.*, 1972; Labbe & Chang, 1995). However, the role of the multiple GSLEs of *B. subtilis* and their potential as sporocidal targets is still unknown. In this study we have analysed the combined function of the GSLEs of *B. subtilis* in germination under different environmental conditions and in response to sporocidal treatments.

METHODS

Preparation and germination of spores. Spores of *B. subtilis* HR (wild-type) and *B. subtilis* FB101 (*sleB*; Boland *et al.*, 2000) were prepared in CCY medium, washed, and stored as previously described (Atrih *et al.*, 1996, 1998).

Germination of spores at different pH values. Washed spores of strain HR and strain FB101 were suspended at a final concentration of 3 mg dry wt ml⁻¹ in distilled water, and heat-shocked at 70 °C for 30 min. Spores were then cooled on ice and used within 1 h (Atrih *et al.*, 1998). The following germination buffer solutions were used at a final concentration of 20 mM in the presence of 20 mM KCl: sodium acetate/acetic acid (pH 3.5–5), potassium phosphate buffer (pH 5–7), and Tris/HCl buffer (pH 8–10). Germination was triggered by L-alanine (1 mM; a saturating concentration for germination) and monitored by recording the decrease of OD₆₀₀ as previously described (Atrih *et al.*, 1998). After 2 h, germination exudates were collected by centrifugation (14000 g, 8 min, 4 °C), boiled for 3 min and the pH was adjusted to 5 before lyophilization. Germination was carried out for 2 h, a time at which > 90% of total OD₆₀₀ loss had occurred. All germination experiments were repeated at least twice and showed less than 10% variation.

Spore heat treatment. Spores were suspended at a final concentration of 3 mg dry wt ml⁻¹ in distilled water and heat-treated at 90 °C for different times. The heat-treated spores were immediately cooled on ice and used for germination experiments within 1 h, without further heat shock.

Spore alkali treatment. Spores (4 mg dry weight ml⁻¹) were suspended in distilled water (on ice), and NaOH was added to give a final concentration of 0.25 M. After treatment the spores were recovered by centrifugation (12000 g, 8 min, 4 °C) and washed three times by centrifugation and resuspension with 20 mM phosphate buffer at pH 6, then twice with distilled water at 4 °C. Spores were heat shocked at 65 °C for 25 min and germination was then performed as indicated above.

Lysozyme recovery of heat- or alkali-treated spores. Lysozyme was added to molten nutrient agar (48 °C) to give a final concentration of 0.15, 0.3, 0.6 and 1.0 µg ml⁻¹ prior to pouring the plates. The experiments were performed in duplicate and plate counts were determined after 48 h. The lysozyme concentrations used in this study do not affect the total viable count of HR untreated spores.

Muropeptide purification and amino acid analysis. Muropeptide purification and quantification in the germination exudate were performed as previously described (Atrih *et al.*, 1996, 1998). Diaminopimelic acid (A_{2pm}), a peptidoglycan-specific amino acid, was used to measure the amount of peptidoglycan released in the exudate by the Pico-Tag method

Fig. 2. Analysis of mucopeptides by reverse-phase HPLC. Mucopeptide-containing samples of germination exudate (2 h) from an equivalent amount of dry weight of (a) *B. subtilis* untreated spores, and (b) *B. subtilis* heat-treated spores (90 °C for 1 h) were digested with Cellosyl and the resulting mucopeptides were separated by HPLC. After this heat treatment 8% of the spores retained viability. The numbered mucopeptides are products of the following enzymes: G1–G7, likely epimerase activity (Atrih *et al.*, 1998); G8, G8A, G8B and G8C, glucosaminidase (Atrih *et al.*, 1998; Boland *et al.*, 2000); G9, G10, G12 and G13, lytic transglycosylase (Atrih *et al.*, 1998). X peaks represent non-peptidoglycan-derived products.

(Atrih *et al.*, 1996, 1998). All experiments were carried out in duplicate. The results shown are representative; there was < 5% variability between experiments.

RESULTS

Effect of pH on germination

Heat shocked spores were germinated over a pH range of 3.5 to 10. Optimal germination (57–60% drop in OD₆₀₀ readings) occurred at pH 6–10, with a sharp decline to only a 23% loss in the OD₆₀₀ value at pH 3.5 (Fig. 1a). Microscopic analysis (phase contrast) showed that at pH 3.5 only a small proportion of the spore population had germinated (results not shown). Glucosaminidase and lytic transglycosylase activities during germination result in products which appear in significant quantities in only the germination exudate (Atrih *et al.*, 1998). Epimerase products are present in roughly equal amounts in the exudate and the spore-associated material. Alteration in spore-associated epimerase products matched that in the exudate under all treatments used in this study (results not shown). Analysis of exudates from spores germinated at different pH values (2 h, 37 °C) revealed the pattern of enzyme activities involved in cortex hydrolysis and modification. Enzyme activities were measured by the amount of products generated. Fig. 1(a) shows that glucosaminidase and epimerase products were maximal at pH 5 [7.9 nmol and 8.5 nmol mucopeptides (mg dry wt spores)⁻¹ after 2 h, respectively]. The highest activities of these enzymes at pH 5 were apparent in both acetate and phosphate buffers (results not shown). Conversely, lytic transglycosylase was less active at acidic pH. It was

maximal at pH 8, generating a total of 2.2 nmol anhydro-mucopeptides (mg dry wt spores)⁻¹ (Fig. 1a). GSLE hydrolytic activities resulted in fragments of peptidoglycan released into the exudate, which could be assayed as A₂₀₂pm. Optimal GLSE hydrolytic activities [19.7 nmol A₂₀₂pm (mg dry wt spores)⁻¹] occurred at pH 5 after 2 h germination.

Germination of heat-treated spores

Heat treatment of spores at 90 °C for 130 min did not affect the dormant spore mucopeptide profile, and the spores remained phase bright (results not shown). However, it did result in a loss of approximately 20% of the initial OD₆₀₀ of the dormant spore suspension (results not shown). After germination of the heat-treated spores > 90% became phase dark. The reverse-phase HPLC profile of mucopeptides in the germination exudate of untreated and heat-treated spores (90 °C for 1 h) germinated at pH 5 for 2 h is shown in Fig. 2. Surprisingly, germinated heat-treated spores demonstrated a complete absence of the mucopeptides generated by glucosaminidase (G8, G8A, G8B and G8C; Atrih *et al.*, 1998; Boland *et al.*, 2000) and likely epimerase activity (G1–G7; Atrih *et al.*, 1998). Germinated heat-treated spores also showed an increase in mucopeptides generated by lytic transglycosylase activity (G9, G10, G12 and G13; Atrih *et al.*, 1998).

To examine the relative heat sensitivity of peptidoglycan hydrolytic and modification activities, spores were heat treated at 90 °C for 15, 30 and 60 min and then germinated at pH 5 for 2 h prior to mucopeptide analysis. Quantification of the enzyme products revealed

Fig. 3. GSLE activity during germination of *B. subtilis* heat-treated spores at pH 5. (a) Strain HR (wild-type). (b) Strain FB101 (*sleB*). □, Germination of *B. subtilis* spores expressed in terms of the percent decrease in OD₆₀₀ readings; ○, glucosaminidase products [nmol mucopeptide (mg dry wt spores)⁻¹]; ▲, epimerase products [nmol mucopeptide (mg dry wt spores)⁻¹]; ●, lytic transglycosylase products [nmol anhydro-muropeptide (mg dry wt spores)⁻¹]; ■, hydrolytic activity of GSLEs in terms of A₂pm [nmol A₂pm (mg dry wt spores)⁻¹]. Muropeptides and A₂pm were quantified in germination exudates.

that 88% and 87%, respectively, of glucosaminidase and epimerase products disappeared within 15 min heat treatment at 90 °C (Fig. 3a). Conversely, lytic transglycosylase activity increased after heat treatment. Indeed, after 1 h treatment at 90 °C the total amount of lytic transglycosylase products was 6.6-fold more than that in untreated spores (Fig. 3a). Quantification of A₂pm in the exudate of 1 h heat-treated spores revealed that even after prolonged heat treatment, 45% of the material was still released compared to the control (Fig. 3a).

Germination and cortex hydrolysis/modification capabilities of heat-treated spores were also examined at pH 7. After 15 min heat treatment at 90 °C, germination exudate (2 h germinated spores at pH 7) was completely devoid of glucosaminidase and epimerase products (results not shown). Lytic transglycosylase was, however, more active at pH 7 than pH 5 as evidenced by a 12% increase in the amount of anhydro-muropeptides found in the exudate of 1 h heat-treated spores (results not shown).

Fig. 4. GSLE activity during germination of *B. subtilis* HR alkali-treated spores at pH 5. □, Germination of *B. subtilis* spores expressed in terms of the percent decrease in OD₆₀₀ readings; ○, glucosaminidase products [nmol mucopeptide (mg dry wt spores)⁻¹]; ▲, epimerase products [nmol mucopeptide (mg dry wt spores)⁻¹]; ●, lytic transglycosylase products [nmol anhydro-muropeptide (mg dry wt spores)⁻¹]; ■, hydrolytic activity of GSLEs in terms of A₂pm [nmol A₂pm (mg dry wt spores)⁻¹]. Muropeptides and A₂pm were quantified in germination exudates.

Germination of alkali-treated spores

Treatment of dormant *B. subtilis* HR spores with 0.25 M NaOH for 130 min did not affect the peptidoglycan structure significantly, although a minor change of a 1.5% increase of muropeptides with an open lactam was noted (results not shown) (Atrih *et al.*, 1996). Alkali-treated spores were evaluated for their germination ability and cortex hydrolysis. The loss of OD₆₀₀ during germination actually increased after 1 h in 0.25 M NaOH treatment, from 41% (untreated) to 47%, a slight but reproducible effect (Fig. 4). The loss of epimerase and glucosaminidase products in alkali-treated spores followed a similar pattern to heat-treated spores: 88% and 89% of epimerase and glucosaminidase products, respectively, were lost within 15 min (Fig. 4). Lytic transglycosylase activity was not only alkali resistant, but actually increased after treatment (Fig. 4). The amount of A₂pm released from 1 h alkali-treated spores was 40% lower than that released from untreated spores. Heat- and alkali-treated spores were also germinated in the presence of AGFK and similar results to those obtained with L-alanine were noted (results not shown).

Germination of *sleB* mutant spores

Spores of *B. subtilis* FB101 (*sleB*) have a defect in cortex hydrolysis during germination, due to the lack of the lytic transglycosylase activity (Boland *et al.*, 2000). Fig. 1(b) shows that the optimum loss of OD₆₀₀ during germination occurred at pH 7, whereas release of peptidoglycan material was greatest at pH 5 (Fig. 1b). As expected, both glucosaminidase and epimerase ac-

Fig. 5. Effect of heat or alkali treatment on dormant spore viability of *B. subtilis* HR and *B. subtilis* FB101 (*sleB*). The symbols used represent: ○, *B. subtilis* HR heat-treated at 90 °C; □, *B. subtilis* HR alkali-treated with 0.25 M NaOH; ●, *B. subtilis* FB101 heat-treated at 90 °C; ■, *B. subtilis* FB101 alkali-treated with 0.25 M NaOH. Cell recovery was calculated using the equation (treated c.f.u. ml⁻¹/untreated c.f.u. ml⁻¹) × 100, and expressed as percentage values.

tivities are maximal at pH 5. Under no conditions was any lytic transglycosylase activity observed. Heat-treated spores of FB101 (*sleB*) were dramatically affected in germination and cortex hydrolysis. After 1 h treatment at 90 °C, glucosaminidase and epimerase were scarcely detected after germination at pH 5 for 2 h (Fig. 3b). The release of A₂pm into the exudate also decreased dramatically, with only 6% of the value of the untreated sample released (Fig. 3b). FB101 (*sleB*) alkali-treated spores also showed a similar pattern in GSLE inactivation (results not shown).

Effect of heat or NaOH on spore survival

As GSLEs are crucial enzymes in cortex hydrolysis their inactivation, by heat or NaOH, may affect spore survival. Heat treatment (90 °C) of *B. subtilis* HR spores led to a decline in viable cell numbers (as measured by c.f.u.) resulting in only 0.2% recovery after 130 min treatment. Spores of FB101 (*sleB*) were considerably more heat sensitive, with only 0.01% recovery after 130 min heat treatment (Fig. 5). Wild-type spores of *B. subtilis* were not affected by alkali treatment (0.25 M) for up to 130 min (Fig. 5). However, the lack of *SleB* in strain FB101 resulted in sensitivity to alkali. Indeed, 97% of spores had lost viability within the 130 min treatment (Fig. 5).

Lysozyme recovery of heat- and alkali-treated spores

If cortex lytic enzymes are damaged during treatment, spores may not be able to outgrow and divide to form a colony on nutrient agar. Exogenous lysozyme can substitute for endogenous enzymes and may allow recovery to occur (Duncan *et al.*, 1972; Labbe & Chang, 1995). Lysozyme has no effect on the colony-forming ability of HR (wild-type) spores with or without heat treatment (results not shown). Unexpectedly, lysozyme resulted in a reproducible 1.5-fold increase in recovery of even untreated spores of FB101 (*sleB*) (Table 1). Indeed our results suggest on average 33% of *sleB* spores were unable to form colonies on nutrient agar plates (Table 1). Lysozyme treatment of FB101 (*sleB*) resulted in comparable c.f.u. per unit OD₆₀₀ as wild-type (results not shown). Lysozyme was also able to recover FB101 strain (*sleB*) spores after heat and NaOH treatment. This recovery was lysozyme concentration dependent. Treatment with 1 µg lysozyme ml⁻¹ resulted in a five- and tenfold increase in recovery after heat or NaOH treatment for 60 min and 130 min, respectively.

Table 1. Effect of lysozyme on recovery of untreated, and heat- or alkali-treated spores of FB101 (*sleB*)

All spores were treated at OD₆₀₀ 2.5. The results are the mean of two experiments with two different preparations of spores, which showed less than twofold variability between each run.

Lysozyme concn (µg ml ⁻¹)	c.f.u. ml ⁻¹ for untreated spores	Heat-treated spores (90 °C, 60 min)		Alkali-treated spores (0.25 M, 130 min)	
		c.f.u. ml ⁻¹	Recovery (%)*	c.f.u. ml ⁻¹	Recovery (%)*
0.00	8.7 × 10 ⁶	8.0 × 10 ³	0.10	3.0 × 10 ⁵	3.45
0.15	1.1 × 10 ⁷	4.0 × 10 ⁴	0.36	3.0 × 10 ⁶	27.27
0.30	1.2 × 10 ⁷	5.0 × 10 ⁴	0.42	3.5 × 10 ⁶	29.17
0.60	1.3 × 10 ⁷	5.6 × 10 ⁴	0.43	3.9 × 10 ⁶	30.00
1.00	1.3 × 10 ⁷	6.5 × 10 ⁴	0.50	4.8 × 10 ⁶	36.92

* Determined from viable counts obtained after incubation of plates at 37 °C for 48 h. Counts obtained for untreated spores at each lysozyme concentration were considered as 100% recovery. Percentage recovery for heat- or alkali-treated samples was calculated for each lysozyme concentration, using the equation (treated c.f.u. ml⁻¹/untreated c.f.u. ml⁻¹) × 100.

DISCUSSION

Cortex hydrolysis of *B. subtilis* spores is complex and at least three enzyme activities are involved in germination (Atrih *et al.*, 1998): glucosaminidase, lytic transglycosylase and non-hydrolytic, likely epimerase, activities. The individual roles of the enzymes responsible for the observed activities are unknown. In order to unravel the complex interrelationship between the enzymes, the effects of germination conditions and sporicidal treatments were assessed *in vivo*.

Glucosaminidase activity has been previously reported to have a minor role in cortex hydrolysis at pH 7 (Atrih *et al.*, 1998). Here we have shown that glucosaminidase activity is optimum at pH 5. Germination at pH 5 also resulted in a maximal release of peptidoglycan fragments as shown by the amount of A₂pm in the exudate. This is in contrast with the lower loss of OD₆₀₀ compared to spores germinated at pH 7–10. The loss of OD₆₀₀ has already been shown to occur in spores without any cortex hydrolysis (Atrih *et al.*, 1998; Popham *et al.*, 1996; Sekiguchi *et al.*, 1995). Therefore, it is likely to be due to the loss of other spore components such as dipicolinic acid, and the uptake of water (Atrih & Foster, 1999; Sekiguchi *et al.*, 1995). The effect of pH on germination of *B. megaterium* and *B. subtilis* has been previously investigated (Ciarciaolini *et al.*, 2000; Stewart *et al.*, 1981). In both organisms optimum absorbance loss occurs at slightly basic pH 7–9, which correlates with our results.

Another important feature of *B. subtilis* GSLEs is their activities over a pH range of 4–10. These observations concur with previous reports on various purified GSLEs from other spore-forming bacteria (Chen *et al.*, 1997, 2000; Makino *et al.*, 1994). The differential activities of the GSLEs at different pH values may be important in underlining their potential role in spore germination in diverse environments.

GSLEs isolated from spore-forming bacteria are not unusually heat resistant and are generally denatured at temperatures lower than those required in inactivating whole spores (Chen *et al.*, 2000; Makino *et al.*, 1994). The extensive studies of Warth (1980) also indicate that enzymes of central metabolism are inactivated at temperatures 24–46 °C lower than those required to inactivate the same enzymes within intact spores.

Heat or alkali treatment of *B. subtilis* spores resulted in rapid loss of epimerase and glucosaminidase activities. These results suggest that the enzymes themselves, or their activation mechanism(s), are sensitive to heat and alkali. The fact that the two distinct treatments affect the same enzymes suggests that the localization or the mechanisms of activation of these enzymes are different to that of the lytic transglycosylase. The NaOH effect implies a close association or location of some germination components with the alkali-soluble proteins, which are removed by such treatment (Duncan *et al.*, 1972; Gould *et al.*, 1970). The lytic transglycosylase is not affected by heat or NaOH and thus this enzyme

is protected to a much greater degree than the other enzymes. Alternatively, the glucosaminidase and epimerase may be protected, but their activation mechanism(s) is not.

In *B. subtilis*, *sleB* and the adjacent downstream *ypeB* gene form a bicistronic operon (Moriyama *et al.*, 1999), and both genes are necessary for cortex hydrolysis (Boland *et al.*, 2000). SleB is most likely a lytic transglycosylase (Boland *et al.*, 2000) and is located in a mature form on the outside of the cortex in the dormant spore (Moriyama *et al.*, 1999). This localization of the enzyme does not correlate with its exceptional resistance to heat and alkali, suggesting that it may have a unique protection mechanism. Studies using FB101 (*sleB*) highlight the pH dependence of germination enzyme activities. In FB101 (*sleB*) the maximal release of peptidoglycan material during germination occurred at pH 5, the pH at which glucosaminidase activity is greatest. The role of the likely epimerase in cortex hydrolysis is not clear, as it is not a hydrolytic enzyme. However, it has been previously suggested to cause an alteration in peptidoglycan conformation which may affect the activity of the GSLEs (Atrih *et al.*, 1998). The present study suggests that the epimerase is not necessary for cortex hydrolysis since the lytic transglycosylase is active in the absence of significant epimerase activity.

CwlJ is another apparent GSLE involved in cortex hydrolysis (Ishikawa *et al.*, 1998). The hydrolytic bond-specificity of this enzyme is still unknown and the double mutant *sleB/cwlJ* is unable to hydrolyse the cortex as evidenced by the absence of peptidoglycan fragments in the germination exudate (Ishikawa *et al.*, 1998). Thus, the epimerase and glucosaminidase activities are unable to initiate cortex hydrolysis. Also, CwlJ activity or its activation mechanism must be destroyed by heating, as FB101 (*sleB*) spores show reduced heat resistance compared to the parent. The lytic transglycosylase is probably the only significant enzyme which survives heat treatment and is able to hydrolyse the cortical peptidoglycan to allow outgrowth. The heat sensitivity of FB101 (*sleB*) spores is not due to the core dehydration level, as the core wet density of the mutant is comparable to that of wild-type spores (results not shown).

From previous work it was not apparent whether the products of glucosaminidase or lytic transglycosylase activities are acted on by the epimerase (Atrih *et al.*, 1998). In this study, the lytic transglycosylase products are not altered by epimerase because their retention time is identical in the presence or absence of epimerase. Similarly, partially purified glucosaminidase from *B. megaterium* generated products with identical retention time to those obtained in the presence of epimerase (Atrih *et al.*, 1999). It is possible that epimerase products are resistant to the action of other enzymes, and that the subtle modification of the peptidoglycan may direct hydrolysis to optimize the hydrolytic effect of the other GSLEs. Thus, the increase in lytic transglycosylase activity in both the heat- and alkali-treated spores could

be explained by the availability of substrate in the absence of the other enzymes.

The loss of spore viability during heat treatment cannot be correlated directly with GSLE activities. Indeed, after 1 h treatment of spores at 90 °C only 8% of spores remained viable but the lytic transglycosylase was still fully active. Thus, even though cortex hydrolysis is taking place in this case, it is likely that outgrowth or vegetative growth components have been damaged, thus preventing colony formation. This is confirmed by the inability of lysozyme to recover heat-treated spores of *C. perfringens* (Duncan *et al.*, 1972; Labbe & Chang, 1995). Enzymes and other proteins are the main targets for heat-killing of spores (Belliveau *et al.*, 1992; Marquis *et al.*, 1994; Nicholson *et al.*, 2000); however, it is still unclear which proteins are the critical targets for such treatment (Nicholson *et al.*, 2000).

Alkali treatment of spores, although it affects glucosaminidase and epimerase activities, does not affect spore viability. Also, CwlJ activity must be inactivated by alkali as *sleB* spores are much more sensitive than the wild-type to this treatment. Thus, the glucosaminidase, epimerase and CwlJ activities are not crucial for germination in alkali-treated spores; however *SleB* certainly is. In *C. perfringens* both heat and alkali treatment reduced the apparent viability of spores to a comparable level (Duncan *et al.*, 1972). Furthermore, the loss of viability in alkali-treated spores was linked to inactivation of GSLEs. This implies that although the bacterial spores share a conserved peptidoglycan structure, the GSLEs involved in its hydrolysis show some diversity (Atrih & Foster, 1999, 2001; Foster & Johnstone, 1988; Moriyama *et al.*, 1999).

Previous studies have shown that heat combined with additional controlling factors (pH, organic acids, preservatives) affects *Bacillus* species spore viability (Oloyede & Scholefield, 1994), outgrowth and germination (Ciarciaglini *et al.*, 2000). However, none of these studies identified the molecular target of the treatments. Here we have shown that the major GSLE, *SleB*, is a critical resistance determinant for *B. subtilis* spores in response to heat, and in particular NaOH. Thus, development of inhibitors of GSLEs coupled with sporicidal treatments may provide novel combinatorial approaches to prevent outgrowth of potentially deleterious spore-formers. It is by a further understanding of the molecular mechanisms of spore germination and resistance that rational spore control measures can be developed.

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