Analysis of spore cortex lytic enzymes and related proteins in *Bacillus subtilis* endospore germination

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The location and function of recognized cortex-lytic enzymes of *Bacillus subtilis* have been explored, and the involvement in germination of a number of related proteins tested. The SleB and CwlJ proteins are cortex-lytic enzymes, partially redundant in function, that are required together for effective cortex hydrolysis during *B. subtilis* spore germination. Spores were fractionated, and Western blotting of individual fractions suggests that the CwlJ protein is localized exclusively to the outer layers, or integument. The second spore-lytic enzyme, SleB, is localized both in the inner membrane of the spore and in the integument fraction. Neither protein changes location or size as the spore germinates. The *ypeB* gene is the second gene in a bicistronic operon with *sleB*. The SleB protein is absent from *ypeB* mutant spores, suggesting that YpeB is required for its localization or stabilization. In fractions of wild-type spores, the YpeB protein is found in the same locations as SleB – in both the inner membrane and the integument. As the absence of CwlJ protein does not affect the overall RP-HPLC profile of peptidoglycan fragments in germinating spores, this enzyme’s hydrolytic specificity could not be defined. The effects of inactivation of several homologues of cortex-lytic enzymes of as yet undefined function were examined, by testing null mutants for their germination behaviour by OD₆₀₀ fall and by RP-HPLC of peptidoglycan fragments from dormant and germinating spores. The YaaH enzyme is responsible for a likely epimerase modification of peptidoglycan during spore germination, but the loss of this activity does not appear to affect the spore’s ability to complete germination. Unlike the other cortex-lytic enzymes, the YaaH protein is present in large amounts in the spore germination exudate of *B. subtilis*. Mutants lacking either YdhD or YvbX, both homologues of YaaH, had no detectable alteration in either dormant or germinating spore peptidoglycan, and germinated normally. The *ykvT* gene, which encodes a protein of the SleB/CwlJ family, has no apparent association with germination: the gene is expressed in vegetative cells, and mutants lacking YkvT have no detectable phenotype.

**Keywords:** germination-specific cortex-lytic enzymes, *ypeB*, *yaaH*

**INTRODUCTION**

Bacterial endospores have a unique structure that determines their extreme resistance properties. The structure of *Bacillus subtilis* endospores includes three distinct features, i.e. spore coat, cortex and core. The spore cortex, which is required for spore dormancy and heat resistance, consists of a thick layer of peptidoglycan of a spore-specific structure. Despite their resistance and dormancy, spores will respond to chemical stimuli by germinating. The earliest events in spore germination involve receptor proteins encoded by homologues of the

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Abbreviations: GSLE, germination-specific cortex-lytic enzyme; RP-HPLC, reverse-phase HPLC.
GerAA, AB and AC proteins (Paidhungat & Setlow, 1999; Moir et al., 2002), which are located in the inner membrane (Hudson et al., 2001; Paidhungat & Setlow, 2001). Receptor–germinant interaction results in the triggering of a cascade of biochemical events, including loss of heat resistance and ion movements from the core (Thackray et al., 2001; Southworth et al., 2001). Hydrolysis of spore cortex by cortex-specific lytic enzymes is a later event in germination (Popham et al., 1996; Atrih & Foster, 1999; Boland et al., 2000). The enzymic hydrolysis of spore-cortex peptidoglycan is essential for spores to complete rehydration during germination and to commence outgrowth.

Spore cortex-lytic enzymes have been purified from spores of Bacillus megaterium (Foster & Johnstone, 1987), Bacillus cereus (Makino et al., 1994; Moriyama et al., 1996b; Chen et al., 2000a, b) and Clostridium perfringens (Miyata et al., 1995), and were reviewed by Atrih & Foster (1999). Based on structural analysis of spore peptidoglycan and its dynamics during germination, Atrih et al. (1998, 1999) have suggested that there are at least three different types of enzyme activities involved in spore-cortex hydrolysis and modification during germination: glucosaminidase, lytic transglycosylase and, a possible non-hydrolytic epimerase. SleB and CwlJ proteins have been recognized as important enzymes in normal spore germination of B. subtilis. The SleB protein (Moriyama et al., 1996a) has apparent lytic transglycosylase activity (Boland et al., 2000). The CwlJ protein is required for rapid germination in otherwise wild-type spores, and is strictly essential for cortex hydrolysis if the SleB protein is absent (Ishikawa et al., 1998); recent genetic experiments suggest that it is activated by dipicolinic acid or its calcium chelate (Paidhungat et al., 2001).

SleB has been shown to be responsible for the germination-associated lytic transglycosylase activity (Boland et al., 2000), and spores of an sleB mutant are slow to complete late stages of germination. A ypeB mutant, defective in the gene downstream of sleB, shows the same germination defect as an sleB mutant (Boland et al., 2000).

A cwlJ single mutant is blocked in late stages of germination, failing to phase-darken completely; Chen et al. (2000a) described a cortex-lytic enzyme of B. cereus (SleL) that is found in an active form in the germination exudate. It requires disrupted rather than intact spore peptidoglycan for its activity, and is therefore likely to act on a substrate that has already been cleaved by another lytic enzyme, such as SleB. Its role in germination, however, has not been tested by mutation. The YaaH, YdhD and YvbX proteins of B. subtilis are homologues of SleL (49, 30 and 26% amino acid identity, respectively), the homology extending throughout the protein. All have putative cell-wall-binding motifs. A yaaH mutant was reported as slow to germinate (Kodama et al., 1999), and a ydhD-overexpressing strain made spores of some of which germinated spontaneously, and others of which were reluctant to germinate (Kodama et al., 2000).

In the work described below, we have characterized and localized the CwlJ, SleB and YpeB proteins in fractions of dormant and germinating spores. We have also analysed the function of the uncharacterized B. subtilis homologues of cortex-lytic enzymes, by making null mutations in each gene. This has shown that the likely epimerase activity during germination is dependent on YaaH.

**METHODS**

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are listed in Table 1. B. subtilis mutations were created in, or transferred into, the laboratory wild-type background (HR). Vegetative cells of B. subtilis were grown in nutrient broth or agar (Oxoid). Escherichia coli strains were grown on Luria–Bertani broth or agar. When appropriate, chromosomal drug-resistance markers in B. subtilis were selected with chloramphenicol (5 µg ml⁻¹), kanamycin (10 µg ml⁻¹), spectinomycin (100 µg ml⁻¹) or erythromycin (1 µg ml⁻¹) and lincomycin (25 µg ml⁻¹). All bacterial cultures were grown at 37 °C.

**Spore preparation and germination.** Spores were prepared from cultures allowed to sporulate in CCY medium (Stewart et al., 1981) as previously described (Atrih et al., 1996). Purified spores were heat-activated at 70 °C for 30 min and cooled in ice before the addition of germinants. Germination, at 37 °C, was initiated by the addition of l-alanine (1 mM) or a combination of asparagine (30 mM), glucose (5.6 mM) and fructose (5.6 mM), to spore suspension in 10 mM Tris/HCl (pH 7) containing KCl (10 mg ml⁻¹) (AGK). For peptidoglycan analysis, the germination was carried out using concentrated spore suspensions as described by Atrih & Foster (2001b).

**Reverse-phase (RP)-HPLC analysis of spore peptidoglycan.** Preparation of peptidoglycan from spore cortex and vegetative cells of B. subtilis, muropeptide separation by RP-HPLC, and amino acid analysis were performed as described by Atrih et al. (1996, 1998).

**β-Galactosidase assay.** Levels of β-galactosidase activity were measured using methylumbelliferyl β-d-galactoside as substrate, as described by Horsburgh & Moir (1999).

**DNA isolation and cloning.** Standard cloning technologies (Sambrook et al., 1989) were used. PCR was carried out using Expand (Boehringer Mannheim). Transformation of B. subtilis was as described by Kunst & Rapoport (1995).

**Construction of insertionally inactivated cwlJ and ykvT mutants.** For the construction of a cwlJ mutant, a 1270 bp DNA fragment spanning the cwlJ region was synthesized by PCR using B. subtilis chromosomal DNA as template. Primers 5′-GGCCAAATTTCAGACCAGACCAGATACA-3′ and 5′-GATCCGAGCTTTCCAGCACGCTTCTTTCAT-3′ were used as forward and reverse primers, respectively (the chromosomally derived sequence is italicized and the EcoRI and SacI restriction sites added are underlined). The PCR products were cloned into plasmid pBlueScript IKS (Stratagene) after appropriate restriction digestion and ligation. The recombinant plasmid, pHCJ1, was isolated from a transformant of E. coli DH5α and was used as a template DNA for an inverse-PCR using primers 5′-GACAGGATCCGAGGCC- GAATTAATTCA-3′ and 5′-TACAGGCCCTCGGCTC- GATATGGTGATAG-3′ as forward and reverse primers, respectively (the italicized chromosomal sequences are 120 bp apart in the cwlJ gene; the BamHI and SstI restriction sites 5′-GATCCGAGCTTTCCAGCACGCTTCTTTCAT-3′ and 5′-GATCCGAGCTTTCCAGCACGCTTCTTTCAT-3′ were used as forward and reverse primers, respectively (the chromosomally derived sequence is italicized and the EcoRI and SacI restriction sites added are underlined). The PCR products were cloned into plasmid pBlueScript IKS (Stratagene) after appropriate restriction digestion and ligation. The recombinant plasmid, pHCJ1, was isolated from a transformant of E. coli DH5α and was used as a template DNA for an inverse-PCR using primers 5′-GACAGGATCCGAGGCC- GAATTAATTCA-3′ and 5′-TACAGGCCCTCGGCTC- GATATGGTGATAG-3′ as forward and reverse primers, respectively (the italicized chromosomal sequences are 120 bp apart in the cwlJ gene; the BamHI and SstI restriction sites
Table 1. B. subtilis strains and plasmids

<table>
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<tr>
<th>Strain or plasmid</th>
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<td>Laboratory stock</td>
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* Cm', chloramphenicol resistance; Km', kanamycin resistance; Em', erythromycin resistance; Sp', spectinomycin resistance.

added are underlined). The PCR product was then restricted with BamHI and Stal and ligated with a kanamycin cassette obtained by restriction digestion of pDG792 (Guerout-Fleury et al., 1995) with the same enzymes. E. coli DH5α was transformed with the ligation mixture, and a recombinant plasmid, pHCJ2, containing the insertion of the kanamycin cassette in the cwlJ gene, was isolated. This plasmid DNA was then linearized with ScaI, gel-purified, and used for transforming B. subtilis. HC101, a kanamycin-resistant transformant in which the insertional inactivation copy had replaced the wild-type gene by a double crossover (confirmed by Southern hybridization) was isolated.

A mutant with a spectinomycin-resistance gene insertional inactivating yku'T was constructed in an analogous fashion. A 1248 bp DNA fragment spanning the yku'T region was synthesized by PCR using B. subtilis chromosomal DNA as template. Primers 5'-GGCGCATTTCCAGAAGTGGCAGTATCCAGA-3' and 5'-GGCGCATTTCCAGAAGTGGCAGTATCCAGA-3' were used as forward and reverse primers, respectively. A recombinant plasmid, pHYT1, containing the PCR fragment in pBluescript KS was used as a template DNA for an inverse-PCR using primers 5'-ATGTGGATCCGTTGCTGCTGATAAGTTTACA-3' and 5'-ACGGACGGCTTTAGGCCTGCTGCTGCTGCAAAG-3' as forward and reverse primers, respectively, to leave a 124 bp gap within the yku'T gene. The PCR product was then restricted with BamHI and Stal and ligated with a spectinomycin cassette obtained by restriction digestion of pDG1727 (Guerout-Fleury et al., 1995) with the same enzymes. E. coli DH5α was transformed with the ligation mixture and a recombinant plasmid, pHYT2, containing the insertion of the spectinomycin cassette in the yku'T gene, was isolated. This plasmid was then linearized by ScaI, gel-purified, and used for transforming B. subtilis. A transformant with the correct insertion of the spectinomycin cassette in the yku'T gene, checked by Southern hybridization, was named HC201.

Construction of a yku'T-lacZ fusion strain. A lacZ transcriptional fusion of yku'T was constructed by cloning a 655 bp fragment generated by PCR in pMUTIN4 (Vagner et al., 1998) to yield pHYC2. Primers 5'-CGCGAATTTCTCAGAAGCTGATCAGTCAGA-3' and 5'-ATGTTGATCCGTTGCTGCTGATAAGTTTACA-3' were used as upstream and downstream primers, respectively. A recombinant plasmid, pHC101, contained the yku'T gene, checked by Southern hybridization, was named HC201.

Overexpression and purification of proteins (CwlJ, SleB and YpeB). The CwlJ, SleB and YpeB proteins were overexpressed by cloning the respective genes in E. coli overexpression vector pET24d (Novagen). The primers were designed to make C-
terminal histidine-tagged fusions for affinity purification of the overexpressed proteins.

The *cwlJ* gene was amplified by PCR using 5'-CGTG-CCATGCGGTCCGTAGAGCA-3' and 5'-ACTG-CGCAGGAAATGTTTATATACCTTTATCACAGC-3' as forward and reverse primers, respectively. The *sleB* gene was amplified, without its signal sequence, by PCR using 5'-CATGGCATGCTTTTGGATACAGGC-3' and 5'-CGGTCGGATCACGAAATGTTTACCC-3' as forward and reverse primers, respectively. The *ypeB* gene was amplified by PCR using 5'-CATGCGCATGCTCTAGAGGGAATCTTTAT-AATATCG-3' and 5'-CCGCTCGAGATCGTCTTTATATAGTTCGCTAT-3' as forward and reverse primers, respectively. The PCR products were gel-purified, restriction-digested with NcoI and Xhol and ligated with pET24d DNA, restricted with the same enzymes. The ligation mixtures were used to transform *E. coli* DH5α to kanamycin resistance. Recombinant plasmids, pETHCJ1, pETMOR3 and pETMOR2, carrying the cloned *cwlJ*, *sleB* and *ypeB* genes, respectively, were isolated. *E. coli* BL21(DE3) was transformed with pETHCJ1, pETMOR3 and pETMOR2, and expression was induced by the addition of 0.4 mM IPTG. Inclusion bodies were collected, and solubilized in 8 M urea. The overexpressed proteins were purified using a HisTrap (Amersham Pharmacia) affinity column in urea-containing buffers, and dialysed against PBS. The purity of the preparations was assessed on Coomassie-blue-stained gels as > 95%.

**Raising of antibodies and Western blot analysis.** Polyclonal antibodies against *CwlJ*, *SleB* and *YpeB* were raised in rabbits by injecting purified proteins as previously described (Hudson *et al.*, 2001). Antisera were collected and contaminating *E. coli* antibodies were removed on an immobilized *E. coli* lysate column (Pierce). Proteins were detected after Western blotting by chemiluminescence (Amersham Pharmacia). Primary antibody was generally used at 1/5000 dilution, and secondary antibody (horseradish-peroxidase-linked anti-rabbit IgG F(abbit)2 fragment) at 1/5000 dilution.

**Spore fractionation and protein separation.** Spores were broken by using a Fast-Prep blue kit (BIO-101) with a Fast-Prep system reciprocal shaker (BIO-101), and fractions prepared from 50 mg dry weight of spores as described by Hudson *et al.* (2001). In one experiment, the fractionation process was limited to separation of the integument fraction from the membrane plus soluble material, by omitting the high-speed centrifugation normally used to separate the latter components. Samples for analysis by SDS-PAGE were adjusted to give equivalent loading of protein (usually 10 µg protein per lane). Biotinylated markers (from Sigma or Bio-Rad) were detected with horseradish peroxidase-linked streptavidin.

**RESULTS**

**Localization of CwlJ within spores**

Polyclonal antibodies were raised against overexpressed and purified histidine-tagged *CwlJ*, *SleB* and *YpeB* proteins, and were used in Western blotting experiments. The *SleB* protein was overexpressed in a form that corresponded to the processed, mature form of the enzyme; attempts to overexpress the protein complete with its signal sequence failed. Cross-reacting anti-*E. coli* antibodies were reduced or removed by purification on a column containing immobilized *E. coli* lysate (Pierce). Each antibody preparation was able to recognize its cognate protein antigen overexpressed in *E. coli* (data not shown). Spores of strain HR, the laboratory wild-type 168 strain, or of otherwise isogenic mutants, were used.

Spores were isolated, washed and fractionated into soluble, inner-membrane and integument fractions by the method already described and verified (Hudson *et al.*, 2001). Briefly, washed spores were broken by shaking with silica beads in a PMSF-containing buffer. Fractionation involved low-speed centrifugation to recover a pellet containing the integument fraction (which contains coats, cortex and outer membrane), then at higher centrifugal force to pellet a membrane fraction, which contains inner membrane, leaving the remaining soluble material (Hudson *et al.*, 2001). In equivalent fractions, the GerAA and GerAC proteins were found exclusively in the inner-membrane fraction. Whole spore extracts (Hudson *et al.*, 2001) of protease-negative strain WB600,
Cortex hydrolysis in *B. subtilis* spore germination

Wild-type HR, or of specific mutants were used as positive and negative controls. Western blot analysis of overexpressed CwlJ protein in *E. coli*, and of spore fractions probed with anti-CwlJ antibody, are shown in Fig. 1. The predicted molecular mass of CwlJ is 16 kDa, and a protein of this size is detected in *E. coli* extracts, along with minor additional bands at twice this molecular mass and larger. This suggests that in *E. coli* the overexpressed CwlJ protein may form some oligomers, not entirely dissociated in standard SDS-PAGE loading buffer. In spore fractions, no cross-reaction was seen with proteins in either the soluble or the inner-membrane fraction, but strong signals were obtained from the integument fraction (Fig. 1a). The specificity of the antiserum was verified by its reaction with overexpressed protein in *E. coli* extracts (Fig. 1a, b) with purified CwlJ protein (Fig. 1b), and by the absence of cross-reactive bands in extracts from a *cwlJ* mutant (Fig. 1b). Purified CwlJ from *E. coli* runs as a monomer and in higher molecular mass forms, but, under the conditions used, CwlJ in spore extracts ran as a diffuse band of 32 kDa. This suggests that CwlJ may be present in the spore in dimeric and larger forms, or alternatively, that its mobility is modified, possibly by association with another component. This higher apparent molecular mass was not the consequence of covalent binding to peptidoglycan, as lysozyme treatment neither released the enzyme from the integument, nor altered the apparent molecular size (Fig. 1b).

**Localization of SleB and YpeB within spores**

The *ypeB* gene is located downstream of *sleB* in a bicistronic operon in *B. subtilis*, and a *ypeB* mutant shows the same germination defect as an *sleB* mutant. YpeB has no detectable homology with autolysins, or with any proteins of known function in other organisms, although it has orthologues in other spore-formers, including *B. cereus* and *Clostridium acetobutylicum*. A Western blot of spore fractions was probed sequentially with anti-YpeB and anti-SleB antisera. The results are summarized in Fig. 2. The anti-SleB antibody detects a band of apparent molecular mass 29 kDa, corresponding to the size expected for SleB, in total spore extract, but not in extracts of vegetative cells. Specificity is confirmed by the absence of this band in extracts of *sleB* mutant spores, as shown in the germination data.
presented later. The SleB protein was detected strongly in both integument and membrane fractions.

Probing with anti-YpeB antibodies revealed a 51 kDa band corresponding to the size of YpeB in the membrane and integument fractions of the spore (Fig. 2). Additional cross-reactive bands, of approximately 54 kDa and 116 kDa, were seen, but these were also present in vegetative extracts. The 51 kDa band was present in wild-type spores, and data presented below demonstrate its absence from extracts of a strain carrying a sleB mutation that would be polar on ypeB, confirming it as YpeB-specific. The 51 kDa band also corresponded to the size of the YpeB protein, expressed in E. coli (data not shown). The band corresponding to YpeB was only just visible in tracks representing total spore extracts, or in the integument fraction. A comparison with the stronger signal from SleB in the same extract (Fig. 2, lane 2), suggests that the YpeB protein is not very efficiently detected in total extracts of broken spores or integuments, perhaps because of inefficient solubilization, although it can be detected as a strong signal in the purified membrane fraction.

**YpeB is required for SleB localization**

Anti-SleB serum was used to probe a Western blot of total spore extracts prepared from strain FB102 (ypeB); in this strain, the upstream sleB gene remains intact. No SleB protein was detected in either dormant or germinated spores (compare Fig. 3a with Fig. 3b). The absence of SleB from these spores indicates that expression of YpeB is important for the presence of SleB in the spore; it may be required for the localization of the protein, and/or for its stabilization against proteolysis. Expression of the sleB–ypeB operon, as measured by a lacZ transcriptional fusion, remains high in a ypeB mutant background (Boland et al., 2000).

**Analysis of SleB, YpeB and CwlJ during germination**

Western blot analysis was carried out to study possible changes in SleB that might relate to its activation during germination. Spores were collected at specific intervals (0, 10, 30, 60 and 120 min) after the initiation of germination at 37 °C in l-alanine, with chloramphenicol (10 µg ml⁻¹) to prevent any protein synthesis. The percentage OD₆₀₀ fall (57% represents germination of 100% of the spores for 10, 30, 60 and 120 min was 20, 40, 52 and 57%, respectively. Spores were centrifuged (12000 g for 10 min) and an extract of total spores was prepared after breakage of the pelleted material with silica beads, as described for dormant spore extracts by Hudson et al. (2001). The results for wild-type spores (Fig. 3b) show a strongly cross-reactive band of the expected size of SleB, at 30 kDa, present at all time points. There was no change in size of the protein to suggest a proteolytic activation of the SleB protein during germination, and no evidence of bulk release of the protein from the spore. The YpeB protein profile changed in germinating wild-type spores, however (Fig. 4a). The 51 kDa band is, as in Fig. 2, only faintly visible in dormant spore extracts, and it disappears by 10 min. At this time, a strongly detected 30 kDa band appears; this band is not present in extracts from germinating spores of a strain carrying a mutation in sleB that is polar on ypeB. We deduce, from the strong and specific signal, that this 30 kDa band is likely to correspond to a specific cleavage product of YpeB. Other strong bands in the blot on Fig. 4(a) are not YpeB-specific, as they are still present in a YpeB mutant.

An analogous experiment used the anti-CwlJ antibody. Extracts were prepared from spores germinated for 15 min (18% OD₆₀₀ fall) or 30 min (45% OD₆₀₀ fall).
The bands corresponding to CwlJ protein do not change in either size or intensity during germination, as detected on Western blots (Fig. 5).

Proteins released from spores during germination

The supernatants from germinated spore suspensions of strain HR (germination exudates) were concentrated 50-fold by freeze-drying, and subjected to SDS-PAGE on a 12% gel. Two major protein bands were detected on these gels after Coomassie blue staining. These, with apparent molecular masses of 50 and 22 kDa, were also present in exudates from sleB or cwlJ single mutants (data not shown). The N-terminal sequences of the two proteins [MQYVVKQGDKLAIAXQYRTTTND and NYRNPAANDDM, respectively] indicated that the larger was intact YaaH (discussed in detail below; note also that its initiation codon is four codons earlier than that reported in GenBank), and that the other protein was YhcN, reported by Bagyan et al. (1998) to play a minor role in spore outgrowth; as reported by those authors, we confirmed that a yhcN mutant showed no defect in initiation of germination.

Testing of other potential lytic enzymes for their role in germination

Spore suspensions of null mutants in cwlJ and in potential lytic enzyme genes ykvT, yaaH, ydhD and yvbX (Table 1) were prepared. For each one, germination behaviour, as measured by OD<sub>600</sub> loss and by the muropeptide pattern during germination, was examined.

YaaH. YaaH is 49% identical to SleL of B. cereus, an enzyme demonstrated to have N-acetylglucosaminidase activity (Chen et al., 2000a). Although Chen et al. (2000a) reported a germination defect, spore preparations of a yaaH mutant constructed by transfer of their null mutation into our standard laboratory strain (HR) background showed wild-type germination kinetics in l-alanine, and outgrew normally (data not shown). The composition of dormant-spore peptidoglycan, as revealed by RP-HPLC analysis of muropeptides after Cellosyl digestion, was identical to wild-type traces published previously (Atarih et al., 1998). The germination exudate, however, contained altered muropeptides (Fig. 6). Muropeptides G1–G4 and G6–G7 are absent from the profile. These muropeptides have been previously identified in B. subtilis and in B. megaterium, and have been suggested to be generated by an epimerase (Atarih et al., 1998, 1999). In contrast, muropeptides generated by glucosaminidase activity (G8, G8A–G8C) or lytic transglycosylase activity (G9–G10 and G12–G13), and those already identified in dormant spores (G10–G11 and G20–G21), are found in the yaaH-mutant exudate. The total amount of peptidoglycan released into the germination exudate of the mutant was similar to that of the wild-type. The activity of YaaH is therefore not required for the hydrolysis of the cortex in germination, but is required for the epimerase-like activity.

In the germination exudate from an sleB yaaH double mutant, the muropeptides generated by both lytic transglycosylase and epimerase activities are absent (Fig. 6c). This results in a further increase in the proportion of glucosaminidase products (G8B – pentasaccharide tetrapeptide, G8C – pentasaccharide alanine) relative to those of hexasaccharide tetrapeptide (muropeptide 20) and hexasaccharide alanine (muropeptide 21).

YdhD and YvbX. Both YdhD and YvbX are homologues of YaaH. Like yaaH, both ydhD and yvbX are expressed during sporulation (Kodama et al., 2000). Spores made from ydhD and yvbX null mutants germinated apparently normally. The RP-HPLC profiles of dormant-spore muropeptides, germinating spores, or germination exudate, showed no difference from wild-type (data not shown). Therefore these homologues appear to have no individually unique involvement in cortex peptidoglycan synthesis during sporulation, or in cortex breakdown during germination.

YkvT. SleB has strong similarity to two other proteins in the B. subtilis database: CwlJ and YkvT. The YkvT protein sequence shows 31% identity to the equivalent region of SleB. It has a shorter putative N-terminal wall-binding domain, and, as in SleB, this is connected to the predicted catalytic domain by a linker region. The ykvT gene was inactivated by insertion of a spectinomycin-resistance cassette by a double crossover event. Germination of mutant spores was identical to that of the wild-type (data not shown), and its combination with cwlJ and/or sleB mutations produced the same phenotypes as expected for those mutants without a ykvT mutation. Measurements of the β-galactosidase activity of cells carrying a ykvT::lacZ fusion (strain HC203)
showed that the gene is expressed maximally during vegetative growth, and expression falls in post-exponential phase cells (data not presented). Muropeptide analysis of the vegetative and spore peptidoglycan showed that there was no difference between the ykvT mutant and the wild-type parent (data not shown). The YkvT protein is therefore a vegetatively expressed protein of apparently redundant function.

**CwlJ.** As expected from previous work (Ishikawa et al., 1998), spores of HC101 (cwlJ::kan) germinated more slowly than the wild-type spores in both l-alanine and AGFK, as estimated by a late-germination parameter, i.e. loss of OD₆₀₀ (data not shown). Although the rate of OD₆₀₀ loss in spore suspensions was slow in the first 30 min (28% fall by this time rather than 53%), by 60 min the proportion of the initial OD₆₀₀ lost was almost as high as for the wild-type, and the spores were phase-dark.

The cortex-lysis products from this mutant were examined after germination at different pH values, as it has been demonstrated that the activity of cortex-lytic enzymes is affected by the pH of the germination buffer (Atrih & Foster, 2001). The RP-HPLC profiles of Cellosyl-digested muropeptides, both of germination exudate and of the remaining spore-associated material of cwlJ spores germinated for up to 2 h at pH 5, 7 and 9, were all identical to the profiles seen in the equivalent wild-type control (results not shown).

The possibility remains that CwlJ is a muramidase, whose activity would be masked by the overall muramidase digestion mediated by Cellosyl as part of the muropeptide analysis protocol. To address this, spore-associated material and exudates from spores of AM1596 (cwlJ yaaH) and AM1597 (yaaH) were compared (inclusion of the yaaH mutation simplifies the pattern of peaks observed). Muramidase activity would result in the production of new reducing groups which would be labelled with sodium borohydride. Thus, samples from spores germinated at pH 5 and 10 were borohydride-treated prior to Cellosyl digestion and the muropeptide profiles compared. In no case was there any difference in the reduced muropeptide profile between the strain bearing the cwlJ mutation and its respective parent. Thus CwlJ is unlikely to have significant muramidase activity.
DISCUSSION

In this work, spore-lytic enzymes demonstrated as important for spore germination were localized in the spore. The CwlJ protein is required for spore germination in response to calcium dipicolinate (Paidhungat et al., 2001), which bypasses the normal nutrient germination receptors (Paidhungat & Setlow, 2000), and CwlJ activity appears to be stimulated by the release of intra-spore dipicolinic acid during normal nutrient-stimulated germination (Paidhungat et al., 2001). Our evidence suggests that CwlJ is located in the periphery of the spore, but that it is not covalently associated with peptidoglycan. CwlJ (16 kDa) shows 30% sequence identity to the deduced catalytic domain of B. subtilis SleB, the lytic transglycosylase enzyme, but it lacks the N-terminal putative cell-wall-binding domain found in SleB. The cwlJ gene is expressed during sporulation and is under the control of Eσ^R RNA polymerase (Ishikawa et al., 1998). The precise location of CwlJ in the integument is not obvious, but it is not necessarily in the cortex, as it is expressed in the mother cell and has no apparent signal sequence for export across a membrane. Evidence from the germination behaviour of decoated and cotE mutant spores suggests that it may be lost when the coat is removed chemically or its assembly is disturbed (Paidhungat et al., 2001). The tendency of the protein to dimerize at 32 kDa in our Western blots of extracts from broken spores suggests that it may be present in the spore in dimeric and multimeric forms, or, alternatively, that it may be stably complexed with another molecule.

We have shown previously that sleB is expressed during sporulation and that the expression starts between 2 and 3 h after the onset of sporulation (Boland et al., 2000). Expression in the forespore, the possession of a signal sequence, and evidence of a peptidoglycan-binding domain would suggest a likely location of SleB either on the outside of the inner spore membrane, or in the cortex. Our data show its presence in both inner-membrane and integument fractions of spores. SleB has been shown to be more resistant to spore damage, in particular heat and alkali, than the other germination-specific cortex-lytic enzyme (GSLE) activities (Atrih & Foster, 2001). In fact, spores of a sleB mutant are relatively sensitive to alkali. SleB also has a different pH optimum from the other GSLEs. The present study suggests that the localization of GSLEs in the dormant spore may be involved in their resistance properties. SleB colocalizes, at least in part, with YpeB, which may therefore be responsible for SleB protection in the dormant spore. YpeB has a sequence at its N-terminus likely to represent either a signal sequence or a membrane anchor, and at least some of this protein is located in the inner-membrane fraction.

A dual location of SleB and YpeB is unexpected. Moriyama et al. (1999) used immunoelectron microscopy to demonstrate that the SleB enzymes from B. subtilis and B. cereus are located just inside the spore-coat layer in the dormant spore, but our study provides strong evidence for a second, additional localization in the inner spore membrane. In a cwlJ mutant, the function of SleB protein during germination is essential for colony formation on rich agar medium. Chemical decoating of cwlJ mutant spores makes very little difference to their ability to germinate to form colonies (Paidhungat et al., 2001). Therefore chemical decoating, while it removes CwlJ from spores, does not remove enough SleB to interfere with its role in germination. It is possible, therefore, that the location of some SleB protein at the inner membrane is functionally important. The location of YpeB and SleB in the inner membrane also places them in an appropriate position for their activation by receptor-mediated signal transduction, as the receptor proteins are also inner membrane-located.

How GSLE activity is triggered during germination is still unknown. As SleB may be stabilized by YpeB, destabilization of their association, perhaps related to the proteolytic processing of YpeB, may result in activation of heat-sensitive SleB. The recent observation that CwlJ can be activated by calcium dipicolinate still does not explain how physiological, nutrient germinants act to initiate the process. The question of how germinant binding to receptors triggers activation of these lytic enzymes is currently being addressed.

The other enzymes recognized as germination-specific cortex-modifying enzymes, CwlJ and YaaH, do not require YpeB protein for their localization or function. The role of YaaH is uncertain, but it may alter the activity of other enzymes by the substrate modification it mediates. The enzyme(s) responsible for the glucosaminidase activity during germination and the activity of CwlJ remains elusive. It is only after identification of all the cortex hydrolysis/modification activities during germination that their individual and combined roles can be determined.

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