Characterization of LytH, a Differentiation-Associated Peptidoglycan Hydrolase of *Bacillus subtilis* Involved in Endospore Cortex Maturation

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The cortex peptidoglycan from endospores of *Bacillus subtilis* is responsible for the maintenance of dormancy. LytH (YunA) has been identified as a novel sporulation-specific component with a role in cortex structure determination. The *lytH* gene was expressed only during sporulation, under the control of the mother cell-specific sigma factor σ^K. Spores of a *lytH* mutant have slightly reduced heat resistance and altered staining when viewed by electron microscopy. Analysis of the peptidoglycan structure of *lytH* mutant spores shows the loss of muramic acid residues substituted with L-alanine and a corresponding increase in muramic acid residues substituted with tetrapeptide compared to those in the parent strain. In a *lytH cwlD* mutant, the lack of muramic acid residues substituted with L-alanine and δ-lactam leaves 97% of residues substituted with tetrapeptide. These results suggest that *lytH* encodes an L-Ala–D-Glu peptidase involved in production of single L-alanine side chains from tetrapeptides in the spore cortex. The lack of di- or tripeptides in a *lytH* mutant reveals the enzyme is an endopeptidase.

Bacterial endospores are characterized by their extreme dormancy and high-level resistance to a range of stresses, in particular heat (7). The specialized spore structure determines resistance properties, with the dehydrated spore core being crucial for heat resistance. The spore cell wall peptidoglycan, known as the spore cortex, is essential for the maintenance of the dehydrated core, heat resistance, and dormancy (7). The cortex has a unique, spore-specific structure, and recent analysis has begun to identify features important in its role during differentiation and to reveal components responsible for cortex synthesis, modification during sporulation, and hydrolysis during germination (1, 2). Spore cortex is characterized by an extremely low level of cross-linking, which is important for the maintenance of heat resistance (1, 15). A unique feature of the dormant spore cortex is the presence of muramic acid δ-lactam residues. This modification has a specific role during germination, because the putative germination-specific lytic enzymes SleB and CwlJ, which are responsible for cortex degradation, require the presence of muramic acid δ-lactam for substrate recognition, since cortex lacking this modification is unable to be hydrolyzed during germination (2).

During cortex maturation, a sporulation-specific amidase, CwlD, and a recently described polysaccharide deacetylase, PdaA, are responsible for the formation of the characteristic δ-lactam residues of bacterial endospores (1, 8, 15). Although *cwlD* or *pdaA* mutants and a *cwlD pdaA* double mutant can produce resistant endospores, no cortex hydrolysis is observed during germination, and the spores do not outgrow (1, 2, 8, 16, 18). The final characteristic feature of the cortex is that about 25% of the muramic acid side chains are substituted for with single L-alanine residues (1, 17). These may be formed by the action of an L-Ala–D-Glu endopeptidase on nascent stem peptides. The enzyme or enzymes responsible for the single L-Ala side chains have up to now been unknown, as has the role of this peptidoglycan modification. In this paper, we describe a novel peptidoglycan hydrolase, LytH (formerly YunA), which is required for the production of the single L-Ala side chains in the spore cortex.

MATERIALS AND METHODS

Bacterial strains, growth, and preparation of spores. All *Bacillus subtilis* strains used in this study are shown in Table 1. Mutations were transformed into the laboratory wild-type background (HR) used for muropeptide analysis (1). Vegetative cells of *B. subtilis* were grown in nutrient broth (Oxoid) or on nutrient agar plates. Sporulation was initiated in CCY medium, and spores of *B. subtilis* were prepared as described by Stewart et al. (23). Spores were stored at a concentration of 10 mg (dry weight) ml^{-1} in distilled water at $-20°C$.

Plasmids were constructed in and prepared from *Escherichia coli* strain DH5α grown in Luria-Bertani (LB) broth or on LB agar. When appropriate, chromosomal drug resistance markers in *E. coli* were selected with ampicillin (50 μg ml^{-1}), and those 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 a *lytH* mutation. (i) Plasmid construction. Primers were designed to enable the amplification, by PCR, of a 359-bp fragment of DNA internal to the *lytH* gene. The forward primer GH18 (5′-GCCG**AAGCTT**GGC ACAGATGAAGACAACA-3′) contained a *Hin*dIII site (boldface) followed by 19 nucleotides identical to the DNA sequence between bases 472 and 490 within the *lytH* gene (underlined). The reverse primer GH19 (5′-CGC**GGATCC**TGG GCAAAATAATGATACG-3′) contained a *Bam*HI site (boldface) followed by 19 nucleotides identical to residues 806 to 824 (underlined). The 359-bp DNA fragment corresponding to part of the *lytH* gene was amplified by PCR, purified, digested with *Hin*dIII and *Bam*HI, and then ligated into *Hin*dIII- and *Bam*HI-restricted pMUTIN4 to create plasmid pGJH160.

(ii) Transformation of *E. coli* and *B. subtilis*. Transformation of *E. coli* DH5α was performed as described by Hanahan (9). Transformation of *B. subtilis* 1A304 with pGJH160 was performed by the method of Kunst and Rapoport (10). Disruption of the *lytH* gene by Campbell-type recombination was confirmed by Southern blot analysis with the insert in pGJH160 as a probe. Hybridization, probe labeling, and detection were done with the Boehringer Mannheim non-radioactive DNA labeling and detection kit.

TABLE 1. *B. Subtilis* strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference[a]
Strains		
B. subtilis 168		
1A304	*trpC2 metB5 xin-1* SPβ(s)	BGSC[b]
HR	*trpC2*	Laboratory stock
1A304 background		
GJH160	*trpC2 metB5 xin-1* SPβ(s) *lytH*::pMUTIN4 Em^r	This study
HR background		
SH243	*trpC2* P*spac-sigE* Kan^r	Laboratory stock
SH61	*trpC2* P*spac-sigF* Cm^r	Laboratory stock
SH248	*trpC2* P*spac-sigG* Kan^r	Laboratory stock
SH132	*trpC2* P*spac-sigK* Cm^r	Laboratory stock
1295	*trpC2 spoIIGB::kan*	J. Errington, Oxford University
650	*trpC2 iluB2 leuB16 spoIIAABC::cat*	J. Errington, Oxford, University
618	*trpC2 spoIIIC::cat*	25
1296	*trpC2 spoIIIG::kan*	J. Errington, Oxford University
GJH161	*trpC2 lytH*::pMUTIN4 Em^r	This study
GJH162	*trpC2 spoIIGB::kn lytH*::pMUTIN4 Em^r	1295 → GJH161
GJH163	*trpC2 spoIIAABC::cat lytH*::pMUTIN4 Em^r	650 → GJH161
GJH164	*trpC2 spoIIIG::kn lytH*::pMUTIN4 Em^r	1296 → GJH161
GJH165	*trpC2 spoIIIC::cat lytH*::pMUTIN4 Em^r	618 → GJH161
GJH166	*trpC2* P*spac-sigE lytH*::pMUTIN4 Em^r Kan^r	GJH161 → SH243
GJH167	*trpC2* P*spac-sigF lytH*::pMUTIN4 Em^r Cm^r	GJH161 → SH61
GJH168	*trpC2* P*spac-sigG lytH*::pMUTIN4 Em^r Kan^r	GJH161 → SH248
GJH169	*trpC2* P*spac-sigK lytH*::pMUTIN4 Em^r Cm^r	GJH 161 → SH132
AA107	*trpC2 cwlD::cat*	1
GJH171	*trpC2 cwlD::cat lytH*::pMUTIN4 Em^r	GJH160 → AA107
Plasmids		
pMUTIN4	Suicide vector in *B. subtilis;* Em^r	26
pGJH160	pMUTIN4 containing 359-bp *Hin*dIII-*Bam*HI insert carrying part of *lytH* gene	This study

[a] Arrows indicate transformation of recipient strain with donor chromosomal DNA.
[b] *Bacillus* Genetic Stock Center, Ohio State University, Columbus.

Multiple mutant crosses. Chromosomal DNA isolated from strain GJH160 (*lytH*) was transformed into HR (wild type) to create strain GJH161. The transformation into HR was performed because previous peptidoglycan structural analysis had been carried out in this background (1–3). Chromosomal DNA from strain GJH160 was also transformed into strains carrying various sporulation sigma factor mutations (Table 1). These crosses created strains GJH162, GJH163, GJH164, GJH165, GJH166, GJH167, GJH168, and GJH169, which carry mutations in *sigE*, *sigF*, *sigG*, *sigK*, P*spac sigE*, P*spac sigF*, P*spac sigG*, and P*spac sigK*, respectively. The P*spac* sigma factor strains are mutants in which the genes have been separated from their own promoters and placed under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter, so expression of the genes can be controlled. Chromosomal DNA was isolated from strain AA107 (*cwlD*) and transformed into strain GJH161 (*lytH*), creating strain GJH171 (*lytH cwlD*). All strains were verified by the appropriate drug resistance tests and Southern blotting.

Measurement of spore heat resistance. Spores were resuspended in 1.5 ml of sterile distilled water to a final optical density at 600 nm (OD$_{600}$) of 2.5. The spore samples were then incubated at 90°C in a water bath, and 0.1-ml aliquots were removed at intervals and diluted into 0.9 ml of ice-cold sterile distilled water. Appropriate dilutions were made in sterile distilled water, and samples were spread on nutrient agar in duplicate. The results obtained were the average from two independent experiments.

Spore protoplast wet density. The wet density of the *B. subtilis* spore protoplast was determined by equilibrium density centrifugation on metrizamide (Sigma) gradients as described previously (12). Prior to density determination, spores were decoated for 90 min at 37°C in a mixture of 50 mM Tris-HCl (pH 8.0), 8 M urea, 1% (wt/vol) sodium dodecyl sulfate, and 50 mM dithiothreitol and washed three times with 150 mM NaCl and twice with water by centrifugation (11,000 × *g* for 5 min at room temperature) and resuspension. The decoated spores were equilibrated for 60 min in 50% (vol/vol) metrizamide prior to loading onto

gradients. Results are an average of two different spore preparations, which show variation between each other of <0.001 g/ml.

Analysis of gene expression. Synchronous sporulation was performed by the resuspension method of Sterlini and Mandelstam (22). Samples were harvested every hour after the initiation of sporulation (*t$_0$*) for 7 h, and sporulation morphology was monitored by microscopy. Induced expression of sigma factor genes under the control of P*spac* was carried out by adding IPTG (1 mM final concentration) to cells growing in LB at an OD$_{600}$ of 0.25 (24). β-Galactosidase assays, using MUG (methylumbelliferyl β-D-galactoside) as the substrate, were performed as described by Youngman (27), except that cells were permeabilized by incubation with lysozyme (250 μg ml^{-1}) on ice for 20 min. MUG was used at a final concentration of 600 μg ml^{-1} in dimethyl sulfoxide, and the assay mixture 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 that releases 1 pmol of methylumbelliferone min^{-1} ml^{-1} (25), normalized to a culture OD$_{600}$ of 1.0.

RP-HPLC analysis of spore peptidoglycan. Vegetative peptidoglycan extraction, cortex extraction from dormant and germinating spores, muropeptide separation by reverse-phase high-performance liquid chromatography (RP-HPLC), and amino acid and mass spectrometry analyses were performed as previously described (1–3).

Transmission electron microscopy. Samples for examination were recovered by centrifugation, and the supernatant was removed. The pellet was fixed by resuspension in glutaraldehyde fixative (3% [vol/vol] glutaraldehyde in 0.1 M phosphate buffer). Samples were incubated in fixative for 2 h at room temperature. Following fixing, the samples were washed five times (10 min each) in 0.1 M sodium cacodylate buffer and then postfixed in 2% (wt/vol) aqueous osmium tetroxide. Following a wash in distilled water, samples were incubated in 75% (vol/vol) ethanol (three changes, 5 min each), 95% (vol/vol) ethanol (three changes, 5 min each), and then absolute ethanol (two changes, 10 min each).

Further incubation with dried absolute ethanol (two changes, 10 min each) and propylene oxide (two changes, 10 min each) was followed by overnight incubation in a 1:1 mixture of propylene oxide and araldite. Two 3-h incubations in fresh 100% (vol/vol) araldite were followed by embedding in 100% (vol/vol) araldite and polymerization (60°C, 48 h). Sections from the embedded sample were cut into 80-nm sections on a Reicher Ultracut. The sections were then stained with saturated uranyl acetate in 50% (vol/vol) ethanol for 15 min, washed in distilled water, and then stained in Reynolds lead citrate for 5 min. Specimens were viewed at a range of magnifications on a Phillips CM-10 electron microscope operated at 80 kV. At least 50 spores of each strain were examined.

RESULTS

Role and regulation of *lytH*. The predicted protein encoded by *lytH* (*yunA*) is 349 amino acids (aa) in length with a calculated M_r of 39.5 kDa and an estimated pI of 6.89. A general BLAST search revealed LytH to have the highest homology to peptidase M37 (54% identity, 309 aa) from *Bacillus anthracis*. This is part of the M23/37 family of zinc metallopeptidases, in which are included the Gly-Gly endopeptidases, such as lysostaphin. Smith et al. (20) had previously identified YunA (LytH) as a lysostaphin homologue. Interestingly, the presence of muropeptides containing glycine has been observed previously in spores of a *cwlD* mutant (16) and vegetative cell wall peptidoglycan (3). However, the presence of these structures has been shown to be dependent on the medium (3). The *lytH* gene, which is not located within any potential prophage, is followed by a putative ρ-independent terminator and is preceded by a divergently transcribed gene, *yutB*, which is itself followed by a putative ρ-independent terminator and encodes a protein similar to lipoic acid synthase.

In order to determine the role of *lytH*, a mutation was created by the insertion of the suicide plasmid pGJH160 into the *B. subtilis* chromosome. This led to inactivation of *lytH* and the creation of a *lytH::lacZ* transcriptional fusion. No defects in vegetative growth rate or sporulation kinetics were found for GJH161 (*lytH*) compared to its parent (HR).

Expression analysis. Expression of *lytH* was monitored during vegetative growth and sporulation. No β-galactosidase activity of strain GJH161 was observed during vegetative growth (data not shown). When analyzed for expression during sporulation, β-galactosidase activity was first detected between t_2 and t_3 (2 to 3 h after the onset of sporulation, after asymmetric septation) and was maximal around t_5 in two separate experiments, suggesting that the operon is regulated by a sporulation-specific sigma factor (Fig. 1).

Chromosomal DNA carrying the *lytH::lacZ* fusion was transferred by transformation into strains carrying various *spo* mutations, and β-galactosidase activity was measured during sporulation (Fig. 1). The absence of expression of *lytH::lacZ* in strain GJH165 carrying a mutation in the *spoIIIC* gene (σ^K) but still containing an intact *spoIIIG* gene (σ^G) indicates that σ^K is most likely required for the in vivo expression of *lytH::lacZ*. As expected, expression was blocked in strains GJH163, GJH164, and GJH165, because σ^E, σ^F, and σ^G are all required for later sigma factor activity.

To test the sigma factor specificity of RNA polymerase that could transcribe the *lytH* gene, the *lytH::lacZ* fusion was introduced by transformation into strains SH243 (P*spac-sigE*), SH61 (P*spac-sigF*), SH248 (P*spac-sigG*), and SH132 (P*spac-sigK*) to produce strains GJH166, GJH167, GJH168, and GJH169, respectively. Expression of β-galactosidase activity
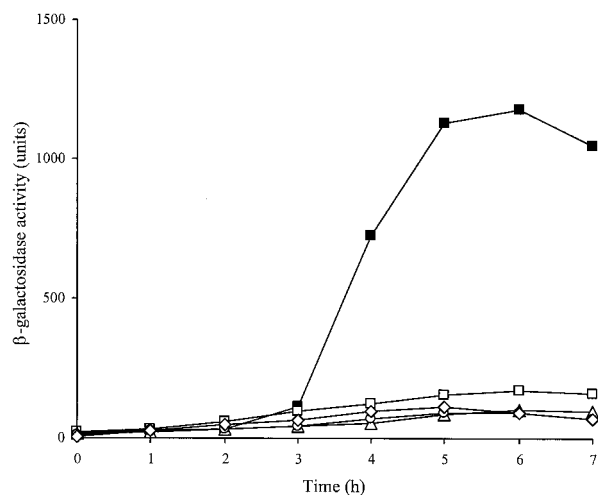

FIG. 1. Expression of *lytH* during sporulation. β-Galactosidase levels were measured following induction of synchronous sporulation in strain GJH161 (*lytH*; ■) and various sigma factor mutant background strains: GJH162 (*sigE*; ○), GJH163 (*sigF*; △), GJH164 (*sigG*; □), and GJH165 (*sigK*; ◇).

was detected on induction of σ^K, showing that production of σ^K is directing the expression from the *lytH* promoter (Fig. 2). Expression of β-galactosidase did not occur following induction of σ^E, σ^F, and σ^G (Fig. 2).

Role of LytH in endospore heat resistance. Endospores of strain GJH161 (*lytH*) were analyzed to determine the role of *lytH* in spore heat resistance. Spores were heated at 90°C, and the comparison of the percentages of loss of CFU showed that GJH161 had slightly reduced heat resistance compared to the parental strain (Fig. 3). After 100 min, GJH161 showed greater than 10-fold-less survival than HR (wild type).
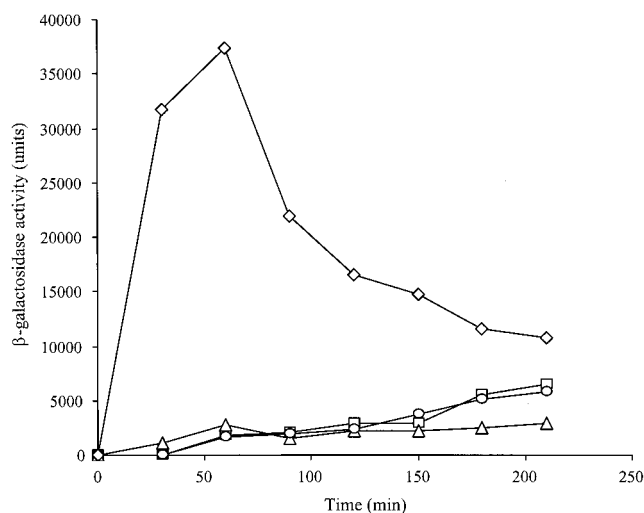

FIG. 2. Expression of *lytH::lacZ* in strains carrying various sporulation-specific sigma factors under P*spac* control during vegetative growth. Strains GJH166 (P*spac-sigE*; □), GJH167 (P*spac-sigF*; △), GJH168 (P*spac-sigG*; ○), and GJH169 (P*spac-sigK*; ◇) were grown in L broth and induced by addition of IPTG to a final concentration of 1 mM.
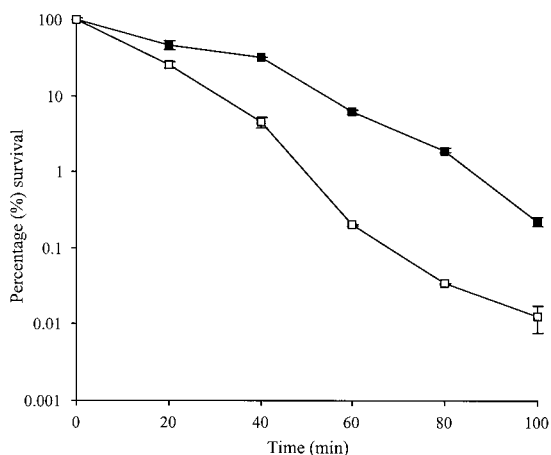
FIG. 3. Heat resistance of spores of strains GJH161 (*lytH*) and HR. Solid squares represent HR (wild type), while open squares represent strain GJH161 (*lytH*). Error bars show the standard deviation. Results are from two independent experiments. Survival was measured as CFU after heat treatment at 90°C.

Role of *lytH* in spore water content. In comparison to a vegetative cell, the protoplast of the spore is relatively dehydrated, even when suspended in water. This dehydration results in metabolic dormancy and is mainly responsible for the heat resistance properties of the spore (4, 13, 15). The spore protoplast wet densities of permeabilized endospores of *B. subtilis* HR (wild type) and mutant strains were measured. Strains HR, GJH161 (*lytH*), AA107 (*cwlD*), and GJH171 (*lytH cwlD*) had protoplast wet densities of 1.346, 1.344, 1.343, and 1.342 g ml^{-1}, respectively.

Transmission electron micrograph studies of *lytH* spores. As observed previously, micrographs of dormant wild-type spores show a densely stained spore protoplast and an electron-transparent spore cortex (Fig. 4). When the mutant strains were viewed by electron microscopy, it was noted that strain GJH161 (*lytH*) showed a slightly more electron-dense spore cortex (Fig. 4). In strain AA107 (*cwlD*), as previously observed by Popham et al. (16), the amount of staining observed in the spore cortex was greater than that in the parent and similar to that seen in a *lytH* mutant (Fig. 4). However, when dormant endospores of strain GJH171 (*lytH cwlD*) were observed, the degree of staining to the spore cortex was dramatically greater than that observed in both the *lytH* and *cwlD* mutants (Fig. 4).

Role of LytH in peptidoglycan structure determination. Strain GJH161 (*lytH*) was analyzed for any changes in the cell wall peptidoglycan structure of vegetative cells, endospores, and during germination by muropeptide HPLC analysis. No change in cell wall peptidoglycan structure of vegetative cells was observed compared to the level in its parent (data not shown). The characteristic appearance of muropeptides during germination also occurred in GJH161 (*lytH*). Analysis of endospore cell wall peptidoglycan in strain GJH161 (*lytH*) compared to that in HR showed the loss of a number of peaks that correspond to muropeptides with a single alanine side chain (Fig. 5A and B, peaks 2, 4, 6, 11, 14, 15, 21, and 24; and Tables 2 and 3) (1). Two other strains, AA107 (*cwlD*) and GJH171 (*lytH cwlD*), were also analyzed for changes in endospore cell

wall peptidoglycan structure. Strain AA107 (*cwlD*) has previously been shown to have a dramatic alteration in peptidoglycan structure, which was due to the total lack of muramic acid δ-lactam residues in the peptidoglycan (Fig. 5C, peaks 4, 5, 6, 7, 12, 14, 15, 16, 17, and 18) (1). A strain carrying mutations in both *lytH* and *cwlD*, GJH171, was also analyzed and showed the loss of a peak corresponding to disaccharide alanine compared to AA107 (*cwlD*) (Fig. 5D, peak 2). Quantification of the spore muropeptides of strains GJH161 (*lytH*) and GJH171 (*lytH cwlD*) (Tables 2 and 3) revealed the percentage of cross-linking index and the muramic acid substitution compared to those in the parental strain. The cross-linking index per diaminopimelic acid and per muramic acid residue of GJH161 (*lytH*) was found to be similar to that of the wild type. This suggests the mutation in *lytH* has no effect on peptidoglycan cross-linking. The cross-linking index per diaminopimelic acid residue of GJH171 (*lytH cwlD*) was also found to be similar to that of the wild type. However, the cross-linking index per muramic acid residue is approximately doubled. A mutation in *lytH* results in a complete loss of muramic acid residues substituted with L-alanine. The loss of muramic acid residues substituted with alanine corresponds with an increase in muramic acid residues substituted with tetrapeptide in GJH161 (28.8 to 50.5%). In GJH171 (*lytH cwlD*), the lack of any muramic acid residues substituted with alanine and δ-lactam leaves 97% of residues substituted with tetrapeptide. These results determine that LytH is likely to be an L-Ala–D-Glu peptidase involved in the production of the single L-alanine side chains from tetrapeptides in the spore cortex. The lack of di- or tripeptides in GJH161 suggests the enzyme is an endopeptidase.

DISCUSSION

The protein encoded by the *lytH* gene was predicted to be a peptidoglycan hydrolase because of its homology to lysostaphin (20). However lysostaphin is a Gly-Gly endopeptidase, and such a combination of residues has not been identified in *B. subtilis* peptidoglycan. Thus, the role of LytH was unsure. The *lytH* gene was found to be expressed specifically during sporulation, under the control of the late mother cell-specific sigma factor, σ^K. Thus LytH has a likely role during differentiation. Two peptidoglycan hydrolases of *B. subtilis* also transcribed by Eσ^K RNA polymerase have previously been identified and characterized (11, 14, 21). These are the amidases CwlC and CwlH, which are involved in mother cell lysis.

Minimal differences in the wet density of spore protoplasts were observed in *lytH* and *cwlD* mutants compared to the parent, whereas the *lytH cwlD* double mutant showed a small but reproducible difference. Also the heat resistance of *lytH* mutant spores was reduced compared to that of the wild type, suggesting a difference in core dehydration and/or cortex structure. The relative dehydration of the spore core is considered the most important factor in the resistance to heat, and the correlation between heat resistance and spore core water content has been shown in a variety of species (5, 13).

Electron microscopy of the wild type and the *lytH*, *cwlD*, and *lytH cwlD* mutants revealed that the *lytH* and *cwlD* mutants showed an increase in staining of the cortex compared with that in the wild type. An increase in staining in a *cwlD* mutant has previously been observed (16). This increase in staining is
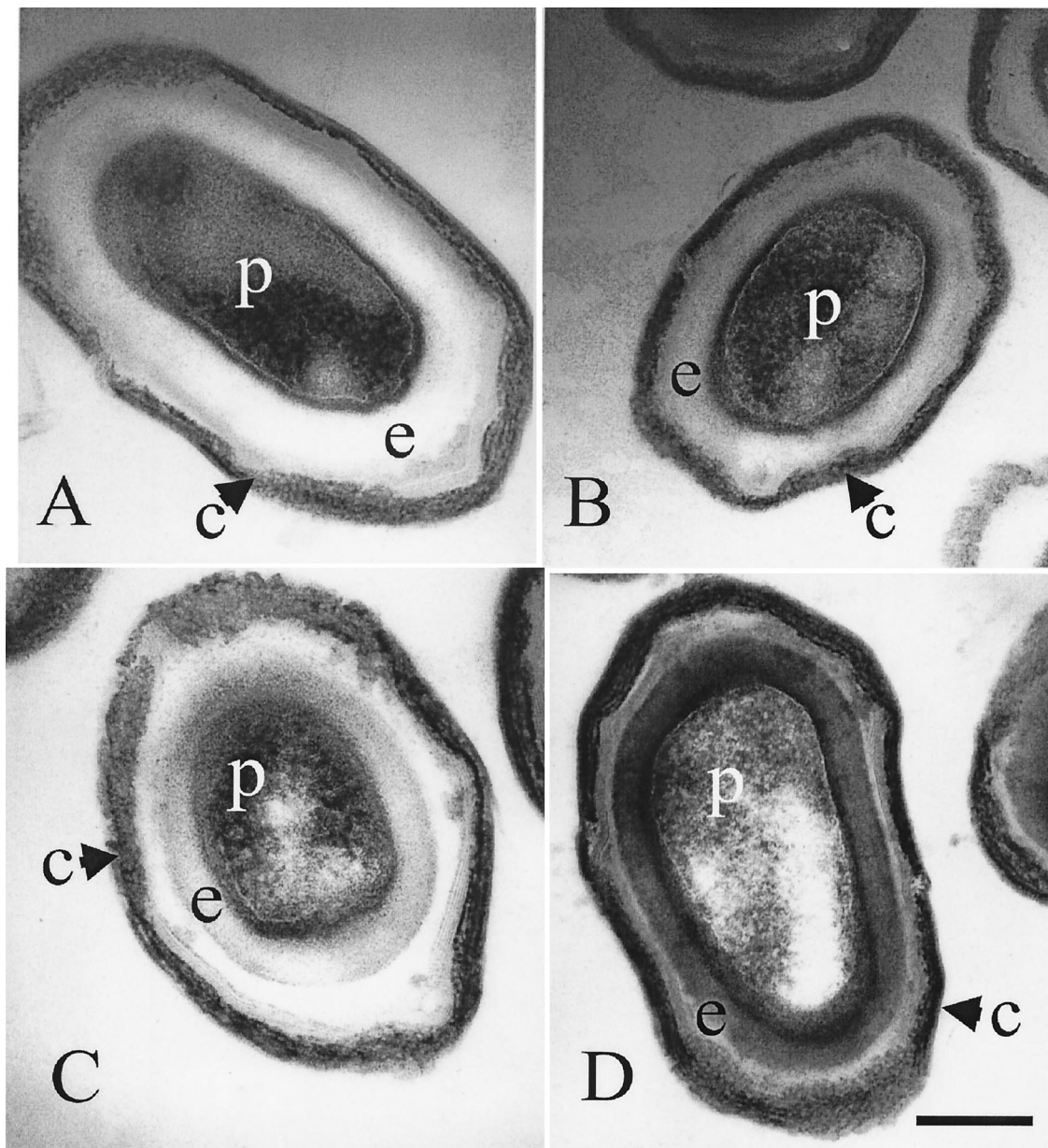
FIG. 4. Transmission electron microscopy of dormant endospores of HR (wild type) (A), GJH161 (*lytH*) (B), AA107 (*cwlD*) (C), and GJH171 (*lytH cwlD*) (D). c, spore coats; e, spore cortex peptidoglycan; p, spore protoplast. Bar, 0.25 μm.

probably due to the changes in the peptidoglycan architecture of the mutants. This in turn suggests the peptidoglycan of the spore cortex of the mutants may be more permeable to the stain, or a charge alteration allows for greater staining.

In order to determine whether LytH has a role in cortex structural determination, muropeptide analysis was carried out. The spore cortex peptidoglycan structure of a *lytH* mutant revealed that peaks corresponding to muropeptides containing single L-alanine side chains were no longer present. This determined that LytH was a likely L-Ala–D-Glu endopeptidase involved in the production of the single L-alanine side chains from tetrapeptides in the spore cortex. Alternatively it is possible that LytH is a carboxypeptidase cleaving D-Ala from position 4 of the peptide side chain, which is then processively

cleaved by another enzyme or enzymes to give single L-Ala side chains. The role of LytH in single L-Ala side chain production was confirmed in a *lytH cwlD* mutant. In this strain, 97% of the muropeptides had tetrapeptide side chains due to the absence of δ-lactam as a result of the lack of CwlD (1, 16) and the absence of single L-alanine side chains from the *lytH* mutation. The lack of L-alanine side chains and an increase in tetrapeptide side chains may bring about a conformational change in the peptidoglycan structure in such a way as to decrease heat resistance. No decrease in heat resistance was observed in a *cwlD* mutant, which besides having no δ-lactam residues also has increased amounts of tetrapeptides. It must therefore be a lack of the single L-alanine residues that results in the reduced heat resistance. The purpose of the production of such single
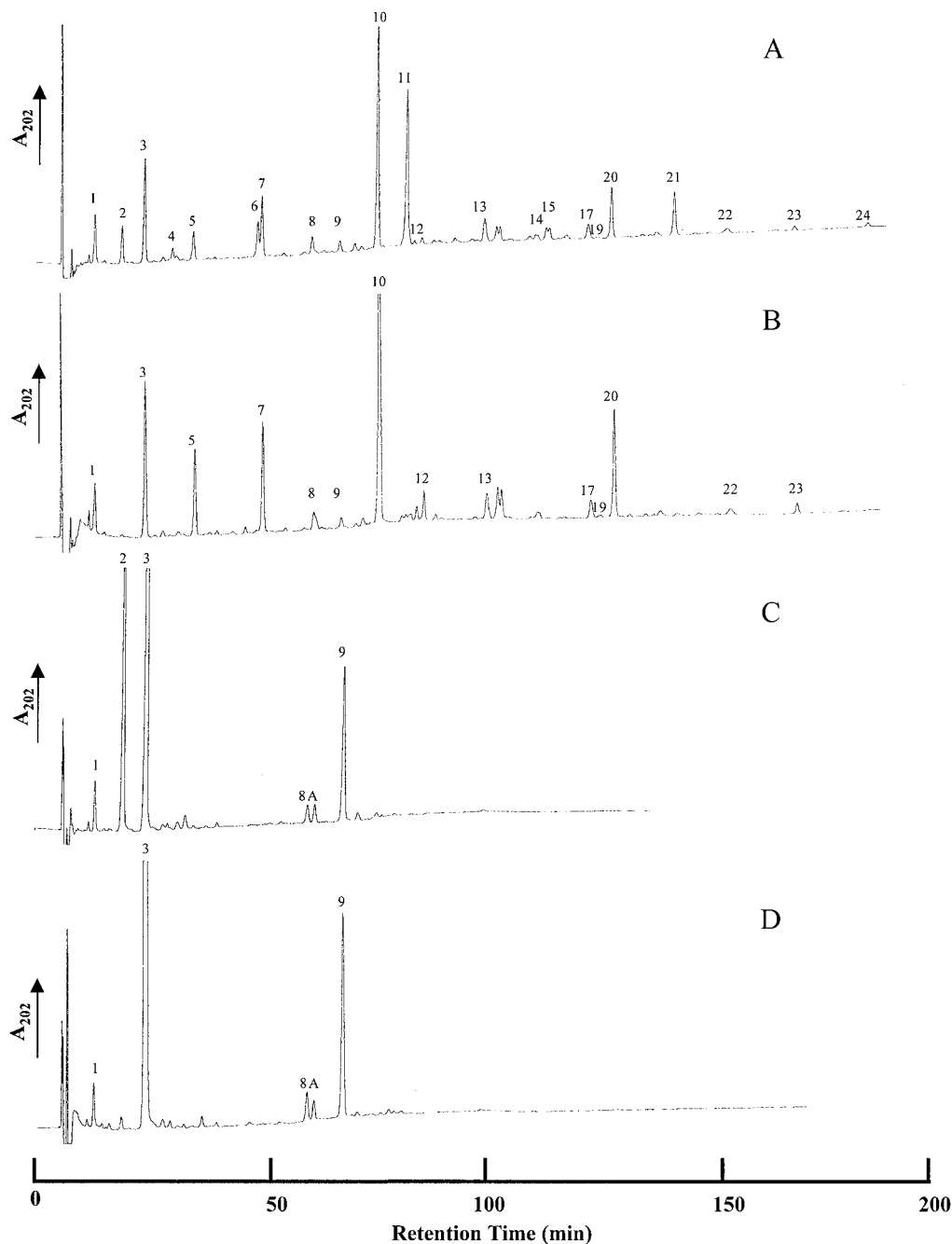
FIG. 5. RP-HPLC muropeptide elution patterns of peptidoglycan from *B. subtilis* 168 endospores. (A) HR (wild type). (B) GJH161 (*lytH*). (C) AA107 (*cwlD*). (D) GJH171 (*lytH cwlD*). Purified Cellosyl-digested peptidoglycan samples were separated on an octadecylsilane column, and the A_{202} of the eluate was measured.

L-alanine residues could be to reduce the number of muramyl peptides per disaccharide, thus decreasing the amount of cross-links. An increase in the amount of cross-linking may reduce the spores' ability to be resistant to heat, as seen in a *dacB* mutant (15); however, a significant increase in cross-linking was not observed in a *lytH* mutant. Another explanation is that single L-alanine residues, the low number of cross-links compared to those in vegetative peptidoglycan, and the ab-

sence of amidation on the D-carboxyl group of diaminopimelic acid account for the fact that the cortical peptidoglycan is more negatively charged than vegetative peptidoglycan (6). It has been suggested that this high net negative charge may be essential for the function of the cortex in heat resistance (19). The *lytH cwlD* mutant exhibits the simplest peptidoglycan profile of any strain so far examined by muropeptide analysis. This is most likely possible as a result of the very specific role of the

TABLE 2. Muropeptide identities and quantification from peptidoglycan of *B. subtilis* HR (wild type), GJH161 (*lytH*), and GJH171 (*lytH cwlD*)

Muropeptide[a]	Identity	Concn (mol%) in:		
		HR (wild type)	GJH161 (*lytH*)	GJH171 (*lytH cwlD*)
1	Disaccharide tripeptide	4.6	4.4	1.9
2	Disaccharide alanine	6.2		
3	Disaccharide tetrapeptide	13.2	21.2	91.4
4	Tetrasaccharide alanine with open lactam	1.2		
5	Tetrasaccharide tetrapeptide with open lactam	0.9	5.3	
6	Tetrasaccharide alanine with reduced lactam	3.1		
7	Tetrasaccharide tetrapeptide with reduced lactam	4.5	10.7	
8	Disaccharide tripeptide disaccharide tetrapeptide	0.9	1.0	0.6
9	Disaccharide tetrapeptide disaccharide tetrapeptide	0.6	1.0	5.3
10	Tetrasaccharide tetrapeptide	21.5	36.0	
11	Tetrasaccharide alanine	21.7		
12	Hexasaccharide tetrapeptide with 1 reduced lactam	1.3	2.3	
13	Disaccharide tetrapeptide tetrasaccharide tetrapeptide	1.6	1.9	
14	Hexasaccharide alanine with 1 reduced lactam	1.3		
15	Hexasaccharide alanine with 1 reduced lactam	1.3		
17	Hexasaccharide tetrapeptide with 1 reduced lactam	1.4	2.1	
19	Tetrasaccharide tetrapeptide tetrasaccharide tetrapeptide	0.8	1.0	
20	Hexasaccharide tetrapeptide	6.7	11.5	
21	Hexasaccharide alanine	6.0		
22	Tetrasaccharide tetrapeptide hexasaccharide tetrapeptide	0.3	0.5	
23	Octasaccharide tetrapeptide	0.5	1.1	
24	Octasaccharide alanine	0.4		
A	Disaccharide tripeptide disaccharide tetrapeptide with 1 amidation			0.8

[a] Muropeptides are numbered as indicated in Fig. 5.

cortex in the maintenance of spore dormancy. How such an apparently simple structure is able to maintain dehydration is a central unanswered question.

The mechanism of production of muropeptides with single L-alanine side chains has been unknown up to now, but several ideas have been previously put forward (1). The mechanism has now been determined as a likely single-step process due to the activity of a sporulation-specific endopeptidase, LytH. LytH is an apparent L-Ala–D-Glu endopeptidase that cleaves nascent side chain to give a single L-alanine substitution. It likely only acts on non-cross-linked chains, and its activity is not dependent on the presence of δ-lactam residues. This modification occurs at a late stage during the maturation of spore cortex via the activity of a mother cell-derived protein. To date, almost the entire complement of components necessary for peptidoglycan structure dynamics during differentiation has now been identified. However, even though the chemistry of

the process has now been established, the functions of components in determining peptidoglycan architecture and how the cortex is able to maintain dormancy have remained elusive.

REFERENCES

1. **Atrih, A., P. Zöllner, G. Allmaier, and S. J. Foster.** 1996. Structural analysis of *Bacillus subtilis* 168 endospore peptidoglycan and its role during differentiation. J. Bacteriol. **178:**6173–6183.
2. **Atrih, A., P. Zöllner, G. Allmaier, M. P. Williamson, and S. J. Foster.** 1998. Peptidoglycan structural dynamics during germination of *Bacillus subtilis* 168 endospores. J. Bacteriol. **180:**4603–4612.
3. **Atrih, A., G. Bacher, G. Allmaier, M. P. Williamson, and S. J. Foster.** 1999. Analysis of peptidoglycan structure from vegetative cells of *Bacillus subtilis* 168 and role of PBP 5 in peptidoglycan maturation. J. Bacteriol. **181:**3956–3966.
4. **Beaman, T. C., J. T. Greenamyre, T. R. Corner, H. S. Pankratz, and P. Gerhardt.** 1982. Bacterial spore heat resistance correlated with water content, wet density, and protoplast/sporoplast volume ratio. J. Bacteriol. **150:**870–877.
5. **Beaman, T. C., and P. Gerhardt.** 1986. Heat resistance of bacterial spores correlated with protoplast dehydration, mineralization, and thermal adaptation. Appl. Environ. Microbiol. **52:**1242–1246.
6. **Buchanan, C. E., A. O. Henriques, and P. J. Piggot.** 1994. Cell wall changes during bacterial endospore formation, p. 167–186. *In* J.-M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier, Amsterdam, The Netherlands.
7. **Ellar, D. J.** 1978. Spore specific structures and their function. Symp. Soc. Gen. Microbiol. **28:**295–325.
8. **Fukushima, T., H. Yamamoto, A. Atrih, S. J. Foster, and J. Sekiguchi.** 2002. A polysaccharide deacetylase gene (*pdaA*) is required for germination and for production of muramic δ-lactam residues in the spore cortex of *Bacillus subtilis*. J. Bacteriol. **184:**6007–6015.
9. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
10. **Kunst, F., and G. Rapoport.** 1995. Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. J. Bacteriol. **177:**2403–2407.

TABLE 3. Cross-linking index and muramic acid substitution of *B. subtilis* spore peptidoglycan[a]

Strain	Cross-linking index (%)[b]		% of muramic acid substitution			
	Mur	A₂pm	Tri	Ala	Tetra	δ-Mur
HR (wild type)	2.1	7.3	2.7	20.5	28.9	47.9
GJH161 (*lytH*)	2.7	5.4	2.8	0	50.5	46.7
AA107 (*cwlD*)[c]	6.5	11.0	3.0	38.2	58.8	0
GJH171 (*lytH cwlD*)	6.4	6.6	3.1	0	96.9	0

[a] Mur, muramic acid; A₂pm, diaminopimelic acid; Tri, tripeptide; Ala, alanine; Tetra, tetrapeptide; δ-Mur, muramic acid δ-lactam.
[b] Calculated as described by Atrih et al. (1).
[c] Values taken from Atrih et al. (1).

11. **Kuroda, A., Y. Asami, and J. Sekiguchi.** 1993. Molecular cloning of a sporulation-specific cell wall hydrolase gene of *Bacillus subtilis*. J. Bacteriol. **175:** 6260–6268.

12. **Lindsay, J. A., T. C. Beaman, and P. Gerhardt.** 1985. Protoplast water content of bacterial spores determined by buoyant density sedimentation. J. Bacteriol. **163:**735–737.

13. **Nakashio, S., and P. Gerhardt.** 1985. Protoplast dehydration correlated with heat resistance of bacterial spores. J. Bacteriol. **162:**571–578.

14. **Nugroho, F. A., H. Yamamoto, Y. Kobayashi, and J. Sekiguchi.** 1999. Characterization of a new sigma-K-dependent peptidoglycan hydrolase gene that plays a role in *Bacillus subtilis* mother cell lysis. J. Bacteriol. **181:**6230–6237.

15. **Popham, D. L., B. Illades-Aguiar, and P. Setlow.** 1995. The *Bacillus subtilis* *dacB* gene, encoding penicillin-binding protein 5*, is part of a three-gene operon required for proper spore cortex synthesis and spore core dehydration. J. Bacteriol. **177:**4721–4729.

16. **Popham, D. L., J. Helin, C. E. Costello, and P. Setlow.** 1996. Muramic lactam in peptidoglycan of *Bacillus subtilis* spores is required for spore outgrowth but not for spore dehydration or heat resistance. Proc. Natl. Acad. Sci. USA **93:**15405–15410.

17. **Popham, D. L., J. Helin, C. E. Costello, and P. Setlow.** 1996. Analysis of the peptidoglycan structure of *Bacillus subtilis* endospores. J. Bacteriol. **178:** 6451–6458.

18. **Sekiguchi, J., K. Akeo, H. Yamamoto, F. K. Khasanov, J. C. Alonso, and A. Kuroda.** 1995. Nucleotide sequence and regulation of a new putative cell wall hydrolase gene, *cwlD*, which affects germination in *Bacillus subtilis*. J. Bacteriol.**177:**5582–5589.

19. **Shibata, H., H. Murakami, and I. Tani.** 1980. Delayed germination of *Ba-*

cillus cereus T spores after treatment with trichloroacetic acid and their reactivation by heating. Microbiol. Immunol. **24:**291–298.

20. **Smith, T. J., S. A. Blackman, and S. J. Foster.** 2000. Peptidoglycan hydrolases of *Bacillus subtilis*: multiple enzymes with multiple functions. Microbiology **146:**249–262.

21. **Smith, T. J., and S. J. Foster.** 1995. Characterization of the involvement of two compensatory autolysins in mother cell lysis during sporulation of *Bacillus subtilis* 168. J. Bacteriol. **177:**3855–3862.

22. **Sterlini, J. M., and J. Mandelstam.** 1969. Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin resistance. Biochem. J. **113:**29–37.

23. **Stewart, G. S. A. B., K. Johnstone, E. Hagelberg, and D. J. Ellar.** 1981. Commitment of bacterial spores to germinate. Biochem. J. **198:**101–106.

24. **Sun, D., P. Stragier, and P. Setlow.** 1989. Identification of a new σ-factor involved in compartmentalized gene expression during sporulation of *Bacillus subtilis*. Genes Dev. **3:**141–149.

25. **Turner, S. M., J. Errington, and J. Mandelstam.** 1986. Use of a *lacZ* gene fusion to determine the dependence pattern of sporulation operon *spoIIIC* in *spo* mutants of *Bacillus subtilis*: a branched pathway of expression of sporulation operons. J. Gen. Microbiol. **132:**2995–3003.

26. **Vagner, V., E. Dervyn, and S. D. Ehrlich.** 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. Microbiology **144:**3097–3104.

27. **Youngman, P.** 1990. Use of transposons and integrational vectors for mutagenesis and construction of gene fusions in *Bacillus* species, p. 221–266. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biology methods for *Bacillus*. Wiley, New York, N.Y.