A Novel Spore Peptidoglycan Hydrolase of *Bacillus cereus*: Biochemical Characterization and Nucleotide Sequence of the Corresponding Gene, *sleL*

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The exudate of germinated spores of *B. cereus* IFO 13597 in 0.15 M KCl–50 mM potassium phosphate (pH 7.0) contained a spore-lytic enzyme which has substrate specificity for fragmented spore cortex from wild-type organisms (cortical-fragment-lytic enzyme [CFLE]), in addition to a previously characterized germination-specific hydrolase which acts on intact spore cortex (spore cortex-lytic enzyme [SCLE]) (R. Moriyama, S. Kudoh, S. Miyata, S. Nonobe, A. Hattori, and S. Makino, J. Bacteriol. 178:5330–5332, 1996). CFLE was not capable of degrading isolated cortical fragments from spores of *Bacillus subtilis* ADD1, which lacks muramic acid 6-lactam. This suggests that CFLE cooperates with SCLE in cortex hydrolysis during germination. CFLE was purified in an active form and identified as a 48-kDa protein which functions as an N-acetylglucosaminidase. Immunochemical studies suggested that the mature enzyme is localized on a rather peripheral region of the dormant spore, probably the exterior of the cortex layer. A gene encoding the enzyme, *sleL*, was cloned in *Escherichia coli*, and the nucleotide sequence was determined. The gene encodes a protein of 430 amino acids with a deduced molecular weight of 48,136. The N-terminal region contains a repeated motif common to several peptidoglycan binding proteins. Inspection of the data banks showed no similarity of CFLE with N-acetylglucosaminidases found so far, suggesting that CFLE is a novel type of N-acetylglucosaminidase. The *B. subtilis* genome sequence contains genes, *yaaH* and *ydhD*, which encode putative proteins showing similarity to *SleL*.

The bacterial endospore cortex is responsible for the maintenance of spore dormancy and heat resistance. The cortex peptidoglycan has a unique spore-specific structure which allows it to fulfill its role, and cortex hydrolysis during spore germination is essential to allow spore outgrowth and the formation of a new vegetative cell (9, 21). The enzymes involved in these hydrolytic reactions have been identified from spores of several species.

A germination-specific cortex-lytic enzyme (GSLE) from *Bacillus megaterium* spores (8) and a spore cortex-lytic enzyme (SCLE) from *B. cereus* spores (16, 24), a mature form of SleB, were shown to hydrolyze intact spore peptidoglycan. A counterpart of *B. cereus* SCLE was also found to exist in *Bacillus subtilis* spores (23), though attempts to solubilize it were unsuccessful. The *B. subtilis* SCLE was involved in the response of spores to l-alanine-stimulated germination, and spores lacking this enzyme were unable to complete germination but showed limited cortex degradation (23). This suggests the contribution of a cortex-lytic enzyme(s) in addition to SCLE in the germination of *B. cereus* and *B. subtilis* spores. Indeed, analysis of muropeptide dynamics during germination of *B. megaterium* and *B. subtilis* spores unequivocally revealed that multiple enzymes are implicated in cortex degradation (2, 4). In *Clostridium perfringens* S40 spores, a cortical fragment-lytic enzyme (CFLE) which attacks disrupted cortex but not intact spores cooperates with SCLE in cortex hydrolysis during germination (6, 19, 20). These results led us to investigate the enzyme(s) other than SCLE responsible for cortex degradation in *B. cereus* spores. This report deals with the purification and the characterization of a CFLE from the germination exudate of *B. cereus* IFO 13597 spores. This enzyme functions as an N-acetylglucosaminidase, and the gene, named *sleL*, has been cloned. The specificity of the enzyme indicates that it acts only in spore germination, as the enzyme hydrolyzes peptidoglycan containing muramic acid 6-lactam, a substrate recognition determinant for cortex-lytic enzymes (28).

MATERIALS AND METHODS

**Bacterial strains and plasmids.** Spores and vegetative cells of *B. cereus* IFO 13597 were used as the source of SCLE and chromosomal DNA, respectively. *B. cereus* IFO 13597, *B. subtilis* 168HJ266, *B. subtilis* ADD1 (a *B. subtilis* mutant with an insertionally inactivated *cwID* gene), and *C. perfringens* S40 were cultured as described by Makino et al. (16), Moriyama et al. (23), Sekiguchi et al. (32), and Miyata et al. (19), respectively. *Escherichia coli* XL1-Blue (Stratagene Cloning System, La Jolla, Calif.) was used as a host for screening the genomic library of *B. cereus* DNA. Plasmids pUC118 (Novagen, Inc., Madison, Wis.), pBluescript II KSI (+) (Stratagene), and pGEM T-vector (Promega Co., Madison, Wis.) were used as cloning vectors. *E. coli* was routinely grown at 37°C in 2x YT medium with ampicillin added to 100 µg per ml for plasmid-carrying strains (30).

**Preparation of spores, decoated spores, isolated cortical fragments, and isolated vegetative cell walls and disruption of spores and vegetative cells.** Spores and decoated spores of *B. cereus*, *C. perfringens*, and *B. subtilis* and *B. subtilis* ADD1 were prepared by methods described previously (references 16, 19, and 23, respectively).

Decoated spores (1 g [wet weight]) from each organism were disrupted at 0 to 4°C with a bead beater in a 20-ml tube containing 10 ml of 50 mM Tris-HCl (pH 8.0) and 10 g of glass beads (diameter, 0.1 mm). The disrupted spores were recovered by centrifugation (13,000 × g for 5 min at 4°C) and heated at 90 to 95°C for 1 h in 1 M NaCl–50 mM Tris-HCl (pH 8.0) containing 2% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol. After being washed extensively with 50 mM Tris-HCl (pH 8.0), the precipitate was treated with tosylamide phenylethyl chloromethyl ketone (TPCK)-trypsin (Sigma-Aldrich, Tokyo, Japan) (0.1 mg/ml in 20 mM Tris-HCl [pH 8.0] containing 10 mM CaCl2) at 37°C for 16 h. The trypsin-treated cortical fragments were heated at 90 to 95°C for 15 min in 20 mM Tris-HCl (pH 8.0) containing 1% SDS. The cortical fragments were then washed in 20 mM Tris-HCl (pH 8.0) until no trace of SDS could be detected in the wash supernatant fluid (11) and used as substrate for CFLE. Dormant...
spores and vegetative cells were also disrupted with a bead beater containing glass beads in 0.15 M KCl-50 mM potassium phosphate (pH 7.0) as described above, and the supernatants obtained by centrifugation (13,000 × g for 5 min at 4°C) were used for enzyme assay.

**Sporolytic enzyme assay.** Peptidoglycan-degrading enzymes were assayed by measuring the decrease in optical density at 600 nm (OD600) of suspensions of decocoted spores and/or isolated peptidoglycan in a cell with a 1-mm light path at 25°C with a Jasco UV spectrophotometer (Japan Spectroscopic Co., Tokyo, Japan), as described previously (16). One unit of activity was defined as a decrease in OD600 of 0.100 per hr.

**Purification of CFLE from germination exudate.** B. cereus spores, which were washed with 50 mM potassium phosphate (pH 7.0) containing 2 M urea to remove spore surface-bound subtilisin-like protease (25), were heated at 75°C for 30 min in deionized water. The spores (5 g [packed weight]) were then germinated at 32°C for 1 h in 100 ml of 0.15 M KCl-50 mM potassium phosphate (pH 7.0) containing 10 mM l-cysteine and 4 mM adenosine; the germination exudate contained activities which digest decorated spores and cortical fragments. After centrifugation (8,000 × g for 10 min at 4°C), the germination exudate was dialyzed at 4°C for 20 h against 60 mM potassium phosphate (pH 8.0) containing 1 mM sodium thiosulfate (buffer A). The dialyzed fluid was applied to an SP-Sephadex C25 column (2.2 by 15 cm; Pharmacia, Uppsala, Sweden), which had been equilibrated with buffer A at 4°C, and adsorbed materials were eluted at 4°C with a 200-ml linear gradient of up to 0.4 M KCl in buffer A. A CFLE was recovered in fractions eluted at a KCl concentration of 0.15 to 0.2 M. The fractions were dialyzed against 40 mM potassium phosphate (pH 7.0) and then applied to a hydroxyapatite column (2.2 by 10 cm; Wako Pure Chemicals, Osaka, Japan) which had been equilibrated with 40 mM potassium phosphate (pH 7.0). CFLE was eluted in 0.2 M potassium phosphate with a 160-ml linear gradient of 0.4 M to 0.04 M of potassium phosphate (pH 7.0). Active fractions were concentrated under low-pressure centrifugation and further purified by a Superoxose 12 gel exclusion column (1.5 by 25 cm; Pharmacia) in 0.15 M KCl-50 mM potassium phosphate (pH 7.0).

**Preparation of antisera and immunoprecipitation.** Preparation of mouse anti-CFLE antisera and immunoprecipitation of the extract from dormant spores with the antisera were carried out as described previously (6).

**Mode of action of CFLE.** Lysophilized cortical fragments from B. subtilis C. perfringens spores (8 mg each) were suspended in 2.5 ml of 0.1 M potassium phosphate (pH 6.0), and the suspension was treated with the purified enzyme (3.0 U; 25 μl) at 5°C, monitoring the change in OD600. Aliquots (300 μl) were centrifuged (13,000 × g for 10 min at 4°C) at 32°C, monitoring the absorbance at 205 nm, and muropeptides were detected by monitoring the absorbance at 205 nm, and muropeptides were detected.

**RESULTS**

**Purification of CFLE.** In addition to SCLE activity acting on decoated spore cortex described previously (16), CFLE activity hydrolyzing disrupted spore cortex was detected in the germination exudate of B. cereus spores. These activities were separated by SP-Sephadex C25 column chromatography (Fig. 1A). This demonstrates the presence of cortex-lytic enzymes differing in the recognition of substrate morphologies. Neither enzyme is synthesized de novo during germination, as shown by the release of activity during germination in the presence of chloramphenicol.

The steps in the purification procedure and the yields obtained for CFLE are shown in Table 1. The enzyme, which was finally purified by gel exclusion chromatography, showed a single band in SDS-polyacrylamide gel electrophoresis with an apparent molecular mass of 48 kDa (Fig. 1B, lane 4). The enzyme was purified with an 18-fold purification was achieved, with a final yield of enzyme activity of 14.9%. The N-terminal amino acid sequence of the enzyme was determined to be MIQIVTVRSGDSVYSLASKY with 95% accuracy. The N-terminal amino acid sequence of the enzyme was determined to be MIQIVTVRSGDSVYSLASKY with 95% accuracy.

**Cloning of the slc gene.** Purified CFLE (30 μg) was digested with TPCK-trypsin (2.5 μg) for 16 h at 30°C in 0.1 ml of 5 mM Tris-HCl (pH 8.0) containing 10 mM CaCl2 and applied to an octadecyl silane-2PU column (4.6 by 250 mm; particle size, 5 μm; Mitsubishi Chemical Co., Tokyo, Japan). Peptides were eluted from the column with a linear gradient of 5 to 95% acetonitrile in the presence of 0.1% trifluoroacetic acid. Five peak fractions were collected, and the amino-terminal sequences of the peptides were determined. Based on the N-terminal sequence of purified CFLE and one of the peptides (peptide C [see Fig. 5]), a JAC primed PCR was performed using the following primers: N1 (5'-ATGGAATACGATCTATTACAGT-3') and N2 (5'-GTGCCATCTCTTTTTGCCTC-3') and S1L2R1 (5'-CGC TAAAGATTTACGAGTATTTTGGTT-3'), were synthesized. The single-specific-primer-PCR method as described by Shyamala and Ames (33) was performed with the purified peptide described above by use of the pairs M13 universal primer (5'-TTTCACACAGGAACAGCTTAGAC-3') and S1L2R1 (5'-CGC TAAAGATTTACGAGTATTTTGGTT-3'), to generate the PCR product containing the slc gene. The product was analyzed.

**Nucleotide sequencing and analysis.** Nucleotide sequencing was performed by the dideoxynucleotide chain termination method of Sanger et al. (31), using the ABI PRISM genetic analyzer (Perkin-Elmer, Applied Biosystems, Foster City, Calif.). The nucleotide and amino acid sequence analysis and sequence comparison with DNAs and proteins registered in the databases (GenBank, EMBL, PIR, and SWISS-PROT) were performed with MACKADIS software (Hitachi Software Engineering, Tokyo, Japan).

**Other procedures.** Protein concentrations were determined by the methods of Lowry et al. (15) and/or Groves et al. (10), with bovine serum albumin as the standard. SDS-polyacrylamide gel electrophoresis (12.5% polyacrylamide) and immunoblotting were carried out as described elsewhere (6). Analyses of N-terminal amino acid sequences were carried out on a protein sequencer (model 470A, Applied Biosystems, Foster City, Calif.).

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB029921.
The appearance of new free amino groups and reducing sugars during enzymatic hydrolysis of cortical fragments by the purified CFLE was monitored (Fig. 2). After a 30-min treatment with the enzyme, the OD$_{600}$ of the suspension had been reduced by 40%. An equivalent amount of the enzyme boiled for 10 min before use had no effect on the suspension as measured by loss of OD$_{600}$. Concomitant with the decrease in OD$_{600}$, there was a marked increase in reducing sugars. In contrast, over this time period, no free amino group appeared. The results suggest a cleavage of the cortex polysaccharide by the enzyme.

The bond specificity of the enzyme was further studied by using fragmented cortex from _B. subtilis_ spores, whose peptidoglycan structure has been explored (3, 27). The fragments were digested with the purified CFLE, and the resulting soluble muropeptides were separated by reversed phase HPLC. The two major fractions, A and B, which were eluted at the retention times of 41 and 46 min, respectively, were collected (20.2 and 27.7% of total products, respectively). Half of each fraction was reduced with sodium borohydride. The nonreduced and reduced materials were hydrolyzed with 6 N HCl, and the amino acid and amino sugar compositions were analyzed (Table 3). Nonreduced samples from both fractions were shown to contain glucosamine and muramic acid in a molar ratio of 1:1. After reduction, the glucosamine content was reduced by about 50% and was replaced by glucosaminitol, whereas the amounts of muramic acid, alanine, glutamic acid, and diaminopimelic acid in fraction A and muramic acid and alanine in fraction B remained unchanged. Thus, the enzyme is shown to be an N-acetylgalactosaminidase.

**Effects of pH, temperature, salt concentration, and chemicals on activity.** The enzyme was active over a pH range of 3.0 to 9.0, with an optimum at pH 6.0 (Fig. 3A). Heat treatment of the isolated enzyme at 40°C for 30 min led to a loss of activity (~90%) (Fig. 3B), while the enzyme in the dormant spore resisted heat treatment at 75°C for 30 min. The enzyme activity depended on the NaCl concentration of the medium, with maximum activity at 0.1 M and a rapid decrease at both lower and higher salt concentrations (Fig. 3C). The same was true with KCl and CaCl$_2$. The addition of dipicolinic acid (DPA; 10 mM), which is released with Ca$^{2+}$ from the spore core at an early stage of germination (12), to 0.1 M NaCl (or KCl)–5 mM Tris-HCl (pH 7.6) had no effect on the CFLE activity. However, the activity observed in 50 mM CaCl$_2$–5 mM Tris-HCl (pH 7.6) was reduced to 70% by the addition of DPA (10 mM). This may be due to a change in ionic strength based on a formation of chelate complex between DPA and Ca$^{2+}$. However, it is not obvious how DPA and Ca$^{2+}$ ion might affect activities of cortex-lytic enzymes in vivo.

Although _p_-nitrophenyl-N-acetyl-β-d-glucosaminidase and _p_-nitrophenyl-tetra-N-acetyl-β-chitotetraoside (synthetic substrates of hexosaminidase and egg white lysozyme, respectively) were not hydrolyzed by CFLE, their structural similarity to the spore peptidoglycan caused partial inhibition of the enzyme (21 and 30% at 1 mM, respectively). ZnCl$_2$ inactivated the enzyme, but no inhibitory effect was observed with MgCl$_2$, MnCl$_2$, or HgCl$_2$ (1 mM each), as well as CaCl$_2$. Enzyme inhibitors, such as diisopropylfluorophosphate, sodium thioglycollate, _N_-ethylmaleimide, and EDTA (1 mM each), had no effect on CFLE activity. However, the enzyme was completely inhibited by 1 mM diethylpyrocarbonate, and the addition of hydroxylamine (0.2 M; incubation at 25°C for 2 h) restored activity (55%) which had been lost with 1 mM diethylpyrocarbonate, and the addition of hydroxylamine (0.2 M; incubation at 25°C for 2 h) restored activity (55%) which had been lost with 1 mM diethylpyrocarbonate.

**Detection of CFLE in spore fractions.** An anti-CFLE antiserum was used to detect CFLE-related proteins in spore frac-

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**TABLE 1. Purification of CFLE of _B. cereus_**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Sp act (U/mg of protein)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germination exudate</td>
<td>0.50</td>
<td>0.014</td>
<td>288</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>SP-Sephadex C$_25$ column</td>
<td>0.53</td>
<td>0.054</td>
<td>1,022.6</td>
<td>47.8</td>
<td>35.5</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>0.11</td>
<td>0.339</td>
<td>3,081.8</td>
<td>29.9</td>
<td>107.0</td>
</tr>
<tr>
<td>Superoxide 12</td>
<td>0.031</td>
<td>0.169</td>
<td>5,451.6</td>
<td>14.9</td>
<td>189.3</td>
</tr>
</tbody>
</table>

*B. cereus_ spores (5 g [packed weight]) were germinated as described in Materials and Methods. After centrifugation (8,000 × g for 10 min at 4°C), the supernatant was used as a germination exudate.
The repeated sequence (nucleotides 1652 to 1664 and 1669 to 1681) a TGA stop codon (nucleotides 1661 to 1663). An inverted site (GAAAGGAG; nucleotides 356 to 363), and terminates at orf3 terminally truncated present between the C-terminally truncated orf1 terminally truncated.

LX1VGQX1LX1V (residues 7 to 47 and 56 to 96; X has a repeated sequence, VX2GDSX1YX5YX8KX1NX1LX5 CFLE were found. The CFLE lacks cysteine residues and peptide sequences obtained from a purified preparation of determined to be the N-terminal amino acid sequence of gel electrophoresis of purified CFLE. A stretch of 20 residues which was similar to that estimated from SDS-polyacrylamide 430 amino acid residues with a molecular mass of 48,136 Da, related protein was detected in vegetative cells collected from is present in an active form in the dormant spore. No CFLE-indicate that the 48-kDa protein in the extract is CFLE, which appearance of the 48-kDa protein (Fig. 4, lane 4). These results CFLE serum lost enzymatic activity, in parallel with the disap-

Normal B. cereus IFO 13597 spores.................................................. <1
Isolated B. cereus IFO 13597 spore cortical fragments............. 100
Isolated B. subtilis 168 AJ12866 spore cortical fragments...... 92
Isolated C. perfringens S40 spore cortical fragments.......... 85
Isolated B. subtilis ADD1 spore cortical fragments............ <5
Decoated B. cereus IFO 13597 spores.............................. <3
Decoated B. subtilis 168 AJ12866 spores......................... <3
Decoated C. perfringens S40 spores............................... <3
Decoated B. subtilis ADD1 spores.................................. <3
Isolated B. cereus IFO 13597 cell walls.............................. <1
Isolated B. subtilis 168 AJ12866 cell walls....................... <1

* Lytic activity was measured by the decrease in OD_{540}. The control value (0.05 U for isolated B. cereus IFO 13597 spore cortical fragment) was taken as 100%.

Nucleotide and predicted amino acid sequences of the slel gene. The nucleotide sequence for the SleLF4-SleLR2 region of B. cereus chromosomal DNA is shown in Fig. 5. The nucleotide sequence, consisting of 1,860 bp, had three open reading frames. The B. cereus slel gene (nucleotides 371 to 1660) was present between the C-terminally truncated orf1 and the N-terminally truncated orf3, which encode unknown proteins. The slel gene starts at an ATG initiation codon (nucleotides 371 to 373), which is preceded by a potential ribosome-binding site (GAAAGGAG; nucleotides 356 to 363), and terminates at a TGA stop codon (nucleotides 1661 to 1663). An inverted repeated sequence (nucleotides 1652 to 1664 and 1669 to 1681) was found near the stop codon.

The slel gene encoded a highly basic (pI 9.7) polypeptide of 430 amino acid residues with a molecular mass of 48,136 Da, which was similar to that estimated from SDS-polyacrylamide gel electrophoresis of purified CFLE. A stretch of 20 residues determined to be the N-terminal amino acid sequence of CFLE was found in the sequence predicted from the nucleotide sequence starting at an initiation codon. Five tryptic peptide sequences obtained from a purified preparation of CFLE were found. The CFLE lacks cysteine residues and has a repeated sequence, VX2GDSX1YX5YX8KX1NX1LX5 LX1VGQX1LX1V (residues 7 to 47 and 56 to 96; X represents an alignment of any amino acid X consisting of n resi-

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Lytic activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal B. cereus IFO 13597 spores</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Isolated B. cereus IFO 13597 spore cortical fragments</td>
<td>100</td>
</tr>
<tr>
<td>Isolated B. subtilis 168 AJ12866 spore cortical fragments</td>
<td>92</td>
</tr>
<tr>
<td>Isolated C. perfringens S40 spore cortical fragments</td>
<td>85</td>
</tr>
<tr>
<td>Isolated B. subtilis ADD1 spore cortical fragments</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Decoated B. cereus IFO 13597 spores</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Decoated B. subtilis 168 AJ12866 spores</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Decoated C. perfringens S40 spores</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Decoated B. subtilis ADD1 spores</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Isolated B. cereus IFO 13597 cell walls</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Isolated B. subtilis 168 AJ12866 cell walls</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

**TABLE 3. Muropeptide analysis**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Materials</th>
<th>Composition (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Nonreduced</td>
<td>1.00 0.00 0.99 0.00 0.54 1.01 0.53</td>
<td></td>
</tr>
<tr>
<td>Reduced</td>
<td>1.00 0.00 0.53 0.48 0.51 0.98 0.50</td>
<td></td>
</tr>
<tr>
<td>B Nonreduced</td>
<td>1.00 0.00 0.99 0.00 &lt;0.01 0.50 &lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Reduced</td>
<td>1.00 0.00 0.52 0.50 &lt;0.01 0.52 &lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

* Cortical fragments from B. subtilis (~1 mg) were digested with CFLE, and the resulting muropeptides were separated by HPLC as described in Materials and Methods. The major peak fractions, A and B, were collected. Their nonreduced and reduced materials were analyzed for amino acids and amino sugars. These values are normalized, with the result for muramic acid set at 1, and are presented in a molar basis. Mur, muramic acid; MurOH, muramitol; Glc, glucosamine; GlcOH, glucosaminitol; Gla, glutamic acid; Ala, alanine; Dpm, diaminopimelic acid.

**FIG. 2.** Release of reducing groups during digestion of cortical fragments with CFLE. Cortical fragments of C. perfringens spores (0.0 mg) were suspended in 2.5 ml of 0.1 M potassium phosphate (pH 6.0). CFLE (3.0 U; 25 µg) was added to the suspension, and the digestion was performed at 32°C. Aliquots were taken at the indicated times for determination of the turbidity at 600 nm in a cell of 1 mm light path (C), the release of reducing sugars (C), and the release of amino groups (C). No decrease in turbidity was seen when heat-denatured enzyme was used (I). The same results were obtained when B. subtilis spore cortical fragments were used as a substrate.

**DISCUSSION**

In this study, we identified a novel spore-lytic enzyme, CFLE, in addition to a known spore-lytic enzyme, SCLE (16, 24), in the exudate of germinated spores of B. cereus. They differ from each other in the recognition of the morphology of substrates; CFLE requires disrupted spore peptidoglycans for its activity, and SCLE preferentially hydrolyzes intact spore peptidoglycan, although it has minimal activity on cortical fragments (the degradation rate is <5% of that for decoated spores [24]). However, the enzymes possess common characteristics as follows: (i) they recognize muramic acid β-lactam residues specific to spore peptidoglycans, which serve as a major determinant for germination-specific spore-lytic enzymes (28); (ii) they are extracted from spores by treatment with reduced alkaline solution containing SDS, a procedure to strip the spore coat (16), suggesting a close localization of CFLE and SCLE in the dormant spore, possibly on the exterior of the cortex layer (22); and (iii) the sensitivities of CFLE activity to temperature, pH,
and ionic strength (Fig. 4) are similar to those of SCLE (16), suggesting that in vivo the activities function in the same environment. These findings suggest that in B. cereus spores, both SCLE and CFLE are germination-specific spore peptidoglycan hydrolases which cooperatively function during germination and that peptidoglycan degradation during germination consists of a cascade of hydrolytic reactions controlled by the morphologies of substrates. This cascade might be common to cortex hydrolysis during germination of B. megaterium and B. subtilis spores, as suggested by the presence of SleB and a putative protein homologous to B. cereus CFLE in B. subtilis (see below) and by a similar pattern of enzyme activities involved in cortex hydrolysis in B. megaterium and B. subtilis (2, 4).

Atrih et al. (2, 4) indicated the involvement of three enzyme activities in spore cortex hydrolysis, based on structural analysis of spore peptidoglycan and its dynamics during germination of B. megaterium and B. subtilis spores. These enzymes are a lytic transglycosylase, an N-acetylglucosaminidase, and an activity generating the subtle modification suggested to be an epimerization of muramic acid. The present study confirmed the existence of N-acetylglucosaminidase in B. cereus spores; this enzyme is suggested to function in a late stage of a cascade of cortex hydrolytic reaction during germination, as itcleaves only broken spore peptidoglycans. It is most probable that GSLE in B. megaterium and/or SCLE in B. cereus and B. subtilis, which have specificity for intact spores, contributes to the initial step of cortex hydrolysis, allowing dissolution of cortex structure. These enzymes were suggested to be amidases (8, 16, 24). However, as mentioned above, an amidase activity has not been detected from the muropeptide analysis of germinated spores of B. megaterium and B. subtilis in the form of amidase products, although the possibility that the activity occurs has not been excluded (2, 4). Indeed, Atrih et al. have questioned the reliability of the classical methods for identification of lytic enzyme activities (2) using decoated spores as a substrate (8, 16). On the other hand, a lytic transglycosylase has not yet been identified from B. megaterium and B. subtilis spores. Identification of a lytic transglycosylase and reexamination of the hydrolytic bond specificity of GSLE and SCLE are needed for further understanding of the role of lytic enzymes in cortex hydrolysis during the germination of spores of Bacillus species.

Comparison of the putative amino acid sequence of B. cereus CFLE with the N-terminal amino acid sequence of purified enzyme indicates that the enzyme is produced in a mature form. The enzyme is probably present in an active form in the dormant spore, as suggested by the presence of activity in the extract made from disrupted dormant spores (Fig. 4). Furthermore, the enzyme did not act on intact cortex. Thus, expression of a CFLE activity which does not need activation must be regulated by the requirement for disrupted cortex as a substrate for hydrolysis. This is also the case for C. perfringens CFLE (6).

A computer search for sequence similarity with B. cereus CFLE

![FIG. 3. Effects of pH, temperature, and NaCl concentration on the activity of CFLE. The CFLE activity is shown relative to the maximum activity. (A) Enzyme (5 μl with 0.08 U) in 5 mM Tris-HCl (pH 7.6) was mixed with 135 μl of 0.1 M buffer solutions to obtain the indicated pH. After incubation at 32°C for 10 min, 5 μl of C. perfringens cortical fragments was added and the enzyme activity was measured. The following buffers were used: CH₃COOH-CH₂COONa (1), Na₂HPO₄-NaH₂PO₄ (6), and NaHCO₃-Na₂CO₃ (-). (B) Enzyme (5 μl with 0.08 U) in 5 mM Tris-HCl (pH 7.6) was mixed with 135 μl of 0.1 M NaCl and incubated for 30 min at the indicated temperature. Then 5 μl of cortical fragments was added, and the residual activity was assayed at 32°C. (C) Enzyme (5 μl with 0.08 U) in 5 mM Tris-HCl (pH 7.6) was mixed with 135 μl of 5 mM Tris-HCl (pH 7.6) containing the desired concentration of NaCl. After incubation at 32°C for 10 min, 5 μl of cortical fragments was added and the enzyme activity was measured. Similar results were obtained when KCl was replaced with NaCl.

![FIG. 4. Immunological detection of CFLE-related proteins in dormant spores and vegetative cells. Spores and vegetative cells were disrupted in and extracted with 0.15 M KC1–50 mM potassium phosphate (pH 7.0), and the coat fraction was recovered as described in the text. The germination exudate, the spor extracts, and the coat fraction were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting. Before electrophoresis, all the samples were dialyzed against 10 mM Tris-HCl (pH 8.0) containing 0.2% SDS and 5 mM 2-mercaptoethanol, and approximately 5 to 30 μg of protein was loaded on the gel. Lanes: 1, purified CFLE; 2, germination exudate; 3, extract made from disrupted dormant spores; 4, the same extract as in lane 3 but pretreated with anti-CFLE serum; 5, extract made from disrupted vegetative cells; 6, coat fraction; 7, extract made from disrupted decoated spores. Prior to electrophoresis, the CFLE activity of the samples was examined. Symbols: +, positive hydrolytic activity; –, no hydrolytic activity.](http://jb.asm.org/Downloadedfrom)
CFLE revealed no similarity with N-acetylglucosaminidases found so far, including LytD, a cell wall N-acetylglucosaminidase of *B. subtilis* (29), indicating that the *B. cereus* CFLE is a novel type of N-acetylglucosaminidase. However, the *B. subtilis* genome sequence revealed the presence of two genes, yaaH and ydhD (14), which encode putative proteins showing high identity to *B. cereus* CFLE (SleL), as shown in Fig. 6. Besides the presence of the proposed cell wall-binding motif in the...
N-terminal region, both YaaH and YdhD showed significant similarity to the C-terminal region of *B. cereus* CFLE, and YaaH has been indicated to be a component of the system involved in the L-alanine-stimulated germination of *B. subtilis* spores (13). A *B. cereus* CFLE was inhibited with diethylpyrocarbonate, which reacts with histidine residues (18). The involvement of histidine in the catalytic activity of N-acetylglucosaminidase has been indicated in the enzymes from *B. subtilis* (LytD) (29) and *Bacillus* sp. strain NCIM 5120 (1). Among 4 His residues of *B. cereus* CFLE, His-379 in the C-terminal region is conserved in YaaH and YdhD. The possible involvement of YaaH and YdhD in cortex hydrolysis during spore germination and of the histidine residue in the catalytic activity of CFLE are currently being investigated.

In addition to SleL, the nucleotide sequence for a SleLF4-SleLR2 region of *B. cereus* chromosomal DNA encoded two unknown proteins, the C-terminally truncated Orf1 (70 amino acid residues) and the N-terminally truncated Orf3 (55 amino acid residues), counterparts for which are found in *B. subtilis*; the former has homology with the N-terminal 70 amino acid residues of a putative protein, YneP (identity, 51.7%) (14), and the latter has homology with the C-terminal 55 amino acid residues of a minor, small acid-soluble protein, Tlp (identity, 48.1%) (5, 14). In contrast to the nucleotide sequence of the *B. cereus* genome, in which sleL is intercalated in an opposite direction between orf1 and orf3, no open reading frame is found in the region between the tlp and the yneP genes of the *B. subtilis* genome. This observation is in agreement with a recent finding that the genome organization is not conserved between *B. cereus* and *B. subtilis* (26).

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