Identification and Characterization of Novel Cell Wall Hydrolase CwlT

A TWO-DOMAIN AUTOLYSIN EXHIBITING N-ACETYLGMURAMIDASE AND DL-ENDOPEPTIDASE ACTIVITIES*§

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Tatsuya Fukushima,‡ Toshihiko Kitajima,‡ Hiroyuki Yamaguchi,‡ Qin Ouyang,‡ Kazumi Furuhata,‡ Hiroki Yamamoto,‡ Toshio Shida,‡ and Junichi Sekiguchi‡§

From the ‡Department of Bioscience and Textile Technology, Interdisciplinary Graduate School of Science and Technology, and §Division of Gene Research, Department of Life Sciences, Research Center for Human and Environmental Sciences, Shinshu University, 3-15-1 Tokida, Ueda, Nagano 386-8567, Japan

A cell wall hydrolase homologue, Bacillus subtilis YddH (renamed CwlT), was determined to be a novel cell wall lytic enzyme. The cwlT gene is located in the region of an integrative and conjugative element (ICEBs1), and a cwlT-lacZ fusion experiment revealed the significant expression when mitomycin C was added to the culture. Judging from the Pfam data base, CwlT (cell wall lytic enzyme T (Two-catalytic domains)) has two hydrolase domains that exhibit high amino acid sequence similarity to DL-endopeptidases and relatively low similarity to lytic transglycosylases at the C and N termini, respectively. The purified C-terminal domain of CwlT (CwlT-C-His) could hydrolyze the linkage of D-γ-glutamyl-meso-diaminopimelic acid in B. subtilis peptidoglycan, suggesting that the C-terminal domain acts as a DL-endopeptidase. On the other hand, the purified N-terminal domain (CwlT-N-His) could also hydrolyze the peptidoglycan of B. subtilis. However, on reverse-phase HPLC and mass spectrometry (MS) and MS-MS analyses of the reaction products by CwlT-N-His, this domain was determined to act as an N-acetylmuramidase and not a lytic transglycosylase. Moreover, the site-directed mutagenesis analysis revealed that Glu-87 and Asp-94 are sites related with the cell wall lytic activity. Because the amino acid sequence of the N-terminal domain of CwlT exhibits low similarity compared with those of the soluble lytic transglycosylase and muramidase (goose lysozyme), this domain represents “a new category of cell wall hydrolases.”

Many microorganisms have peptidoglycan as a major component of the cell wall, which consists of glycan strands cross-linked by peptides. Bacteria have various cell wall lytic enzymes. For Bacillus subtilis, more than 30 candidate peptidoglycan hydrolases are proposed on the basis of amino acid sequence similarity (1), and these enzymes are important in various cellular processes during vegetative growth, sporulation, and germination (1, 2). Several groups of peptidoglycan hydrolases are enzymatically identified as follows: N-acetylmuramidinasides (digesting GlcNAc-MurNAc linkage), N-acetylmuramoyl-l-alanine amidases (digesting MurNAc-l-alanine linkage), DL-endopeptidases (digesting d-glutamic acid-meso-A2pm linkage) (1, 2), and LD-endopeptidase (digesting l-alanine-d-glutamic acid linkage) (3). However, the characterization of the group of DD-endopeptidase (digesting the cross-linked d-alanine-meso-A2pm linkage) has not been reported in B. subtilis. Interestingly, the groups of muramidase and lytic transglycosylase (digesting MurNAc-GlcNAc linkage) in B. subtilis are still not characterized, even many hydrolases in those groups, including hen egg white lysozyme, have already been identified.

Previously Atrih et al. (4) described that the vegetative peptidoglycan in B. subtilis includes (1→6)-anhydro-N-acetylmuramic acid. Thus, it is possible that lytic transglycosylase, which digests MurNAc-GlcNAc linkage with synthesis of a 1,6-anhydro bond in the N-acetylmuramic acid (5), hydrolyzes the vegetative peptidoglycan. However, as seen in the Pfam data base, the solute lytic transglycosylase (SLT) family contains not only lytic transglycosylase but also goose lysozyme (muramidase), and it remains unknown whether the candidate of hydrolase is lytic transglycosylase or muramidase. Therefore, we studied the SLT domain.

In this study, we found that the C-terminal domain is a DL-endopeptidase that hydrolyzes the linkage of D-γ-glutamyl-meso-diaminopimelic acid of peptidoglycan and that the N-terminal domain is an N-acetylmuramidase, which digests the linkage of MurNAc-GlcNAc even though it is predicted as an SLT domain. Moreover, we report that the active sites for hydrolysis of the N-terminal domain are Glu-87 and Asp-94.

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§ Both authors contributed equally to this work.

1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed. Tel.: 81-268-21-5344; Fax: 81-268-21-5345; E-mail: jsekigu@shinshu-u.ac.jp.

3 The abbreviations used are: MurNAc, N-acetylmuramic acid; RP-HPLC, reverse phase-high performance liquid chromatography; aa, amino acid(s); DNP, dinitrophenyl; A2pm, diaminopimelic acid; MurNAc, MurNAc with a reduced end; ES1-MS, electrospray ionization-mass spectrometry; MS-MS, tandem MS; QIT, quadrupole ion trap; ICE, integrative and conjugative element; SLT, soluble lytic transglycosylase; GEWL, goose egg white lysozyme; HEWL, hen egg white lysozyme; T4L, bacteriophage T4 lysozyme; PDB, Protein Data Bank; MES, 4-morpholineethanesulfonic acid.
**Novel *B. subtilis* Autolysin Consists of 2 Catalytic Domains**

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—The strains of *B. subtilis* and *Escherichia coli* used are listed in supplemental Table 1. The primers used in this study are shown in supplemental Table 2. For the LacZ assay, *B. subtilis* was incubated on a nutrient agar plate (6) at 30 °C, and then the cells were inoculated to DSM (Schaeffer) medium (7), followed by incubation at 37 °C. For purification of several proteins, *E. coli* was grown in LB medium (8) or 2X YT medium (9). If necessary, erythromycin (final concentration, 0.3 mg/ml) and ampicillin (final concentration, 50 μg/ml or 100 μg/ml) were added to *B. subtilis* and *E. coli*, respectively.

**Construction of a cwIT-deficient Mutant That Has a cwIT-lacZ Fusion Gene**—A part of the cwIT (yddH) gene was amplified with the h-YDDH and b-YDDH primers with *B. subtilis* 168 DNA as a template. The amplified fragment was digested with HindIII and BamHI and then ligated to the corresponding sites of pMUTIN2, resulting in pM2-DDH. *B. subtilis* 168 (wild type) was transformed with the plasmid from *E. coli* C600(pM2-DDH) to obtain a ddH strain (cwIT:pM2-DDH) through Campbell-type recombination. The constructed strain had a cwIT gene transcriptionally fused with lacZ. Proper integration of this plasmid was confirmed by PCR.

**Construction of Plasmids for Overexpression of a Truncated CwlT Protein**—A truncated cwIT fragment (the C-terminal domain) was amplified by PCR with the BF-YDDH and KR-YDDH primers. The obtained fragment was digested with BamHI and KpnI, and then ligated to the corresponding sites of pQE-30, resulting in pQEDDH. The plasmid was used for overexpression of CwlT-C-His (containing the region from amino acid (aa) 207 to 329 of CwlT).

A truncated cwIT fragment (the N-terminal domain) was amplified by PCR with the yddH-ΔSU and yddH-ΔSDH primers. The amplified fragment was digested with EcoRI and SalI and then ligated to the corresponding sites of pGEM3Zf (+), resulting in pGMddHN. The created pGMddHN was digested with EcoRI and PstI and then ligated to the corresponding sites of pQE-30, resulting in pQE-ddH-N. The plasmid was used for overproduction of CwlT-N-His (from aa 30 to 206 of CwlT).

A cwIT fragment (containing the N- and C-terminal domains) was amplified by PCR with the yddH-ΔSU and yddH-flH primers. The obtained fragment was digested with EcoRI and BamHI and then ligated to the corresponding sites of pGMvcECWB, which has a histidine tag sequence (10), resulting in pGMddHFL. The created pGMddHFL was digested with EcoRI and PstI and then ligated to the corresponding sites of pQE-30, resulting in pQE-ddH-FL. The plasmid was used for overexpression of CwlT-FL-His (from aa 30 to 329 of CwlT).

**Construction of Plasmids for Overexpression of Mutated CwlIT Proteins**—To overexpress the N-terminal domain of mutated CwlIT proteins in *E. coli*, plasmids for site-specific mutations were created using a QuickChange site-directed mutagenesis kit (Stratagene) with the pQE-ddH-N plasmid, according to the manufacturer’s instructions. For PCR amplification to create site-specific mutations in the plasmid, two complementary DNA oligomers as primers (supplemental Table 2) were used. *E. coli* JM109 containing the created plasmids (supplemental Table 1) overexpressed the mutated CwlIT proteins (E87Q, D94N, S98A, S99A, E100Q, S101A, S115A, D133N, and S167A).

**Transformation of *E. coli* and *B. subtilis***—*E. coli* transformation was performed as described by Sambrook et al. (8), and *B. subtilis* transformation was performed by the competent-cell method (11).

**β-Galactosidase Assay**—The β-galactosidase assay was performed as described by Shimotzu and Henner (12).

**Preparation of Cell Wall and Peptidoglycan from *B. subtilis***—Cell wall was prepared from *B. subtilis* ATCC6633 and 168—Cell wall was prepared from *B. subtilis* ATCC6633 (Sigma) or *B. subtilis* 168 as described previously (14, 13). Peptidoglycan from *B. subtilis* ATCC6633 was prepared as described by DeHart et al. (15) and Shida et al. (16).

**SDS-PAGE and Zymography**—SDS-PAGE was performed as described by Sambrook et al. (8). Zymography was performed as described by Leclerc and Asselin (17), with a SDS-polyacrylamide gel containing 0.5 mg/ml purified *B. subtilis* ATCC 6633 or 168 cell wall as a substrate for cell wall hydrolase. Renaturation was performed as described previously (18).

**Purification of Various CwlT Proteins**—Proteins containing the C-terminal and/or N-terminal domains of CwlT were overexpressed in *E. coli*. *E. coli* JM109 harboring pQEDDH, pQE-ddH-N, or pQE-ddH-FL was incubated at 37 °C in LB medium containing 50 μg/ml ampicillin. At an absorbance of 0.7–0.9 at 600 nm, 2 mM isopropyl 1-thio-β-D-galactopyranoside was added to the culture. After 2 h of incubation, the cells were harvested by centrifugation and suspended in 10 mM imidazole NPB buffer (10 mM imidazole, 1 mM NaCl, 20 mM sodium phosphate (pH 7.4)). Purification of CwlT-C-His, CwlT-N-His, and CwlT-FL-His was performed as described previously (10).

For overexpression of various mutated CwlT proteins, *E. coli* JM109 containing a plasmid, pQEYN-XXX (where X is any letter (supplemental Table 1)), was incubated in 2× YT medium with 50 μg/ml ampicillin at 37 °C. At an absorbance of 0.7 at 600 nm, 1 mM isopropyl 1-thio-β-D-galactopyranoside was added to the culture. After 1 h of incubation, the cells were harvested by centrifugation and suspended in 20 mM imidazole NPB buffer (20 mM imidazole, 0.5 mM NaCl, 20 mM sodium phosphate (pH 7.4)). The various mutated CwlT proteins were purified as described previously (10).

**Determination of the Optimum pH Values and Temperatures of CwlT-N-His, CwlT-C-His, and CwlT-FL-His**—Determination of the optimum pH values and temperatures of the truncated CwlT proteins was performed as described previously (3, 13, 18). One unit of hydrolase activity was defined as the amount of enzyme necessary to decrease the absorbance at 540 nm by 0.001 in 1 min (3, 13, 18).

**Determination of the Sites of Cleavage of Cell Wall by CwlT-C-His**—Determination of the sites of cleavage of cell wall for CwlT-C-His was performed as described previously (10, 13).

**Preparation of Glycan Strands of GlcNAc-MurNAc Polymer from Peptidoglycan**—To remove peptide side chains from the glycan strands, the digestion of peptidoglycan by h-CwlH, which is an N-acetylmuramoyl-1,3-alanine amidase (19), was performed as described previously (9). To separate the glycan strands and peptide side chains, the sample was applied to a Sep-Pak Plus (C18) cartridge (Waters) equilibrated with 0.05%
trifluoroacetic acid. The cartridge was washed with 0.05% trifluoroacetic acid, and glycan strands were eluted with a stepwise gradient of 5, 10, 20, and 30% CH₃CN containing 0.05% trifluoroacetic acid. The fractions were checked by gel filtration on a TSKgelG2000SW column (TOSOH) as described by Fukushima et al. (9) to determine whether or not the glycan strands could be separated from the peptide side chains. Because the fraction eluted with 20% CH₃CN containing 0.05% trifluoroacetic acid contained glycan strands, the eluate was freeze-dried and used for digestion of glycan strands with CwlT-N-His.

**Digestion of Glycan Strands of GlcNAc-MurNAc Polymer by CwlT-N-His**—After dried glycan strands had been dissolved in 300 µl of 40 mM sodium phosphate buffer (pH 6.0), 0.1 mg/ml CwlT-N-His was added to the mixture, which was then kept at 37 °C overnight. The mixture was divided into two (samples 1 and 2), and phosphoric acid was added to one-half (sample 1) to adjust the pH to 2–3. (This sample is the "reduced sample.") On the other hand, after the other half (sample 2) and 150 µl of 0.5 M borate buffer (pH 9.0) had been mixed, 12.5 mg/ml NaBH₄ was added to the sample, which was then kept at 37 °C for 30 min to reduce the reducing ends of amino sugars. Finally, the pH of the sample was adjusted at 2–3. (This sample is the "reduced sample.")

The samples were separated by RP-HPLC as described previously (9). For this HPLC, a Symmetry Shield PR18 column (Waters) was used, and elution buffer A consisting of 0.05% trifluoroacetic acid and elution buffer B consisting of 0.05% trifluoroacetic acid and 40% CH₃CN were prepared. Elution was performed for 10 min with buffer A (nongradient) and then for 30 min with a linear gradient of buffer B (from 0 to 50%).

**Identification of Separated Peaks on RP-HPLC by ESI-MS and AXIMA-QIT**—The peaks obtained on RP-HPLC were freeze-dried. For analysis by ESI-MS (Agilent 1100 series LC/MSD Trap VL), samples were dissolved in 0.05% trifluoroacetic acid. The fragment ions were detected in the negative mode. For analysis with an AXIMA-QIT (Shimadzu), after the samples had been dissolved in 10 µl of 0.1% acetic acid, 0.5 µl of the sample and 0.5 µl of a dihydroxybenzoic acid matrix solution (10 mg of dihydroxybenzoic acid dissolved in 1 ml of 40% CH₃CN and 0.1% trifluoroacetic acid) were mixed on a plate for MS, and then the mixture was dried. The fragment ions were detected in the positive and negative modes, and these fragment ions were used for MS-MS analysis.

**Identification of the Active Site of the N-terminal Domain of CwlT**—To identify the active sites of the N-terminal domain of CwlT, 2.5 µg of wild-type CwlT-N-His and each mutated CwlT protein (E87Q, D94N, S98A, S99A, E100Q, S101A, S115A, D133N, and S167A) was applied to a gel. For zymography, as described above.

For quantification of the cell wall hydrolase activities of CwlT-N-His and the mutated CwlT proteins, the purified proteins were precipitated with 2.5% (w/v) (final concentration) of trichloroacetic acid on ice for 25 min to remove salts from the protein samples. After the samples were centrifuged, the pellets were washed with 70% ethanol and then dissolved in SDS-PAGE sample buffer. The samples were used for zymography as described above, and then the intensity of the hydrolytic bands by zymography was quantified with the NIH image program.

**RESULTS**

**Amino Acid Sequence of CwlT**—It is predicted that CwlT has two domains associated with cell wall lytic activity. Fig. 1 shows the domain structure of CwlT. With the Pfam database, its N-terminal and C-terminal domains were assigned as an SLT and NlpC/P60 (DL-endopeptidase), respectively. With the BLAST software program, the similarity between the C-terminal domain of CwlT (from aa 211 to 327) and the DL-endopeptidase domain of LytF (from aa 375 to 486) is 37%; however, the C-terminal domain of CwlT (from aa 212 to 327) is also similar to a part of PgdS (from aa 169 to 285),

![Diagram of gene map and cell wall lytic activities of CwlT-N-His](image-url)

**FIGURE 2**. Cell wall lytic activities of CwlT-N-His (the N-terminal domain of CwlT), CwlT-C-His (the C-terminal domain of CwlT), and CwlT-FL-His (both domains of CwlT). A, SDS-12% PAGE (lanes 1–3) and zymography (lanes 4–6) of the purified CwlT derivatives. The purified CwlT-FL-His (lanes 1 and 4), CwlT-N-His (lanes 2 and 5), and CwlT-C-His (lanes 3 and 6) were applied to a gel. For zymography, all proteins were incubated in a gel containing cell wall for 3 h at 37 °C. Lane M, protein standards (Bio-Rad). B, cell wall lytic activities of CwlT-N-His (squares), CwlT-C-His (triangles), and CwlT-FL-His (circles). 0.1 nmol of various enzymes and 0.33 mg of purified B. subtilis cell wall were used. The activity was monitored at 540 nm under the optimum conditions (at 32 °C (pH 6.5)).
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which is a poly-γ-glutamate depolymerase (20) (the similarity is 43%). On the other hand, the N-terminal domain of CwlT is not very similar to an SLT domain (25.9%). It is also predicted that CwlT has a lipoprotein signal peptide, and the cleavage site may be \(20^{\text{FVL}} \downarrow ^{23}\) (the arrow and numbers indicate the cleavage point and positions of amino acids, respectively) described by Tjalsma et al. (21).

**Transcription of CwlT with Mitomycin C**—The cwlT gene was predicted to be located in the prophage region (1, 22). Recently this region was determined to be an integrative and conjugative element, ICEBs1 (23). To analyze the expression of cwlT, a cwlT-lacZ transcriptional fusion gene was constructed, and the transcription of cwlT was determined as \(\beta\)-galactosidase activity. When the constructed strain was incubated in Difco sporulation medium (DSM), there was no \(\beta\)-galactosidase activity (data not shown). When mitomycin C, which is an inducer of ICEBs1 (23), was added to DSM, the activity was increased around 2 Miller units (supplemental Fig. 1). This finding suggested that cwlT is expressed through induction of ICE with mitomycin C.

**Cell Wall Lytic Activity of the N-terminal and C-terminal Domains**—To confirm that both the N-terminal and C-terminal domains are cell wall hydrolases, CwlT-N-His, CwlT-C-His, and CwlT-FL-His were purified by affinity chromatography. Fig. 2A shows the SDS-PAGE and zymography of the purified proteins. All of the purified proteins (CwlT-N-His (21.0 kDa), CwlT-C-His (15.0 kDa), and CwlT-FL-His (34.8 kDa)) gave only a single band on the SDS-polyacrylamide gel, and these bands exhibited cell wall lytic activity. This finding suggests that both the N-terminal and C-terminal domains of CwlT have cell wall hydrolase activity.

The enzymatic properties of CwlT derivatives were determined. Fig. 2B shows the activities against cell wall under the optimum conditions (the optimum temperature and pH for all proteins were 32 °C and 6.5, respectively). CwlT-N-His and CwlT-C-His had almost the same cell wall lytic activity (CwlT-N-His, 90 units/nmol (4,290 units/mg protein); CwlT-C-His, 90 units/nmol (6,000 units/mg protein)). The lytic activity of CwlT-FL-His was 212 units/nmol (6,090 units/mg protein).

Because there was a possibility that the activity of CwlT-FL-His may only depend on one of the two catalytic domains, the amino terminal and reduced sugar terminal amounts released by the activities of \(\beta\)-endopeptidase and muramidase...
from the cell wall were determined with ninhydrin and by the Nelson-Somogyi method (24), respectively (supplementary Table 3). These results clearly indicated CwlT-FL-His has both DL-endopeptidase and muramidase activities.

Determination of the Sites of Cleavage of B. subtilis Cell Wall by the C-terminal Domain—The sites of cleavage of peptidoglycan by the C-terminal domain of CwlT were determined by the dinitrophenyl method described by Yamaguchi et al. (10) and Ohnishi et al. (13). The purified cell wall of B. subtilis was digested with CwlT-C-His and then the free amino groups of the digested sample were labeled with 1-fluoro-2,4-dinitrobenzene, followed by hydrolysis of glycoside and amido linkages of the cell wall with HCl. Finally, the dinitrophenyl (DNP)-amino acids were separated by RP-HPLC. Fig. 3 shows the amounts of detected DNP-amino acids. Mono-DNP-A2pm and bis-DNP-A2pm increased during the enzymatic reaction; however, DNP-L-Ala, DNP-D-Ala, and DNP-D-Glu were not detected (Fig. 3; data not shown). From this experiment, CwlT-C-His is not a DD-endopeptidase that can digest the cross-linkage between D-alanine and meso-diaminopimelic acid because the DNP derivative on DD-endopeptidase digestion should be only mono-DNP-A2pm. Thus, the results suggest that the C-terminal domain of CwlT has the DL-endopeptidase activity.

Determination of the Sites of Cleavage of B. subtilis Cell Wall by the N-terminal Domain—With the Pfam data base, the N-terminal domain of CwlT was assigned as an SLT. Because lytic transglycosylases digest the linkage of peptidoglycan between MurNAc and GlcNAc, the glycan strands (mainly \([\text{GlcNAc-MurNAc}]_n\)) derived from peptidoglycan of B. subtilis were purified as a substrate as described under “Experimental Procedures.” The purified glycan strands without reduction of the ends of amino sugars were digested with CwlT-N-His, and then the sample was separated by RP-HPLC as described by Fukushima et al. (9). As a result, several peaks were detected (Fig. 4A), indicating that CwlT-N-His could digest the glycan strands.

A lytic transglycosylase produces a 1,6-anhydro bond in MurNAc, and the resulting product does not have a reducing end on the amino sugar (25). To determine whether the

(see below) from the cell wall were determined with ninhydrin and by the Nelson-Somogyi method (24), respectively (supplemental Table 3). These results clearly indicated CwlT-FL-His has both DL-endopeptidase and muramidase activities.
digested sample has a reducing end on amino sugar or not, glycan strands digested with CwlT-N-His were reduced with sodium borohydride, and then the sample was separated by RP-HPLC. The detected peaks (Fig. 4B) were different from those of the nonreduced sample (Fig. 4A). This result suggests that glycan strands digested with CwlT-N-His have a reducing end on amino sugar and thus this enzyme is not a lytic transglycosylase. In other words, it may be a muramidase or glucosaminidase because the enzyme could digest the glycan strands.

In Fig. 4B, peaks A–E were collected, and then MS analysis was performed with ESI-MS. As a result, MS analysis of these materials revealed fragment ions at m/z 975 (peak A), 726 (peak B), 965 (peak C), 1,204 (peak D), and 1,443 (peak E) in the negative mode. These values for peaks A–E corresponded to [M – H]⁻ of a tetrasaccharide (consisting of GlcNAc and MurNAc with a reduced end; the reducing end of the oligosaccharide was reduced with NaBH₄) (Mr, 975), [M – 2H]²⁻ of a hexasaccharide with a reduced end (Mr, 726), [M – 2H]²⁻ of an octasaccharide with a reduced end (Mr, 965), [M – 2H]²⁻ of a decasaccharide with a reduced end (Mr, 1,205), and [M – 2H]²⁻ of a dodecasaccharide with a reduced end (Mr, 1,444), respectively.

To clarify whether CwlT-N-His is a muramidase or glucosaminidase, peak A (tetrasaccharide with a reduced end) and peak B (hexasaccharide with a reduced end) in Fig. 4B were identified by MS-MS analysis with an AXIMA-QIT. MS analysis of peak A in the positive and negative modes gave fragment ions at m/z 999.4 and 975.2, respectively. These values correspond to [M + Na]⁺ and [M – H]⁻ of a tetrasaccharide with a reduced end, respectively. Moreover, as shown in Fig. 5A (positive MS-MS analysis) and Fig. 5B (negative MS-MS analysis) on MS-MS analysis, peak A represented a tetrasaccharide with a reduced end (GlcNAc-MurNAc-
GlNAC-MurNAc) (Fig. 5C). For peak B, MS analysis in the positive and negative modes showed fragment ions at m/z 1,477.6 and 1,453.2, respectively. On MS-MS analysis of peak B, peak B represented a hexasaccharide with a reduced end ([GlCNAC-MurNAc]2-GlcNAc-MurNAc) (supplemental Fig. 2). Because a muramidase produces the GlcNAc-MurNAc oligosaccharides, these results suggest that the N-terminal domain of CwlT is a muramidase.

Moreover, to confirm that CwlT-N-His is a muramidase, peaks B and C in Fig. 4B were digested with hen egg white lysozyme (muramidase), and the reducing ends of amino sugars were reduced with NaBH₄ followed by RP-HPLC. As a result, because both samples (peaks B and C) digested with lysozyme contained only a reduced disaccharide and tetrasaccharide (no trisaccharide) (data not shown), these results indicate that the N-terminal domain of CwlT is a muramidase.

Identification of the Catalytic Amino Acid Residues of the N-terminal Domain—As shown in Fig. 6A, the N-terminal domain of CwlT identified as an N-acetylmuramidase (Figs. 4 and 5) is not very similar to SLT of E. coli (the similarity is only 25.9%) and is also not similar to goose-type lysozymes in Cygnus atratus (swan), Anser anser anser (goose), and Gallus gallus (chicken), which belong to the SLT family (Pfam data base). It is possible that the N-terminal domain of CwlT represents “a new category of cell wall hydrolases.” To understand this muramidase activity, the N-terminal domain of CwlT was compared with proteins that are similar to this domain, and then conserved amino residues, except hydrophobic amino acids and glycine, were chosen as follows: Glu-87, Asp-94, Ser-98, Ser-99, Glu-100, Ser-101, Ser-115, Asp-133, and Ser-167 (Fig. 6A). The selected amino acids were mutated, and these mutated CwlT proteins (E87Q, D94N, S98A, S99A, E100Q, S101A, S115A, D133N, and S167A) were overexpressed in E. coli and then purified. As shown in Fig. 6B, the results of zymography with the mutated CwlT proteins (E87Q and D94N) appeared to be less hydrolytic. The other mutated CwlT proteins (E100Q and S115A) appeared to have less activity than the N-terminal domain of CwlT. To confirm the cell wall hydrolytic activity, the relative activity compared with that of the N-terminal domain of CwlT was measured based on the results of zymography. Judging from the results in Fig. 6C, CwlT (E87Q) and CwlT (D94N) did not show significant hydrolyse activity (less than 2% of the hydrolytic activity of the N-terminal domain of CwlT), and CwlT (E100Q) and CwlT (Ser-115) had ~10% of the activity of the CwlT-N-His protein (9.8 and 11.3%, respectively). These results suggest that two amino residues, Glu-87 and Asp-94, are the catalytic amino residues for muramidase activity.

**DISCUSSION**

It is clear that B. subtilis CwlT has two hydrolytic domains, which are a D.L-endopeptidase (Fig. 3) and an N-acetylmuramidase (Figs. 4 and 5), at its C terminus and N terminus, respectively. The N-terminal domain is not a lytic transglycosylase.

Catalytic Amino Acid Residues of New Categorized Muramidase—From Figs. 4 and 5, it is very clear that the N-terminal domain of CwlT is a muramidase. Interestingly, in the primary amino acid sequence, the N-terminal domain of CwlT (muramidase) is not very similar to a goose lysozyme (GEWL; lysozyme G) or an SLT (Fig. 6A). Thus, it is predicted that CwlT could represent “a new category of muramidase.” Supplemental Fig. 3 shows the identified secondary structures of GEWL in A. anser anser and SLT in E. coli by crystallography. These secondary structures are similar (supplemental Fig. 3), and the predicted secondary structure of the N-terminal domain of CwlT with the PSIPRED software program seems to be similar to the secondary structures of GEWL and SLT. Interestingly, Glu-505 is the only catalytic amino acid residue of SLT (Glu-478 in crystallized SLT (without signal peptide of SLT) (26). On the other hand, the catalytic residues of GEWL seem to be Glu-73 and Asp-86 (27). Because the catalytic amino acid residues of the N-terminal domain of CwlT are Glu-87 and Asp-94 (Fig. 6, B and C), it is suggested that this domain is a lysozyme-like catalytic domain rather than a lytic transglycosylase-like one.

The catalytic residue of CwlT, Glu-87, should be a critical amino acid residue for hydrolysis, because it is known that a glutamic acid residue of HEWL (28, 29), bacteriophage T4 lysozyme (T4L) (30), and GEWL (31) is the critical amino acid residue. Because the catalytic amino acid of SLT is Glu-505 (26), and because Thunnissen et al. (32) proposed that the glutamic acid residue for the catalytic activity of HEWL and SLT is common, it is suggested that all muramidases, including CwlT and SLT, use the glutamic acid residue for the hydrolysis of the linkage between MurNAc and GlcNAc.

Grutter et al. (27) compared HEWL (PDB 1HEL), T4L (PDB 255L), and GEWL (PDB 153L), and there are parts of four α-helices together with β-strands, and these common structures include the catalytically essential elements. In particular, one catalytic residue, glutamic acid (Glu-35 in HEWL, Glu-11 in T4L, and Glu-73 in GEWL), is located at the edge of the

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**FIGURE 6. The active sites of the N-terminal domain of CwlT.** A, alignment of the N-terminal domain of CwlT and homologous gene products in the Pfam data base. The identical amino acid residues are shown by shading, and the numbers indicate the positions with respect to the N-terminal amino acid residue. The mutated amino acid residues are indicated by arrows. The boxes indicate the identified and/or predicted active sites of a goose lysozyme (27) and SLT (26). CwlT, B. subtilis cell wall lytic enzyme T (CwlT); CwlT13_LNTFA, Enterococcus faecalis protein; CmLYM58, Bacillus cereus protein; BcEM02, Oceiih, Oceanobacillus iheyensis protein; Q9ANF0-CLOPE, C. perfringens protein; Q8Y815, Listeria monocytogenes protein; Q9CHW6, LACLA, Lactococcus lactis subsp. lactis protein; Q8DU69, STRMU, Streptococcus mutans protein; Q9VP46, STRN, Streptococcus gordonii protein; PVA50, STRN, Streptococcus pneumoniae pneumococcal vaccine antigen A (PvA); LGY, CHICK, C. atratus lysozyme G (goose-type); LGY, ANSAN, A. anser anser lysozyme G; LGY, CHICK, G. gallus lysozyme G; SLT, Ecoli, E. coli SLT; MLC, Ecoli, E. coli membrane-bound lytic transglycosylase C (MltC); VIVD, BPT7, bacteriophage T7 internal virion protein D; and TBA, ALTMAN, Alteromonas sp. (strain M-1) tributyltin chloride resistance protein B, SDS-PAGE (top panel) and zymography (bottom panel) of the wild-type and mutated CwlT proteins. 2.5 μg of the purified CwlT-N-His (lane 1) and each mutated CwlT protein (E87Q, D94N, S98A, S99A, E100Q, S101Q, S115A, D133N, and S167A) correspond to lanes 2–10, respectively; were used for SDS-PAGE and zymography. C, measurement of hydrolytic activity by zymography. CwlT-N-His and the mutated CwlT proteins (each 2.5 μg) desalted by trichloroacetic acid precipitation as described under “Experimental Procedures” were applied to an SDS-polyacrylamide gel containing purified cell wall. For zymography, all proteins were incubated in the gel for 1 h at 37°C. The hydrolytic activities were determined from the zymographic data with the NIH image program and are shown with the relative activity of the control protein, CwlT-N-His. Hydrolysis activity of less than 2% was within experimental error.
α-helix, and another catalytic residue, aspartic acid (Asp-52 in HEWL, Asp-20 in T4L, and Asp-86 in GEWL), is located near the β-strand (27). It is predicted that the catalytically essential elements of CwlT are the same as those of these lysozymes because Glu-87 and Asp-94 in CwlT also seem to be located at the edge of the α-helix and near the β-strand, respectively (supplementary Fig. 3). However, recently, several researchers described that the catalytic residue, aspartic acid, is associated with substrate specificity rather than the critical muramidase activity on the basis of the results of crystallography and mutagenesis analysis (28, 29). Weaver et al. (33) described that GEWL lacks a catalytic aspartate residue on the basis of crystallography with complexes of the protein and substrate. Thus, it is possible that the catalytic amino acid residue Asp-94 in CwlT may not be critically associated with the catalytic activity or that this residue may have the other role in hydrolyase activity. Because CwlT (D94N) exhibited no hydrolytic activity on zymography (Fig. 6, B and C), at least Asp-94 in CwlT should be associated with hydrolysis as a mutamidase directly or indirectly.

The cwlT Gene Localized in ICE—cwlT was predicted to be in the prophage 2 region (1, 22, 34). However, this region was recently determined to be an integrative and conjugative element, ICEBsI (23). Tn916 is the best characterized ICE (35), and orf14 in it comprising 333 amino acid residues (36) exhibits very high sequence similarity with the entire region of CwlT (43% identity; from 13 aa to 329). Among ICEs orf14 of CW459 from Clostridium perfringens (37) also exhibits very high similarity with the entire region (43% identity) of CwlT, and orf82 of SXT from Vibrio cholerae (38) and orf06 of R391 from Providencia rettgeri (39) exhibit weak but significant similarities with the N-terminal domain (SLT) of CwlT. Thus it is very interesting to know a function of cell wall lytic enzymes for ICEs. Moreover, the catalytically essential Glu-87 and Asp-94 in CwlT are considered at the corresponding sites (Glu-80 and Asp-87, respectively) in the orf14 of Tn916 (data not shown). To our knowledge, this is the first report to be characterized about the cell wall lytic enzymes in ICEs. As next step experiment, we are studying the role of cell wall lytic enzyme in ICEs.

Hydrolysis Activity of CwlT—The cell wall hydrolytic activities of CwlT-N-his and CwlT-C-his were determined to be 90 units/mg protein (4,290 units/mg protein) and 90 units/mg protein (6,000 units/mg protein), respectively. Our group previously reported that cell wall hydrolyase CwlV1 from Paenibacillus polymyxa (previously Bacillus polymyxa) exhibited higher hydrolytic activity (5,700 units/mg protein (16)) than that of LytF (CwlE; 1,560 units/mg protein (13)) or CwlS (1,500 units/mg protein (18)). Both domains of CwlT also had high activity. Interestingly, the CwlT-FL-His protein (containing both domains of CwlT) retained higher hydrolytic activity (212 units/nmol (6,090 units/mg protein)). Moreover, the CwlT-FL-His retains both β,γ-endopeptidase and muramidase activities (supplemental Table 3). Therefore, this enzyme may be more useful for cell wall degradation than the hen egg white lysozyme. To our knowledge, this is also the first study of a polypeptide to exhibit two different peptidoglycan degrading activities in the genus Bacillus.

We completely digested purified glycan strands with the N-terminal domain of CwlT. As a result, tetra-, hexa-, and octasaccharides were detected (Fig. 4B); however, we could only detect a very low amount of disaccharide (GlcNAc-MurNAc) (arrow in Fig. 4B). In contrast, when the glycan strands were digested with a hen egg white lysozyme, a large amount of disaccharide and a low amount of tetrasaccharide were detected (data not shown). Thus, recognition of that substrate (oligosaccharide) by the N-terminal domain of CwlT may be different from the recognition by a lysozyme and/or the other muramidase. At least, the N-terminal domain of CwlT is valuable for easily creating oligosaccharides derived from peptidoglycan. These substrates are useful for studying cell wall hydrolyases in detail, especially crystallography of the complexes between hydrolyases and oligosaccharides.

Neither GEWL nor HEWL has two hydrolyase domains, and only a few proteins of phage lysozyme (such as UniProt Q2BPX4_JANSC, A0G9C2_9BURK) contain two hydrolyase domains. However, no protein can have muramidase and β,γ-endopeptidase domains at its N and C termini, respectively, like CwlT. Because, in addition to orf14 of Tn916 (36), some predicted proteins containing an SLT domain (such as UniProt Q4V1L6_BACCZ, Q3W920_9ACTO, and Q4EIZ3_LISMO) have this module structure (SLT domain (muramidase or lytic transglycosylase) and β,γ-endopeptidase domains at the N and C termini, respectively), we are curious to know the hydrolyase activities and catalytic amino acid residues of these proteins.

This is the first study about newly identified muramidase in B. subtilis. In particular, the finding of the N-terminal domain of CwlT as a new category of cell wall hydrolyases should be remarkable in this field. Moreover, the N-terminal domain will be a useful reagent for creating peptidoglycan-derived oligosaccharides. Furthermore, the CwlT protein containing both the N- and C-terminal domains will be a useful tool for cell lysis because the enzyme activity is very high.

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