Characterization of a New Sigma-K-Dependent Peptidoglycan Hydrolase Gene That Plays a Role in Bacillus subtilis Mother Cell Lysis

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Received 16 April 1999/Accepted 30 July 1999

Bacillus subtilis produces a 30-kDa peptidoglycan hydrolase, CwlH, during the late sporulation phase. Disruption of yqeE led to a complete loss of CwlH formation, indicating the identity of yqeE with cwlH. Northern blot analysis of cwlH revealed a 0.8-kb transcript after 6 to 7.5 h for the wild-type strain but not for the Δσ70, Δσ54, or Δσ32 mutants. Expression of the σ70-dependent cwlH gene depended on gerE. Primer extension analysis also suggested that cwlH is transcribed by EσK RNA polymerase. CwlH produced in Escherichia coli harboring a cwlH plasmid is an N-acetylmuramoyl-l-alanine amidase (EC 3.5.1.28) and exhibited an optimum pH of 7.0 and high-level binding to the B. subtilis cell wall. A cwlC cwlH double mutant led to a lack of mother cell lysis even after 7 days of incubation in DSM medium, but the single mutations led to mother cell lysis after 24 h.

The B. subtilis genome contains many cell wall hydrolase gene homologs (43, 20). To determine the cellular functions of these homologs, it is necessary to determine the expression phases for the genes and to construct disruptants. Moreover, the amino acid sequence similarity of these homologs is a clue for determination of their cellular functions. For instance, amidases in B. subtilis can be divided into three classes, I (CwlA, XlyA, XlyB, and BlyA), II (CwlB, CwlC, and CwlD), and III (SleB and CwlJ) (15). Class I seems to be associated with phage lysins, but classes II and III play roles in various cellular functions and germination, respectively. The yqeE gene belongs to class I (28), but there is no information on its gene expression or cellular function.

In this study, we identified yqeE as a new cell wall hydrolase gene, cwlH, during the sporulation phase of B. subtilis, characterized its expression, and determined the cellular function of the gene product, which is unique in class I.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains of B. subtilis and Escherichia coli and the plasmids used in this study are listed in Table 1. B. subtilis was grown on Luria-Bertani (LB) medium (39) or DSM (Schaeffer) medium (40). If necessary, tetracycline, chloramphenicol, and erythromycin were added to the medium to final concentrations of 10, 5, and 0.3 μg/ml, respectively. E. coli was grown in LB medium or 2×YT medium (39). If necessary, ampicillin was added to a final concentration of 50 μg/ml.

Plasmid and mutant construction. The entire cwlH gene was amplified by PCR using two primers, forward primer ECHF (5′-CGCCCCGGGAAGTGAATT-3′) and reverse primer ECCHR (5′-CCTCCGTTATACCTTGATATCCGCTGC-3′), the cwlH sequence is italicized, numbering is with respect to the first A of the translational start codon of cwlH, and the Smal site is underlined) and reverse primer ECHR (5′-CGCCCCGGGAAGTGAATT-3′) and reverse primer ECCHR (5′-CCTCCGTTATACCTTGATATCCGCTGC-3′), the sequence complementary to the downstream region from cwlH is italicized, and the SalI site is underlined), with B. subtilis 168 DNA as a template. The PCR fragment was digested with Smal and SalI, followed by ligation into the corresponding sites of pUC18. The DNA solution was used for the transformation of E. coli JM109 cells; then the resulting plasmid, pUCH2, was digested with SalI and SalI, followed by agarose gel electrophoresis. A 0.5-kb DNA fragment extracted from the gel was ligated to the SalI and SalI sites of a histidine-tagged plasmid, pQE-31, and then used for the transformation of E. coli M15 (pREP4). The resultant plasmid, pOCH2, contained a histidine-tagged sequence (MRGSHHHHHHHTPDHAASVPG) at its N terminus fused with the structural gene cwlH. Therefore, the histidine-tagged cwlH (h-cwlH) gene encodes a 270-amino-acid (aa) polypeptide with an M, of 29,741.

To construct the B. subtilis cwlH-laclZ strain, an internal fragment of the cwlH
gene was amplified by PCR using two primers, forward primer CWLHF (5'-G CCGGATCCGGATCAGAAAAGGCGAGCTTGAGGCTCACGC135; the internal sequence of the cwlH region is italicized, numbering is with respect to the first A of the translational start codon of cwlH, and the HincIII site is underlined) and reverse primer CWLHR (5'-CCGGGATCCGGATCAGAAAAGGCGAGCTTGAGGCTCACGC135; the sequence complementary to the internal region of cwlH is italicized, and the BamHI site is underlined), with B. subtilis 168 DNA as a template. The PCR fragment was digested with HincIII and BamHI. pMUTIN2 was digested with HincIII and BamHI and then ligated to the digested PCR fragment, followed by transformation of E. coli JM109. The resulting plasmid, pMCWLH, was used for the transformation of E. coli C600 to produce concatemeric DNAs (4). pGEM-3zf(+) was digested with HincIII and BamHI and then ligated to the digested PCR fragment, followed by transformation of E. coli JM109. The resulting plasmid, pMUTIN2, was used for the transformation of E. coli C600 to produce concatemeric DNAs (4). pGEM-3zf(+) was digested with HincIII and BamHI and then ligated to the digested PCR fragment, followed by transformation of E. coli JM109. The resulting plasmid, pMUTIN2, was used for the transformation of E. coli C600 to produce concatemeric DNAs (4). pGEM-3zf(+) was digested with HincIII and BamHI and then ligated to the digested PCR fragment, followed by transformation of E. coli JM109. The resulting plasmid, pMUTIN2, was used for the transformation of E. coli C600 to produce concatemeric DNAs (4). pGEM-3zf(+) was digested with HincIII and BamHI and then ligated to the digested PCR fragment, followed by transformation of E. coli JM109. The resulting plasmid, pMUTIN2, was used for the transformation of E. coli C600 to produce concatemeric DNAs (4).

Preparation of cell walls. Cell walls of B. subtilis 168S and Micrococcus luteus ATCC 4698, unless otherwise noted, were prepared essentially as described previously (8, 17).

Measurement of the optimal pH. B. subtilis cell wall was suspended in 0.1 M imidazole-NPB solution (10 mM imidazole and 1 M NaCl in 20 mM sodium phosphate buffer [pH 7.4]). After ultrasonication, the suspension was centrifuged, and the supernatant (25 ml) was filtered through a 0.45-μm-pore-size membrane filter (Nalgene), followed by application onto a HiTrap chelating column (1 ml; Pharmacia). The column was washed with 10 mM imidazole-NPB solution (20 ml), and then the h-CwlH protein was eluted with the NPB solution containing a stepwise gradient of imidazole from 30 to 60 mM.

Preparation of cell walls. Cell walls of B. subtilis 168S and Micrococcus luteus ATCC 4698, unless otherwise noted, were prepared essentially as described previously (8, 17).

SDS-PAGE and zymography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed in 10% or 12% (wt/vol) polyacrylamide gels as described by Laemmli (21). Zymography was performed essentially as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Laemmli (21). Zymography was performed essentially as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Laemmli (21). Zymography was performed essentially as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Laemmli (21). Zymography was performed essentially as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Laemmli (21). Zymography was performed essentially as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Laemmli (21). Zymography was performed essentially as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23), using SDS-polyacrylamide gels containing 0.1% (wt/vol) polyacrylamide gels as described by Leclerc and Asselin (23).
SDS-PAGE, the amount of protein in the supernatant was measured by densitometric analysis.

Identification of the specific substrate bond cleaved by the cell wall hydrolase. The amino groups released during enzyme digestion of *B. subtilis* cell wall were labeled with 1-fluoro-2,4-dinitrobenzene (18). Dinitrophenyl (DNP) derivatives were separated by ODS column (Wakosil-II5C18) chromatography, and D and L isomers of DNP-alanine were separated by Sumichiral OA-2500S column chromatography (18).

β-Galactosidase assay. The β-galactosidase assay was performed basically as described by Shimotsu and Henner (44). One unit of β-galactosidase activity was defined as the amount of enzyme necessary to release nmol of 2-nitrophenol from 2-nitrophenyl-β-D-galactopyranoside in 1 min.

Northern blot and primer extension analyses. *B. subtilis* cells (15 OD600 units) cultured in DSM medium were harvested and then suspended in 1 ml of chilled killing buffer (20 mM Tris-HCl [pH 7.5] containing 5 mM MgCl2 and 20 mM Na2N3 (14). After centrifugation at 11,000 g for 2 min, the pellet was suspended in 1 ml of SET buffer containing lysozyme (final concentration 6 mg/ml) (14). After incubation at 10 min at 0°C, the suspension was centrifuged at 11,000 g for 2 min. The pellet was used for RNA preparation with Isogen (Nippon Gene) according to the manufacturer’s instructions. Agarose-formaldehyde gel electrophoresis was performed as described by Sambrook et al. (39). The transfer of RNAs onto a nylon membrane (Magnagraph; Micron Separations) was performed with a vacuum blotter (model BE-600; BIOCRAFT). The DNA fragment used for preparing an RNA probe was amplified by PCR with M13(21) and M13RV as primers and pGCWLH DNA, containing the internal region of *cwlH*, as a template. The amplified fragment was digested with *Hind*III.

**FIG. 1.** Gene organization of *yqeE* (designated *cwlH*) and its flanking regions, and nucleotide sequence of the upstream region of *cwlH*. The peptides are numbered with respect to the translational start codon (+1) of each protein. -35 and -10 represent the -35 and -10 regions of a σ70 promoter, and SD represents a Shine-Dalgaro sequence. The arrowhead indicates the transcriptional start point of *cwlH*. The GerE consensus sequence and restriction sites are shown by underlining and overlining, respectively, of the nucleotide sequence.

**FIG. 2.** SDS-polyacrylamide (12%) gel electrophoretic analysis of expression of the CwlH protein in *E. coli* (pREP4, pUCH2) (A) and zymography of CwlH with *B. subtilis* 168 cell wall (B). (A) *E. coli* M15(pREP4, pUCH2) cells were cultured in 100 ml of LB medium at 37°C. When the cell density reached 0.8 at OD660, 2 mM (final concentration) IPTG was added to the culture, followed by further incubation for 3 h. The cells were washed and sonicated, and then the broken-cell suspension was centrifuged. Proteins in the supernatant (equivalent to the 0.1 OD660 cells) were loaded on lane 2. Lane 1 is a sample without IPTG induction. Lane 3 is the purified CwlH protein (7.5 μg) after nickel affinity column chromatography. M, marker proteins (Bio-Rad broad-range markers [1.5 μg of each]; from top to bottom, 97.4, 66.2, 45.0, 31.0, and 21.5 kDa). (B) The purified CwlH (1 μg) was loaded onto lanes 1 and 2. Lanes M (marker proteins) and 1, SDS-PAGE; lane 2, zymography with strain 168 cell wall. Zymographic patterns toward the *M. luteus* cell wall, 168 spore cortex, and muramic acid lactam-deficient EDD1 cortex were very similar to that toward the 168 cell wall (B, lane 2; reference 31).

**FIG. 3.** Digestion of *B. subtilis* cell wall with the purified CwlH protein. *B. subtilis* cell wall (2.0 mg) and the purified CwlH (9.6 μg) were mixed in 6 ml of a 1% potassium borate solution (pH 7.0) and then incubated at 37°C. Aliquots (500 μl of each) was removed at various intervals for determination of turbidity at 540 nm (○), DNP-diaminopimelic acid (▲), DNP-L-alanine (●), and DNP-D-alanine (▲).
the resulting fragments were purified by phenol and chloroform treatments and precipitated with ethanol. The RNA probe was prepared with a DIG (digoxigenin) RNA labeling kit (Boehringer Mannheim), and Northern (RNA) hybridization was performed according to the manufacturer’s instructions. Primer extension analysis was performed as described previously (41), using primer PEH1. Dideoxy DNA sequencing reaction mixtures with the same primer were electrophoresed in parallel (lanes G, A, T, and C). The arrows indicate the nucleotide at the transcriptional start site. The nucleotide sequence of the transcribed strand is given beside the sequence ladder.

**RESULTS**

Paralog analysis of many cell wall hydrolase genes in *B. subtilis* revealed that the yqeE gene is located at 226° on the *B. subtilis* chromosome. This gene encodes a 250-aa polypeptide with an *M*₉ of 27,571 (20). Figure 1 shows the gene organization of the yqeE gene and its flanking regions and the nucleotide sequence of the upstream region of *yqeE*. The amino acid sequence of YqeE (designated CwlH) exhibits 59.6, 44.7, 42.8, and 27.1% identity with those of *B. subtilis* XlyA (297 aa), *B. subtilis* CwlA (272 aa), *B. subtilis* XlyB (317 aa), and *B. subtilis* BlyA (367 aa), respectively (5, 9, 17, 24, 36).

Expression of h-CwlH in *E. coli*. pQCH2 containing a histidine-tagged sequence followed by the cwlH gene was introduced into *E. coli* cells as described in Materials and Methods, and the h-CwlH protein (270 aa) was expressed by IPTG induction. Figure 2A shows the results of SDS-PAGE analysis of the expression of h-CwlH. A large amount of h-CwlH was accumulated in *E. coli* cells (lane 2), and the *M*₉ of 31,000 was in good agreement with that calculated from the sequence (29,741). The h-CwlH protein was purified by nickel affinity chromatography, and the purified protein was run in lane 3.

Characterization of the h-CwlH protein. The substrate specificity of the purified h-CwlH protein was determined by zymography with *B. subtilis* and *M. luteus* cell walls and the wild-type and modified (muramic acid lactam-deficient) cortex. The h-CwlH protein gave a clear hydrolyzing band with all of the above substrates on zymography (Fig. 2B) (31). The optimal pH was 7.0 (relative activities of 5.1% at pH 5.0, 47.5% at pH 6.0, 100% at pH 7.0, 94.9% at pH 8.0, 55.9% at pH 9.0, and 47.5% at pH 10.0). The specific activity of h-CwlH under the

![Image](http://jb.asm.org/Downloaded)
optimal conditions was 1.6 × 10⁴ U per mg of protein. The h-CwlH protein was completely bound to B. subtilis cell wall in distilled water.

The purified h-CwlH protein was added to the B. subtilis cell wall, and the mixture was incubated at 37°C for various time periods. The products resulting from the enzyme reaction were investigated by labeling of free amino groups with 1-fluoro-2,4-dinitrobenzene, followed by chromatography as described in Materials and Methods. Only DNP-L-alanine increased during the enzyme reaction (Fig. 3). Therefore, this protein is an N-acetylmuramoyl-L-alanine amidase.

Regulation of the cwlH gene. A transcriptional fusion between cwlH and lacZ was constructed with a 8.9-kb plasmid, pMCWLH, containing the internal region of CwlH in the B. subtilis chromosome. The cwlH lacZ strain, CWLHd, expressed significant lacZ activity during the late sporulation stage (Fig. 4A). Northern hybridization analysis of the cwlH gene with a specific RNA probe consisting of the internal region (HindIII-BamHI region) of cwlH revealed a single 0.8-kb signal band at t₆ (6 h after onset of sporulation) for the wild-type 168 strain, but no hybridizing band was observed for the sigE, sigF, sigG, and sigK-deficient strains (Fig. 4B). Since some of the SigK-dependent genes are also regulated by the gerE gene, the GerE dependence of the cwlH gene was determined with the gerE-deficient strain, 1G12. No 0.8-kb transcript was observed for the 1G12 strain (Fig. 4C). These results show that the cwlH gene is transcribed by EσK RNA polymerase and regulated by the GerE protein.

Primer extension analysis of the cwlH gene with the primer, PEH1, corresponding to the 5' end of cwlH, revealed one transcriptional start point, A, at position −14 with respect to the translational start point of cwlH (t₁₆₈ sample for the 168 strain in Fig. 5). RNAs of the 168 strain at t₃ and the σ₅²-deficient strain at t₁₆₈ did not show the transcriptional start point (Fig. 5). A significant ρ-independent terminator exists in the downstream region of the cwlH gene (ΔG = −15.9 kcal/mol). The 0.8-kb transcript shows good correspondence in size from the transcriptional start point to the terminator region of the cwlH gene (Fig. 1). TC and CATTCTTAT sequences, which are very similar to the consensus sequences of −35 and −10 sequences (AC and CATANNNTA) (11, 29), were observed at −32 to −31 bp and at −13 to −5 bp, respectively, relative to the transcriptional start point of cwlH (Fig. 1). The GerE consensus sequence (TPuGGPy [Pu, purine; Py, pyrimidine]) was also observed in the region from −152 to −148 bp (TAGGT in Fig. 1). These results confirmed that the cwlH gene is transcribed by EσK RNA polymerase and regulated by GerE.

Production of the CwlH protein during the late sporulation phase. Since the cwlH gene is expressed during the late sporu-
lation phase, surface proteins were extracted in that phase from strains AC327, ANC1 (cwlC), and ACH (cwlC cwlH) and analyzed by zymography (Fig. 6). The wild-type strain gave a cell wall hydrolyzing band corresponding to a molecular size of 28 kDa. Since CwlC is a 28-kDa polypeptide, the size being very similar to that of CwlH, the cwlC gene was disrupted and then analyzed by zymography. A 28-kDa band was still observed, but to a much lesser extent. For the cwlC cwlH mutant,
however, no 28-kDa band was detected. Therefore, the 28-kDa band corresponded to CwIH.

**Role of CwIH in cell morphology.** Cell wall hydrolases play various roles in cell morphology, but the role of CwIH has not been determined. CwIH disruption affected neither vegetative cell growth, sporulation frequency, nor germination frequency (31). The combined effect of CwIH double mutation but weakly by the cwlH cwlB double mutation (Fig. 7). The single cwlH, cwlC, and cwlB mutations did not affect mother cell lysis. The combined effect of CwIH and CwIC is very similar to that of CwIB and CwIC (Fig. 7) reported by Smith and Foster (47).

**DISCUSSION**

The CwIH protein exhibited extensive amino acid sequence similarity with class I (CwIA type) N-acetylmuramoyl-l-alanine amidases (Fig. 8). XlyA, XlyB, and BlyA are autolysins of phages PBS1 and SPβ (24, 36, 5). CwIA is assumed to be a phage lysin, because the cwlA gene is located in the skin element (17, 28). In contrast to these phage lysins, CwIH plays a role in mother cell lysis in concert with CwIC. It is exceptional in class I, because CwIH is produced during the life cycle and has a specific function in cells (Fig. 7). The catalytic domains of class I amidases are located in the N-terminal region, and the amino acid sequences of the C-terminal domains are also very similar to each other except that of BlyA (Fig. 8). Recently, Regamy and Karamata reported that BlyA bound to cell wall very strongly and that it was difficult to release the enzyme from the cell wall with a 5 M LiCl solution (36). This property probably depends on the large difference in the C-terminal amino acid sequence between BlyA and other class I amidases. CwIH exhibits a broad substrate specificity, including hydrolyzing activity for spore cortex (31), which was also found for CwA and CwIC (9, 16). It is interesting that CwIH is able to hydrolyze immature cortex without muramic acid lactam (31).

The **cwlH** gene is transcribed by ErK RNA polymerase and affected by GerE (Fig. 4). The **cwlC** gene is also transcribed by ErK RNA polymerase and affected by GerE (43), and the gene product also plays a role in mother cell lysis in concert with CwIH or CwIB (Fig. 7). The major autolysin (CwIB [LytC]) is produced actively at the end of the exponential growth phase (19, 22) and remains until the late stationary phase (18) and also until the late sporulation phase (Fig. 6 and reference 10). In the late sporulation phase, CwIC is the major hydrolytic enzyme, as judged by zymography, CwIB and CwIH being minor ones (Fig. 6 and reference 10). The results correspond well with the effects of these proteins on mother cell lysis. The total activity of these three peptidoglycan hydrolases may be most important. However, we are unable to eliminate the possibility that the enzymatic property of CwIC in concert with CwIB or CwIH is most important for mother cell lysis.

Combined effects of cell wall and cortex hydrolases are often found for cellular functions. Cell separation is greatly affected by a combination of two endopeptidases (14, 26, 32). Vegetative cell lysis is affected by the major autolysin, CwIB (3, 18, 46), and greatly by four autolysins, including minor ones (CwIF [LytE] and CwIE [LytF]) (26). Motility and cell wall turnover were also affected by combinations of cell wall hydrolases (27, 34, 46), and recently germination was found to be completely blocked with the lack of two deduced class II amidases (15). The combined effect on mother cell lysis observed in this study further supports the idea that peptidoglycan and cortex hydrolases play essential roles in cellular functions in concert.

**ACKNOWLEDGMENTS**

We thank Tatsuya Fukushima for determination of the substrate bond specificity of the enzyme.

This research was supported by a Grant-in-Aid for Scientific Research on Priority Areas (no. 296) from the Ministry of Education, Science, Sports and Culture and by grant JSPS-RFFT96L010015 from the Japan Society for the Promotion of Science.

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