JOURNAL OF BACTERIOLOGY, Oct. 1999, p. 6230–6237 Vol. 181, No. 20
0021-9193/99/$04.00+0

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

FAJAR AJI NUGROHO,[1] HIROKI YAMAMOTO,[1] YASUO KOBAYASHI,[2] AND JUNICHI SEKIGUCHI[1]*

Department of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, Ueda-shi, Nagano 386-8567,[1] and Faculty of Agriculture, Tokyo University of Agriculture & Technology, Fuchu-shi, Tokyo 183-8509,[2] Japan

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 σ^F, σ^E, σ^G, and σ^K mutants. Expression of the σ^K-dependent *cwlH* gene depended on *gerE*. Primer extension analysis also suggested that *cwlH* is transcribed by Eσ^K 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 mutation 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.**

Bacillus subtilis produces a complement set of enzymes capable of hydrolyzing the shape-maintaining and stress-bearing peptidoglycan layer of its own cell wall (6, 37, 45). Some of these peptidoglycan hydrolases can trigger cell lysis and therefore can be called autolysins or suicide enzymes (37). Autolysins have been implicated in several important cellular processes, such as cell wall turnover, cell separation, competence, and flagellation (motility), in addition to cell lysis, and they act as pacemaker and space maker enzymes for cell wall growth (2, 7, 8, 33, 37). Therefore, fine-tuning of autolysin activity through efficient and strict regulation is a must for bacterial survival (13).

Two major vegetative-phase autolysins, (i) a 50-kDa *N*-acetylmuramoyl-L-alanine amidase (amidase), CwlB (LytC), and (ii) a 90-kDa endo-β-*N*-acetylglucosaminidase (glucosaminidase), CwlG (LytD), were initially purified from *B. subtilis* and characterized (12, 18, 22, 25, 35, 38). A study on the physiological functions of CwlB and CwlG revealed that CwlB is responsible for cell lysis in the stationary phase (18) and after cold shock treatment (49) and that both proteins, but only in concert, are required for the motility function (34). Several other amidase genes and their homologs have been cloned from *B. subtilis*. Four prophage-encoded amidase genes (*cwlA*, *xlyA*, *xlyB*, and *blyA*) (5, 9, 17, 24, 36), a sporulation-specific amidase gene (*cwlC*) (10, 16), a cortex maturation-specific and deduced amidase gene (*cwlD*) (41), and germination-specific and deduced amidase genes (*sleB* and *cwlJ*) (30, 15) have been cloned and studied, in addition to *cwlB* (18). Recently, endopeptidase homologs (CwlF [LytE] and CwlE [LytF]) playing roles in cell separation during vegetative growth were found (14, 26, 27, 32). Regarding the sporulation phase-specific expression of autolysin genes, Smith et al. found minor autolysins (A3, A6, and A7) during the sporulation phase (46). They also reported that a *cwlB cwlC* double mutant was found to be resistant to mother cell lysis during the late stage of sporulation (47).

* Corresponding author. Mailing address: Department of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, 3-15-1 Tokida, Ueda-shi, Nagano 386-8567, Japan. Phone: 81 268 21 5344. Fax: 81 268 21 5331. E-mail: jsekigu@giptc.shinshu-u.ac.jp.

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′-CGCCCCGGG$A_1TGGTAA$ $CCATAAAAAAGG_{20}$; the *cwlH* sequence is italicized, numbering is with respect to the first A of the translational start codon of *cwlH*, and the *Sma*I site is underlined) and reverse primer ECHR (5′-CGCGTCGAC$T_{773}TATCCGTTAA$ $ATCCTGC_{756}$; the sequence complementary to the downstream region from *cwlH* is italicized, and the *Sal*I site is underlined), with *B. subtilis* 168 DNA as a template. The PCR fragment was digested with *Sma*I and *Sal*I, followed by ligation into the corresponding sites of pUC118. The DNA solution was used for the transformation of *E. coli* JM109 cells; then the resulting plasmid, pUCH2, was digested with *Sac*I and *Sal*I, followed by agarose gel electrophoresis. A 0.8-kb DNA fragment extracted from the gel was ligated to the *Sac*I and *Sal*I sites of a histidine-tagged plasmid, pQE-31, and then used for the transformation of *E. coli* M15(pREP4). The resultant plasmid, pQCH2, contained a histidine-tagged sequence (MRGSHHHHHHTDPHASSVPG) 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_r of 29,741.

To construct the *B. subtilis cwlH-lacZ* strain, an internal fragment of the *cwlH*

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Reference or source
Strains		
B. subtilis		
168	*trpC2*	47
AC327	*purB his-1 smo-1*	41
CWLHd	*trpC2 cwlH*::pMUTIN2	This study
ANC1	*purB his-1 smo-1 cwlC*::*cat*	16
ANB1	*purB his-1 smo-1 cwlB*::*tet*	This study
ABC	*purB his-1 smo-1 cwlB*::*tet cwlC*::*cat*	This study
ABH	*purB his-1 smo-1 cwlB*::*tet cwlH*::pMUTIN2	This study
ACH	*purB his-1 smo-1 cwlC*::*cat cwlH*::pMUTIN2	This study
ABCH	*purB his-1 smo-1 cwlB*::*tet cwlC*::*cat cwlH*::pMUTIN2	This study
1G12	*gerE*36 *leu-2*	BGSC[a]
1G1Δ*cwlH*	*gerE*36 *leu-2 cwlH*::pMUTIN2	This study
1S38	*trpC2 spoIIIC94*	BGSC
1S60	*leuB8 tal-1 spoIIG41*	BGSC
1S86	*trpC2 spoIIA1*	BGSC
SpoIIIGΔ1	*trpC2 spoIIIGΔ1*	P. Setlow
ADD1	*purB his-1 smo-1 cwlD*::*cat*	40
EDD1	*trpC2 cwlD*::*cat*	*B. subtilis* 168 transformed with ADD1 DNA
E. coli		
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi-1*	Takara
C600	*supE44 hsdR thi-1 leuB6 lacY1 tonA21*	Laboratory stock
M15	F^- Str^r Δ*lacZ*	QIAGEN
Plasmids		
pUC118	Ap^r	Takara
pQE-31	Ap^r	QIAGEN
pMUTIN2	Em^r *lacI lacZ*	47
pGEM3zf+	Ap^r	Promega
pCLTC	Ap^r *cwlB*::*tet*	33
pMCWLH	Em^r *cwlH-lacZ lacI*	This study
pGCWLH	Ap^r	This study
pUCH2	Ap^r *cwlH*	This study
pQCH2	Ap^r *cwlH*	This study

[a] BGSC, *Bacillus* Genetic Stock Center, Ohio State University.

gene was amplified by PCR using two primers, forward primer CWLHF (5′-G CCGAAGCTTG_{94}*CGAATACAGCCAAAGGC*$_{111}$; 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 *Hin*dIII site is underlined) and reverse primer CWLHR (5′-CGCGGATCCA_{353}*TCAGCTTTCTGATCAGCC*$_{335}$; the sequence complementary to the internal region of *cwlH* is italicized, and the *Bam*HI site is underlined), with *B. subtilis* 168S DNA as a template. The PCR fragment was digested with *Hin*dIII and *Bam*HI. pMUTIN2 was digested with *Hin*dIII and *Bam*HI 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 *Hin*dIII and *Bam*HI and then ligated to the digested PCR fragment, followed by transformation of *E. coli* JM109. The resulting plasmid, pGCWLH, was used to synthesize an RNA probe. To construct *B. subtilis* ANB1 (*cwlB*), pCLTC, containing the *cwlB*::*lacZ* fusion, was digested with *Eco*RI and then used for transformation of *B. subtilis* AC327. *B. subtilis* ABC (*cwlB cwlC*) was constructed by transformation of *B. subtilis* ANB1 with *B. subtilis* ANC1 (*cwlC*) DNA. *B. subtilis* 1G1Δ*cwlH* (*gerE cwlH*) was constructed by transformation of *B. subtilis* 1G12 (*gerE*) with *B. subtilis* CWLHd DNA. Competent cells of *B. subtilis* ANB1, ANC1, and ABC were transformed with *B. subtilis* CWLHd DNA. The resultant mutants, ABH, ACH, and ABCH, respectively, were used for zymographic analysis of cell wall hydrolase activity and also for morphological analysis. The *cwlH* disruption of the mutants was confirmed by long-range PCR using a Gene Amp XL PCR kit (Perkin-Elmer).

Transformation of *E. coli* and *B. subtilis*. *E. coli* transformation was performed as described by Sambrook et al. (39), and *B. subtilis* transformation was performed by the competent cell method (1).

Purification of the CwlH protein. *E. coli* M15(pREP4, pQCH2) was cultured in LB medium containing ampicillin (200 ml) to a cell density (optical density at 600 nm [OD_{600}]) of approximately 0.8 at 37°C. Then 2 mM (final concentration) isopropyl-β-D-thiogalactopyranoside was added to the culture, followed by further incubation for 3 h. The culture was centrifuged, and the pellet was suspended in 50 ml of 10 mM 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) *B. subtilis* cell wall, *M. luteus* cell wall, or *B. subtilis* cortex as a substrate.

Cell wall hydrolase assay. *B. subtilis* cell wall was suspended in 0.1 M TMB buffer (0.1 M Tris, 0.1 M maleic acid, 0.1 M bovic acid; adjusted to pH 7.0) to yield a final absorbance of 0.3 at 540 nm. The purified t-CwlH was added to the solution, followed by incubation at 37°C. One unit of cell wall hydrolase activity was defined as the amount of enzyme necessary to reduce the absorbance at 540 nm by 0.001 per min.

Measurement of the optimal pH. *B. subtilis* cell wall was added to various buffer solutions to yield a final absorbance at 540 nm of 0.3. Purified h-CwlH was added to the buffers, followed by incubation at 37°C for 10 min. The buffers used for pHs 3 to 5 and 5 to 10 were 0.1 M citrate buffer and 0.1 M TMB buffer, respectively.

Cell wall binding ability. The cell wall binding ability of the enzyme was examined in distilled water containing 5.5 μg of the purified protein and 10 mg of *B. subtilis* cell wall. After 30-min incubation at 0°C, the reaction mixture was centrifuged, and then protein in the supernatant was applied to a gel. After

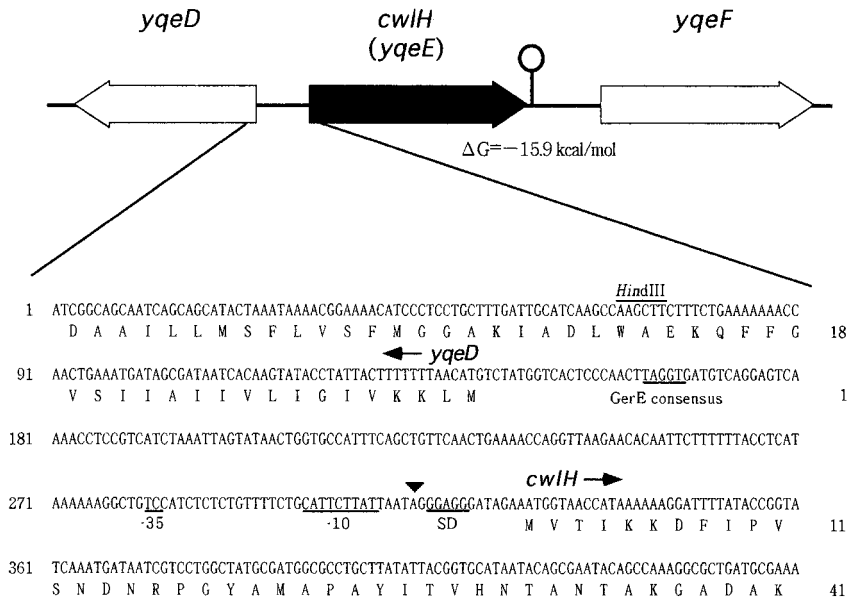

FIG. 1. Gene organization of *yqeE* (designated *cwlH*) and its flanking regions, and nucleotide sequence of the upstream region of *cwlH*. The peptides are numbered with respect to the translational start codon (+1) of each protein. −35 and −10 represent the −35 and −10 regions of a σ^K promoter, and SD represents a Shine-Dalgarno 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.

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 OD_{600} 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 $MgCl_2$ and 20 mM Na_2N_3 (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 *Hin*dIII;

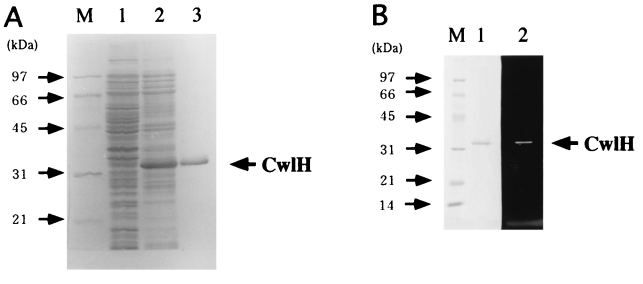

FIG. 2. SDS-polyacrylamide (12%) gel electrophoretic analysis of expression of the CwlH protein in *E. coli*(pREP4, pUCH2) (A) and zymography of CwlH with *B. subtilis* 168 cell wall (B). (A) *E. coli* M15(pREP4, pUCH2) cells were cultured in 100 ml of LB medium at 37°C. When the cell density reached 0.8 at OD_{600}, 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 OD_{600} cells) were loaded onto 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).

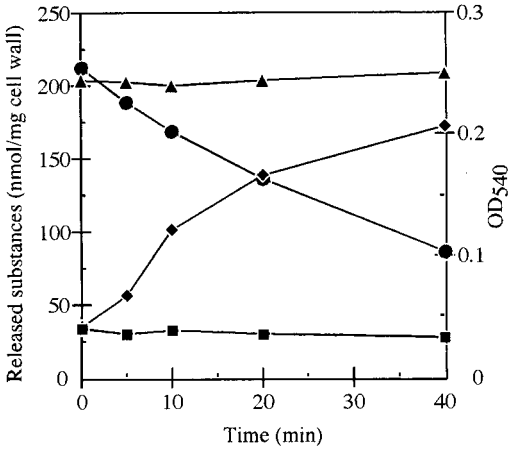

FIG. 3. Digestion of *B. subtilis* cell wall with the purified CwlH protein. *B. subtilis* cell wall (2.0 mg) and the purified CwlH (9.6 μg) were mixed in 6 ml of a 1% potassium borate solution (pH 9.0) and then incubated at 37°C. Aliquots (500 μl of each) was removed at various intervals for determination of turbidity at 540 nm (●), DNP-diaminopimelic acid (▲), DNP-L-alanine (◆), and DNP-D-alanine (■).

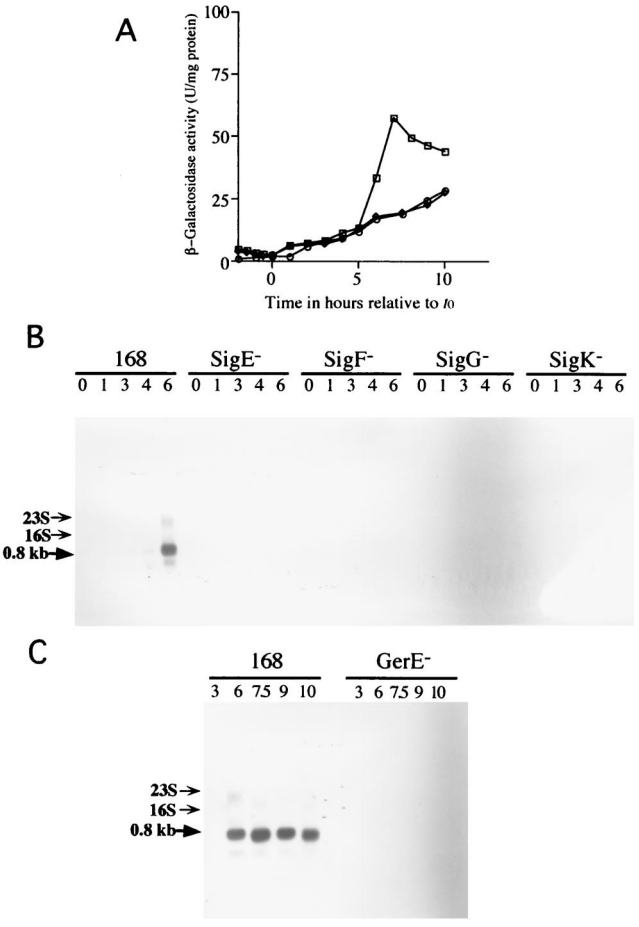

FIG. 4. (A) β-Galactosidase activities of the *cwlH-lacZ* transcriptional fusion strains constructed in the *B. subtilis* chromosome. The strains were grown in DSM medium at 37°C. Squares, strain CWLHd; circles, strain 1G1Δ*cwlH*, diamonds, strain 168. (B and C) Northern hybridization analysis of *cwlH* with a specific RNA probe. *B. subtilis* total RNAs from cultured cells were purified with Isogen (numbers indicate the times [hours] after onset of sporulation), and 10 μg of each RNA was separated on a 1% formaldehyde agarose gel. Signals were detected with the digoxigenin-labeled RNA probe. The *cwlH* mRNA signal is indicated by a thick arrow; positions of 23S and 16S RNAs are indicated by thin arrows. 168, *B. subtilis* 168; SigE⁻, 1S60 (*spoIIG*); SigF⁻, 1S86 (*spoIIA*); SigG⁻, spoIIIGΔ1 (*spoIIIG*); SigK⁻, 1S38 (*spoIIIC*); GerE⁻, 1G12 (*gerE*).

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 (5′-CCATCGCATAGCCAGGACGA; the 5′ and 3′ ends corresponding to the complementary nucleotides at positions 64 and 45 with respect to the 5′ end of the *cwlH* gene).

Microscopic observation and cell density determination. Cells were shake-cultured in a test tube (17-mm diameter) containing 5 ml of DSM medium at 37°C. After 1 and 7 days, 5-μl samples were mixed with 5 μl of 2% agarose on slide glasses, and then the cell morphology was observed by phase-contrast microscopy. The OD_{600} was measured after strong vortexing of samples. In the case of filamentous mutant cells, a small amount of lysozyme was added to the samples just before vortexing.

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_r 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).

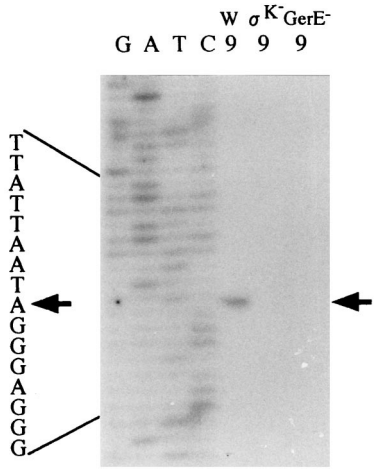

FIG. 5. Determination of transcriptional start sites by primer extension analysis. Total RNAs (50 μg) at t_9 from *B. subtilis* 168 (W), 1S38 (σ^{K-}), and 1G12 ($GerE^-$) were used as RNA samples. Signals were detected with ^{32}P-labeled primer PEH1. Dideoxy DNA sequencing reaction mixtures with the 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.

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_r 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

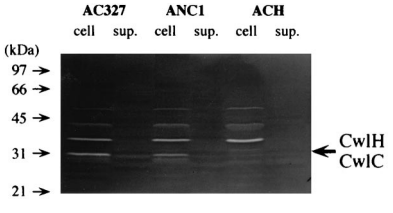

FIG. 6. Zymographic analysis of cell extracts and culture supernatants of *B. subtilis* AC327 (wild type), ANC1 (*cwlC*), and ACH (*cwlC cwlH*) cells. Cultures at t_{10} were centrifuged, and then the supernatants (sup.) of the cultures (2 ml of each) and SDS extracts of the pellets were applied on a 12% polyacrylamide gel containing 0.1% *B. subtilis* cell wall. The cell wall lytic bands of CwlH and CwlC overlapped, as indicated by an arrow. Standard size markers are shown on the left.

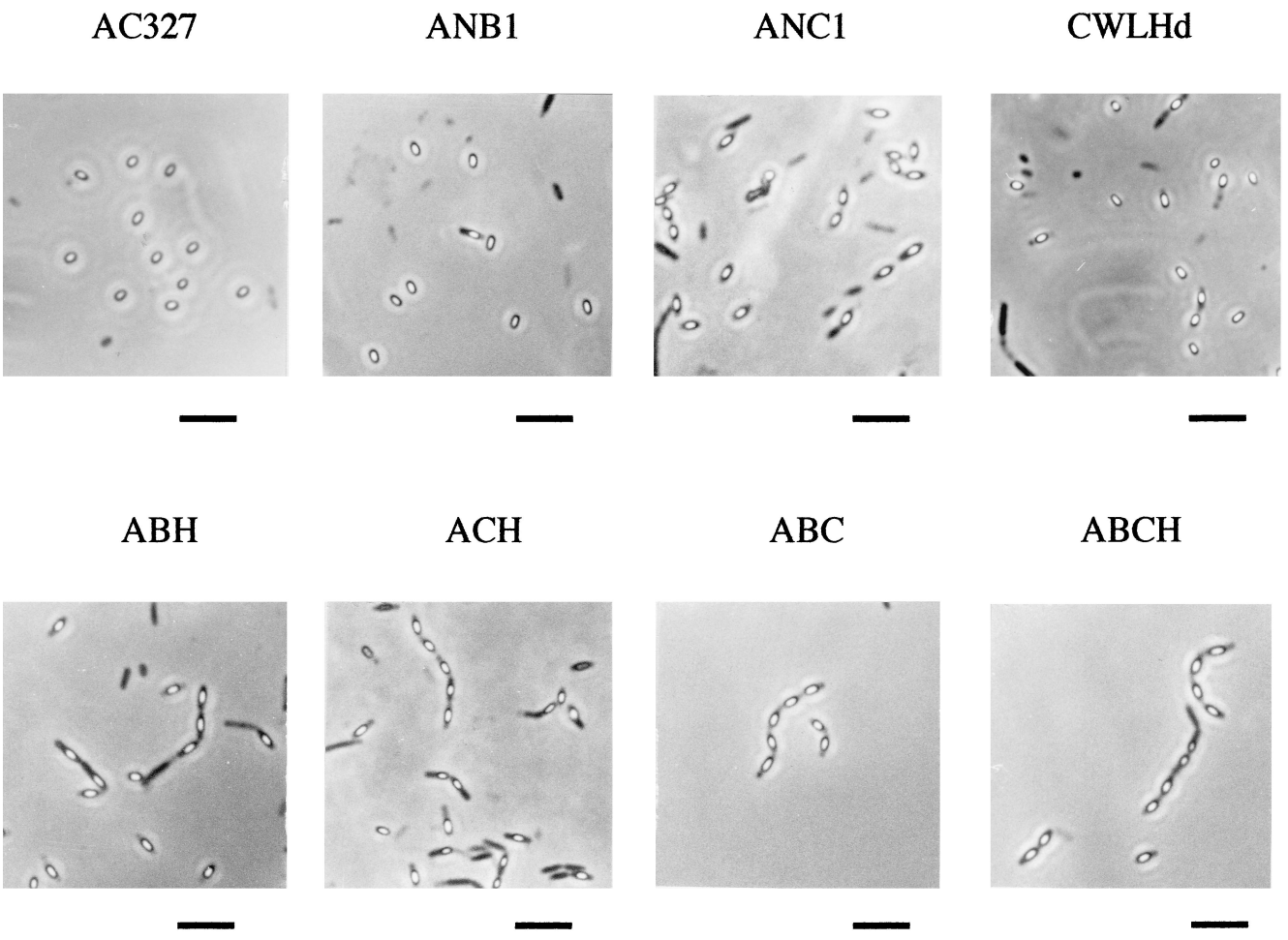

FIG. 7. Phase-contrast micrographs of *B. subtilis* AC327 (wild type), ANB1 (*cwlB*), ANC1 (*cwlC*), CWLHd (*cwlH*), ABH (*cwlB cwlH*), ACH (*cwlC cwlH*), ABC (*cwlB cwlC*) and ABCH (*cwlB cwlC cwlH*) cells after 24 h incubation in DSM medium at 37°C. Bars, 10 μm.

optimal conditions was 1.6×10^4 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 (*Hin*dIII-*Bam*HI region) of *cwlH* revealed a single 0.8-kb signal band at t_6 (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 shows that the *cwlH* gene is transcribed by $E\sigma^K$ 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* ($t_{7.5}$ sample for the 168 strain in Fig. 5). RNAs of the 168 strain at t_3 and the σ^K-deficient strain at $t_{7.5}$ did not show the transcriptional start point (Fig. 5). A significant ρ-independent terminator exists in the downstream region of the *cwlH* gene ($\Delta G = -15.9$ kcal/mol). The 0.8-kb transcript shows good correspondence in size from the transcriptional start point to the terminator region of *cwlH* (about 814 bp), thus indicating monocistronic transcription. 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 $E\sigma^K$ 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-

```
CwlA   1 MAIKVVKNLVSKSKYGLKCPNPMK-AEYITIHNTANDAS---AANEISYMKNNSSSTSFH
XlyA   1 -MVNIIQDFIPVG-ANNRPGYAMT-PLYITVHNTANTAVGADAAAHARYLKNPDTTTSWH
CwlH   1 -MVTIKKDFIPVS-NDNRPGYAMA-PAYITVHNTANTAKGADAKMHAKFVKNPNTSESWH
XlyB   1 MSIPVKKNLVSEAKYALKCPNAMS-AEYITIHNTANDAS---AANEISYMIGNTSSTSFH
BlyA   1 -MSVFTNSYIPVN-KYTRPGLKLQGVKKCVLHYTANPGAG--ADNHRRYFSNAQVYASAH
                                       * ***      *             * *

CwlA  57 FAVDDKQVIQGIPTNRNAWHTGDGT--NGTGN--R-----------KSIGVEICYSKSGG
XlyA  58 FTVDDTEIYQHLPLNENGWHAGDG---NGSGN--R-----------ASIGIEICENADG-
CwlH  58 FTVDDSVIYQHLPIDENGWHAGDGT--NGTGN--R-----------KSIGIEICENADG-
XlyB  57 FAVDDQEVIQGLPLNRNAWHTGDGT--NGPGN--R-----------KSIGVEICYSKSGG
BlyA  57 IFVDKAEAICIIPLNEVAYHANDIQQRDSAGNPYRGVAALKPNANFLSIGVEMCLEKDGS
           **        *      *  *       **  *            *** * *

CwlA 102 VRYKAAEKLAIKFVAQLLKERGWGIDRVRKHQDWNGKYCPHRILSEGR-WIQVKTAIEAE
XlyA 101 -DFAKATANAQWLIKTLMAEHNISLANVVPHKYWSGKECPRKLLDT---WDSFKAGIGGG
CwlH 102 -DFEKATSNAQWLIRKLMKENNIPLNRVVPHKKWSGKECPRKLLDH---WNSFLNGISSS
XlyB 102 PKYEAAEALAISFVAQLLKERGWGIDRVRKHQDWSGKYCPHRILSEGR-WDQVKAAIEKE
BlyA 117 FHSDTVERTEDVFVELCNKFGLDPIDDIVRHYDITHKNCPAPWVSNSQKFVDFKNRVKAK
                                       *     * **

CwlA 161 LK----------------------KLGGKTNSSKASVAKKKTT---------NT-SSKK
XlyA 157 GS--------------QTYVVKQGDTLTSIARAFGVTVAQLQEW---------NN-IEDP
CwlH 158 ------------------------------------------------------DTP
XlyB 161 LNGGVSAKKAAVSSSASEYHVKKGDTLSGIAASHGASVKTLQSI---------NH-ITDP
BlyA 177 MSGKSVSKASPTKPTTSSPSSSSAVSGSLKSKVDGLRFYSKPSWEDKDVVGTVNKGIGFP


CwlA 188 ----------------------------TSYALPSGIFKVKS--PMMRGE---------
XlyA 193 NLIRVGQVL---IVSAPSAA------EKPELYPLPDGIIQLTT--PYTSGE---------
CwlH 161 ----------------P--K------ETSPSYPLPSGVIKLTS--PYRKGT---------
XlyB 211 NHIKIGQVIKLPQTASASKS------HAASSYPLPSGVIKVTS--PLTQGT---------
BlyA 237 TVVEKVKVGSAYQYKVKNSKGTTYYITASDKYVDVTGSVKTSSSAPKTTSTSSSSSSIKS
                                           *        *

CwlA 208 --KVTQIQKALAALYFY-PDKG-AKNNGIDGVYGPKTAD-AIRRFQSMYGLTQDGIYGPK
XlyA 233 --HVFQVQRALAALYFY-PDKG-AVNNGIDGVYGPKTAD-AVARFQSVNGLTADGIYGPA
CwlH 186 --NILQLQKALAVLHFY-PDKG-AKNNGIDGVYGPKTAN-AVKRFQLMNGLTADGIYGPK
XlyB 254 --KVKQVQTALAALYFY-PDKG-AKNHGVDGVYGPKTAN-AVKRFQSVSGLTADGIYGPK
BlyA 297 VGKIKIVGVSSAAIVMDKPDRNSSKNIGTVKLGSTISISGSVKGKNNSNGYW EVIYKGK
                           **       *                              **

CwlA 263 TKAKLEALLK-
XlyA 288 TKEKIAAQLS-
CwlH 241 TKAKLKSKLK-
XlyB 309 TKAKMEEKL--
BlyA 356 RGYISGQFGSTI
```

FIG. 8. Alignment of amino acid sequences of class I *N*-acetylmuramoyl-L-alanine amidases. CwlA is a deduced phage lysin (17, 27), XlyA and XlyB are phage lysins from PBS1 (24, 5), and BlyA is a phage lysin from SPβ (36). Asterisks indicate identical amino acids among the five amidases in class I. Dashes indicate the introduction of gaps in the alignment, and numbers on the left are with respect to the N-terminal amino acid of each amidase.

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 CwlH.

Role of CwlH in cell morphology. Cell wall hydrolases play various roles in cell morphology, but the role of CwlH has not been determined. CwlH disruption affected neither vegetative cell growth, sporulation frequency, nor germination frequency (31). Since the *cwlH* gene is expressed during the late stage of sporulation, we investigated the sporulation and germination more precisely with multiple combinations of mutations of cell wall hydrolase genes. Mother cell lysis was blocked strongly by the *cwlH cwlC* 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 CwlH and CwlC is very similar to that of CwlB and CwlC (Fig. 7) reported by Smith and Foster (47).

DISCUSSION

The CwlH protein exhibited extensive amino acid sequence similarity with class I (CwlA type) *N*-acetylmuramoyl-L-alanine amidases (Fig. 8). XlyA, XlyB, and BlyA are autolysins of phages PBS1 and SPβ (24, 36, 5). CwlA 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, CwlH plays a role in mother cell lysis in concert with CwlC, and this is exceptional in class I, because CwlH 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. CwlH exhibits a broad substrate specificity, including hydrolyzing activity for spore cortex (31), which was also found for CwlA and CwlC (9, 16). It is interesting that CwlH is able to hydrolyze immature cortex without muramic acid lactam (31).

The *cwlH* gene is transcribed by $E\sigma^K$ RNA polymerase and affected by GerE (Fig. 4). The *cwlC* gene is also transcribed by $E\sigma^K$ RNA polymerase and affected by GerE (43), and the gene product also plays a role in mother cell lysis in concert with CwlH or CwlB (Fig. 7). The major autolysin (CwlB [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, CwlC is the major hydrolytic enzyme, as judged by zymography, CwlB and CwlH 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 CwlC in concert with CwlB or CwlH 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, CwlB (3, 18, 46), and greatly by four autolysins, including minor ones (CwlF [LytE] and CwlE [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-RFTF96L00105 from the Japan Society for the Promotion of Science.

REFERENCES

1. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirement for transformation in *Bacillus subtilis*. J. Bacteriol. **81:**741–746.
2. **Ayusawa, D., Y. Yoneda, K. Yamane, and B. Maruo.** 1975. Pleiotropic phenomena in autolytic enzyme(s) content, flagellation, and simultaneous hyperproduction of extracellular α-amylase and protease in a *Bacillus subtilis* mutant. J. Bacteriol. **124:**459–469.
3. **Blackman, S., T. J. Smith, and S. J. Foster.** 1998. The role of autolysins during vegetative growth of *Bacillus subtilis* 168. Microbiology **144:**73–82.
4. **Canosi, U., G. Morelli, and T. A. Trautner.** 1978. The relationship between molecular structure and transformation efficiency of some *Streptococcus aureus* plasmids isolated from *Bacillus subtilis*. Mol. Gen. Genet. **166:**259–267.
5. **da Silva, E., P. F. Longchamp, and D. Karamata.** 1997. Identification of XlyB, the second lytic enzyme of the defective prophage PBSX, abstr. B25. *In* Abstracts of the 9th International Conference on Bacilli. Universite de Lausanne, Lausanne, Switzerland.
6. **Doyle, R. J., and A. L. Koch.** 1987. The functions of autolysins in the growth and division of *Bacillus subtilis*. Crit. Rev. Microbiol. **15:**169–222.
7. **Fein, J. E.** 1979. Possible involvement of bacterial autolysin enzymes in flagellar morphogenesis. J. Bacteriol. **137:**933–946.
8. **Fein, J. E., and H. J. Rogers.** 1976. Autolytic enzyme-deficient mutants of *Bacillus subtilis* 168. J. Bacteriol. **127:**1427–1442.
9. **Foster, S. J.** 1991. Cloning, expression, sequence analysis and biochemical characterization of an autolytic amidase of *Bacillus subtilis* 168 *trpC2*. J. Gen. Microbiol. **137:**1987–1998.
10. **Foster, S. J.** 1992. Analysis of the autolysins of *Bacillus subtilis* 168 during vegetative growth and differentiation by using renaturing polyacrylamide gel electrophoresis. J. Bacteriol. **174:**464–470.
11. **Haldenwang, W. G.** 1995. The sigma factors of *Bacillus subtilis*. Microbiol. Rev. **59:**1–30.
12. **Herbold, D. R., and L. Glaser.** 1975. *Bacillus subtilis N*-acetylmuramic acid L-alanine amidase. J. Biol. Chem. **250:**1676–1682.
13. **Höltje, J.-V., and E. I. Tuomanen.** 1991. The murein hydrolases of *Escherichia coli*: properties, functions and impact on the course of infections *in vivo*. J. Gen. Microbiol. **137:**441–454.
14. **Ishikawa, S., Y. Hara, R. Ohnishi, and J. Sekiguchi.** 1998. Regulation of a new cell wall hydrolase gene, *cwlF*, which affects cell separation in *Bacillus subtilis*. J. Bacteriol. **180:**2549–2555.
15. **Ishikawa, S., K. Yamane, and J. Sekiguchi.** 1998. Regulation and characterization of a newly deduced cell wall hydrolase gene (*cwlJ*) which affects germination of *Bacillus subtilis* spores. J. Bacteriol. **180:**1375–1380.
16. **Kuroda, A., Y. Asami, and J. Sekiguchi.** 1993. Molecular cloning of a sporulation-specific cell wall hydrolase gene of *Bacillus subtilis*. J. Bacteriol. **175:** 6260–6268.
17. **Kuroda, A., and J. Sekiguchi.** 1990. Cloning, sequencing and genetic mapping of a *Bacillus subtilis* cell wall hydrolase gene. J. Gen. Microbiol. **136:** 2209–2216.
18. **Kuroda, A., and J. Sekiguchi.** 1991. Molecular cloning and sequencing of a major *Bacillus subtilis* autolysin gene. J. Bacteriol. **173:**7304–7312.
19. **Kuroda, A., and J. Sekiguchi.** 1993. High-level transcription of the major *Bacillus subtilis* autolysin operon depends on expression of the sigma D gene and is affected by a *sin* (*flaD*) mutation. J. Bacteriol. **175:**795–801.
20. **Kunst, F., et al.** 1997. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. Nature **390:**249–256.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
22. **Lazarevic, V., P. Margot, B. Soldo, and D. Karamata.** 1992. Sequencing and analysis of the *Bacillus subtilis lytRABC* divergon: a regulatory unit encompassing the structural genes of the *N*-acetylmuramoyl-L-alanine amidase and its modifier. J. Gen. Microbiol. **138:**1949–1961.
23. **Leclerc, D., and A. Asselin.** 1989. Detection of bacterial cell wall hydrolases after denaturating polyacrylamide gel electrophoresis. Can. J. Microbiol. **35:**749–753.
24. **Longchamp, P. F., C. Mauël, and D. Karamata.** 1994. Lytic enzymes associated with defective prophages of *Bacillus subtilis*: sequencing and characterization of the region comprising the *N*-acetylmuramoyl-L-alanine amidase gene of prophage PBSX. Microbiology **140:**1855–1867.
25. **Margot, P., C. Mauël, and D. Karamata.** 1994. The gene of the *N*-acetyl-

glucosaminidase, a *Bacillus subtilis* cell wall hydrolase not involved in vegetative cell autolysis. Mol. Microbiol. **12:**535–545.
26. **Margot, P., M. Pagni, and D. Karamata.** 1999. *Bacillus subtilis* 168 gene *lytF* encodes a γ-D-glutamate-*meso*-diaminopimelate muropeptidase expressed by the alternative vegetative sigma factor, σ^D. Microbiology **145:**57–65.
27. **Margot, P., M. Wahlen, A. Gholamhuseinian, P. Piggot, and D. Karamata.** 1998. The *lytE* gene of *Bacillus subtilis* 168 encodes a cell wall hydrolase. J. Bacteriol. **180:**749–752.
28. **Mizuno, M., S. Masuda, K. Takemaru, S. Hosono, T. Sato, M. Takeuchi, and Y. Kobayashi.** 1996. Systematic sequencing of the 283 kb 210°–232° region of the *Bacillus subtilis* genome containing the *skin* element and many sporulation genes. Microbiology **142:**3103–3111.
29. **Moran, C. P., Jr.** 1993. RNA polymerase and transcription factors, p. 653–667. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington, D.C.
30. **Moriyama, R., A. Hattori, S. Miyata, S. Kudoh, and S. Makino.** 1996. A gene (*sleB*) encoding a spore cortex-lytic enzyme from *Bacillus subtilis* and response of the enzyme to L-alanine-mediated germination. J. Bacteriol. **178:** 6059–6063.
31. **Nugroho, F. A., H. Yamamoto, and J. Sekiguchi.** Unpublished data.
32. **Ohnishi, R., S. Ishikawa, and J. Sekiguchi.** 1999. Peptidoglycan hydrolase LytF plays a role in cell separation with CwlF during vegetative growth of *Bacillus subtilis*. J. Bacteriol. **181:**3178–3184.
33. **Pooley, H. M., and D. Karamata.** 1984. Genetic analysis of autolysin-deficient and flagellaless mutants of *Bacillus subtilis*. J. Bacteriol. **160:**1123–1129.
34. **Rashid, M. H., A. Kuroda, and J. Sekiguchi.** 1993. *Bacillus subtilis* mutant deficient in the major autolytic amidase and glucosaminidase is impaired in motility. FEMS Microbiol. Lett. **112:**135–140.
35. **Rashid, M. H., M. Mori, and J. Sekiguchi.** 1995. Glucosaminidase of *Bacillus subtilis*: cloning, regulation, primary structure and biochemical characterization. Microbiology **141:**2391–2404.
36. **Regamey, A., and D. Karamata.** 1998. The *N*-acetylmuramoyl-L-alanine amidase encoded by the *Bacillus subtilis* 168 prophage SPβ. Microbiology **144:** 885–893.
37. **Rogers, H. J., H. R. Perkins, and J. B. Ward.** 1980. Microbial cell walls and membranes. Chapman and Hall, London, England.
38. **Rogers, H. J., C. Taylor, S. Rayter, and J. B. Ward.** 1984. Purification and properties of autolytic endo-β-*N*-acetylglucosaminidase and the *N*-acetylmuramoyl-L-alanine amidase from *Bacillus subtilis* strain 168. J. Gen. Microbiol. **130:**2395–2402.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Schaeffer, P., J. Millet, and J. P. Aubert.** 1965. Catabolite repression of bacterial sporulation. Proc. Natl. Acad. Sci. USA **54:**704–711.
41. **Sekiguchi, J., K. Akeo, H. Yamamoto, F. K. Khasanov, J. C. Alonso, and A. Kuroda.** 1995. Nucleotide sequence and regulation of a new putative cell wall hydrolase gene, *cwlD*, which affects germination in *Bacillus subtilis*. J. Bacteriol. **177:**5582–5589.
42. **Sekiguchi, J., B. Ezaki, K. Kodama, and T. Akamatsu.** 1988. Molecular cloning of a gene affecting the autolysin level and flagellation in *Bacillus subtilis*. J. Gen. Microbiol. **134:**1611–1621.
43. **Sekiguchi, J., S. Ishikawa, and H. Yamamoto.** Unpublished data.
44. **Shimotsu, H., and D. J. Henner.** 1986. Modulation in *Bacillus subtilis* levansucrase gene expression by sucrose, and regulation of the steady-state mRNA level by *sacU* and *sacQ* genes. J. Bacteriol. **168:**380–388.
45. **Stockman, G. D., and J.-V. Höltje.** 1994. Microbial peptidoglycan (murein) hydrolases, p. 131–166. *In* J.-M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier, Amsterdam, The Netherlands.
46. **Smith, T. J., S. A. Blackman, and S. J. Foster.** 1996. Peptidoglycan hydrolases of *Bacillus subtilis* 168. Microb. Drug Resist. **2:**113–118.
47. **Smith, T. J., and S. J. Foster.** 1995. Characterization of the involvement of two compensatory autolysins in mother cell lysis during sporulation of *Bacillus subtilis* 168. J. Bacteriol. **177:**3855–3862.
48. **Vagner, V., E. Dervyn, and S. D. Ehrlich.** 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. Microbiology **144:**3097–3104.
49. **Yamanaka, K., J. Araki, M. Takano, and J. Sekiguchi.** 1977. Characterization of *Bacillus subtilis* mutants resistant to cold shock-induced autolysis. FEMS Microbiol. Lett. **150:**267–275.