JOURNAL OF BACTERIOLOGY, May 1999, p. 3178–3184 Vol. 181, No. 10
0021-9193/99/$04.00+0

Peptidoglycan Hydrolase LytF Plays a Role in Cell Separation with CwlF during Vegetative Growth of *Bacillus subtilis*

RYO OHNISHI, SHU ISHIKAWA, AND JUNICHI SEKIGUCHI*

Department of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, 3-15-1 Tokida, Ueda-shi, Nagano 386-8567, Japan

Received 28 December 1998/Accepted 5 March 1999

Peptidoglycan hydrolase, LytF (CwlE), was determined to be identical to YhdD (deduced cell wall binding protein) by zymography after insertional inactivation of the *yhdD* gene. YhdD exhibits high sequence similarity with CwlF (PapQ, LytE) and p60 of *Listeria monocytogenes*. The N-terminal region of YhdD has a signal sequence followed by five tandem repeated regions containing polyserine residues. The C-terminal region corresponds to the catalytic domain, because a truncated protein without the N-terminal region retained cell wall hydrolase activity. The histidine-tagged LytF protein produced in *Escherichia coli* cells hydrolyzed the linkage of D-γ-glutamyl-*meso*-diaminopimelic acid in murein peptides, indicating that it is a D,L-endopeptidase. Northern hybridization and primer extension analyses indicated that the *lytF* gene was transcribed by $E\sigma^D$ RNA polymerase. Disruption of *lytF* led to slightly filamentous cells, and a *lytF cwlF* double mutant exhibited extraordinary microfiber formation, which is similar to the cell morphology of the *cwlF sigD* mutant.

Bacillus subtilis produces peptidoglycan hydrolases, some of which are autolysins (34, 38). Two vegetative autolysins, a major 50-kDa *N*-acetylmuramoyl-L-alanine amidase (amidase, CwlB [LytC]) and a 90-kDa endo-β-*N*-acetylglucosaminidase (glucosaminidase, CwlG [LytD]), have been studied at the molecular level (20, 24, 26, 31). Recently, two minor autolysins produced during vegetative growth were reported (32). CwlF is a 35-kDa protein, and its production is unaffected by the sigma D and *flaD1* (*sinR*) mutations. The other one, CwlE (LytF), is a 50-kDa protein, and it is not produced by the *sigD* null mutant. CwlE (LytF) overlapped CwlB in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (32). Very recently, it was reported that CwlF is identical to PapQ and LytE (13, 28). The cells of the *cwlF*-deficient mutant were about twice as long as those of the wild-type strain, and the *cwlF sigD* double-mutant cells exhibited extraordinary microfiber formation (13). *B. subtilis* genome-sequencing analysis indicated the existence of many paralogs of cell wall hydrolases (17). One large group among the paralogs includes the cell wall-lytic enzyme, p60, of *Listeria monocytogenes* (4, 16), CwlF (PapQ, LytE), and YhdD.

In this study, we identified *yhdD* as a new peptidoglycan hydrolase gene, *cwlE* (*lytF*), expressed during the vegetative growth phase, characterized the gene expression, and determined the role of cell separation in *B. subtilis*. Moreover, we report that CwlE (LytF) is an endopeptidase which digests the linkage of D-γ-glutamyl-*meso*-diaminopimelic acid in muramic acid peptides.

(Preliminary data were presented at the International Conference on Bacilli, Japan [Osaka, Japan, 12 to 15 July 1998]. After the submission of this paper, Margot et al. [27] published the function of YhdD and designated the gene *lytF*.)

* 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.

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* 168 was the parent strain throughout this study, and mutants having the 168 background were constructed. *B. subtilis* was grown on Luria-Bertani (LB) agar medium (35) at 37°C for about 10 h and was then incubated in Schaeffer medium (36) at 30°C unless otherwise noted. When necessary, chloramphenicol, tetracycline, and erythromycin were added to the medium to final concentrations of 3, 5, and 0.3 μg/ml, respectively. *E. coli* was grown in LB medium (35) at 37°C. When necessary, ampicillin and kanamycin were added to final concentrations of 50 or 100 μg/ml and 25 μg/ml, respectively.

Plasmid construction. To construct a *B. subtilis lytF* (*yhdD*, *cwlE*) mutant, an internal fragment of the *lytF* gene was amplified by PCR with two primers, forward primer h-YHDD (5′-GCGCAAGCTTA_{30}*GCATCTGCGATTGT* CGG_{47}; the internal sequence of the *yhdD* (*lytF*, *cwlE*) region is italicized, the numbering is with respect to the first A of the translational start codon of *lytF*, and the *Hin*dIII site is underlined) and reverse primer b-YHDD (5′-GCGCGGATCCG_{275}*AACTTCCGCTCTTCATG*$_{258}$; the sequence complementary to the internal region of *lytF* is italicized, and the *Bam*HI site is underlined), with *B. subtilis* 168 DNA as a template. The PCR fragment was digested with *Hin*dIII and *Bam*HI. pMUTIN2 was also digested with *Hin*dIII and *Bam*HI and was then ligated to the digested PCR fragment, followed by the transformation of *E. coli* JM109. The resultant plasmid, pM2-HDD, was used for the transformation of *E. coli* C600 to produce concatemeric DNAs (6). To construct a *B. subtilis cwlF* (*papQ*, *lytE*) mutant, an internal fragment of the *cwlF* gene was amplified by PCR with two primers, forward primer cFSDBF (5′-GCGCGGATCCT_{-26}*AGAGTTAACATTTGGGGAG*$_{-7}$; the upstream sequence of *cwlF* is italicized, the numbering is with respect to the first A of the translational start codon of *cwlF*, and the *Bam*HI site is underlined) and reverse primer cFSDSR (5′-GCGCCCCGGGT_{1005}*TAGAATCTTTTCGCACCG*$_{987}$; the sequence complementary to the downstream sequence of *cwlF* is italicized, and the *Sma*I site is underlined), with *B. subtilis* 168 DNA as a template. The PCR fragment was digested with *Bam*HI and *Sma*I and was then ligated to *Bam*HI- and *Hin*cII-digested pUC118, followed by the transformation of *E. coli* JM109. The DNA of the resultant plasmid, pU8cF2, was digested with *Cla*I and *Spe*I, followed by ligation to the *Cla*I- and *Xba*I-digested pDG1515 DNA containing the tetracycline resistance gene. The resultant plasmid, pUCFTET, was used for the construction of *cwlF* mutants. To construct a *lytF* gene encoding a histidine-tagged protein (H-*lytF*), a region corresponding to the catalytic domain of LytF was amplified by PCR with forward primer BF-CWLE2 (5′-GCGCGGATCCA_{1105}*CGAGTGCGAAGATTAACAC*$_{1124}$; the *Bam*HI site is underlined) and reverse primer KR-CWLE (GCGCGGTACCC_{1529}*ATCAACGTCTTTAGGCT* CT_{1512}; the *Kpn*I site is underlined), with *B. subtilis* chromosomal DNA as a template. Then the amplified 445-bp fragment was digested with *Bam*HI and *Kpn*I, followed by ligation to the corresponding sites of pUC118. After the transformation of *E. coli* JM109, an ampicillin-resistant plasmid, pUCEtCTD, was extracted from the transformant. After reconfirmation of the sequence, the *Bam*HI-*Kpn*I fragment of pUCEtCTD was ligated into the corresponding site of a histidine-tag-encoding plasmid, pQE-30 (Qiagen), followed by the transformation of *E. coli* M15(pREP4). *E. coli* cells harboring the resultant plasmid,

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relative genotype(s)	Source or reference[a]
B. subtilis		
168	*trpC2*	42
168S	*trpC2 strA smo-1*	20
327SDC	*purB his-1 smo-1 sigD*::*cat*	M. H. Rashid
327SD1	*purB his-1 smo-1 sigD*::*tet*	M. H. Rashid
168SDC	*trpC2 sigD*::*cat*	327SDC→168
168SD1	*trpC2 sigD*::*tet*	327SD1→168
FTD	*trpC2 cwlF* (*papQ*)::*tet*	pUCFTET→168
FTDSDC	*trpC2 cwlF* (*papQ*)::*tet sigD*::*cat*	pUCFTET→168SDC
AN8	*purB his-1 smo-1 cwlB*::*cat*	A. Kuroda
EN8	*trpC2 cwlB*::*cat*	AN8→168
ED	*trpC2 yhdD* (*lytF*, *cwlE*)::*erm*	pM2-HDD→168
FED	*trpC2 cwlF* (*papQ*)::*tet yhdD* (*lytF*, *cwlE*)::*erm*	ED→168FTD
BED	*trpC2 cwlB*::*cat yhdD* (*lytF*, *cwlE*)::*erm*	AN8→ED
M. luteus ATCC 4698		Sigma
E. coli		
JM109	*recA1 endA1 gyrA96 thi-1 hsdR17 relA1 supE44* Δ(*lac-proAB*)/F′ [*traD36 proAB lacI*^{q} *lacZ* ΔM15]	Takara
C600	*supE44 hsdR17 thi-1 thr-1 leuB6 lacY1 tonA21*	Laboratory stock
M15	Nal^s Str^s Rif^s *lac ara gal mtl* F$^-$ *recA*$^+$ *uvr*$^+$	Qiagen
Plasmids		
pUC118/119	*lacZ bla*	Takara
pMUTIN2	*lacZ lacI erm bla*	42
pDG1515	*lacZ bla tet*	BGSC
pQE-30	*bla cat*	Qiagen
pREP4	*lacI kan*	Qiagen
pM2-HDD	*lacZ lacI erm bla* Δ*yhdD* (*lytF*, *cwlE*)	This study
pU8cF2	*lacZ bla cwlF* (*papQ*)	This study
pUCFTET	*lacZ bla cwlF* (*papQ*)::*tet*	This study
pUCEtCTD	*lacZ bla yhdD* (*lytF*, *cwlE*)	This study
pQECEtCTD	*bla cat* H-*lytF*	This study

[a] Arrows indicate construction by transformation. BGSC, *Bacillus* Genetic Stock Center, the Ohio State University.

pQECEtCTD, were used for the production of H-LytF (134 amino acids, including a 12-histidine-tagged amino acid sequence; M_r, 14,616).

Mutant construction. To construct isogenic strains, DNAs from *B. subtilis* 327SD1 (32) and 327SDC (33) were used for the transformation of *B. subtilis* 168; the resultant strains, 168SD1 and 168SDC, were selected with tetracycline and chloramphenicol, respectively. *B. subtilis* EN8 was also constructed through the transformation of *B. subtilis* 168 with *B. subtilis* AN8 DNA. For the construction of *cwlF* and *cwlF sigD* mutants, *B. subtilis* 168 and 168SDC were transformed with *Sca*I-digested pUCFTET DNA and transformants (FTD and FTDSDC) were selected with tetracycline. To obtain a *lytF* mutant, *B. subtilis* 168 was transformed with pM2-HDD DNA and a transformant (ED) was selected with erythromycin. To obtain a *lytF cwlF* mutant, *B. subtilis* FTD was transformed with *B. subtilis* ED DNA and a transformant (FED) was selected with erythromycin. To obtain a *lytF cwlB* mutant, *B. subtilis* ED was transformed with *B. subtilis* AN8 DNA and a transformant (BED) was selected with chloramphenicol. All of the mutants constructed in this study were confirmed to be properly constructed by PCR or Southern blot analysis.

Transformation of *E. coli* and *B. subtilis*. *E. coli* transformation was performed as described by Sambrook et al. (35), and *B. subtilis* transformation was performed by the competent cell method (1).

Preparation of cell wall binding proteins. To prepare cell wall binding proteins, *B. subtilis* 168, EN8, and BED cells were cultured in modified Spizizen medium (32) at 37°C to an optical density at 600 nm (OD_{600}) of 1.5 to 1.8. Then cultures (40 ml each) were centrifuged at 8,000 × *g* for 5 min at 4°C, and the cells were resuspended in distilled water, followed by the addition of SDS-PAGE sample buffer as described previously (32). The cell suspensions were then boiled for 5 min at 100°C, and the cells were removed by centrifugation. The supernatants were used as SDS-extracted samples (extract S).

Preparation of cell walls. *B. subtilis* 168S and *Micrococcus luteus* ATCC 4698 cell walls were prepared as described previously (18). For determination of the cleavage site of the enzyme, the partially purified *B. subtilis* cell walls were incubated in a 10% trichloroacetic acid solution at 4°C for 2 days. After a washing with deionized water, the cell walls were suspended in 0.1 M Tris-HCl (pH 7.5) containing α-amylase (0.1 mg/ml) and were then incubated at 37°C for 2 h. Then $CaCl_2$ and trypsin were added to final concentrations of 10 mM and 0.1 mg/ml, respectively, followed by incubation at 37°C for 16 h. After the enzymatic reactions, SDS (final concentration, 1%) was added to the solution, followed by boiling for 15 min. After centrifugation, the purified cell wall peptidoglycan was washed with deionized water and 0.1 M EDTA and then with ultrapure water.

Zymography. Zymography was performed essentially as described previously (9, 25, 32), using an SDS-polyacrylamide (12 or 10%) gel (23) containing 0.1% (wt/vol) *B. subtilis* and *M. luteus* cell walls.

Production of H-LytF in *E. coli*. *E. coli* M15(pREP4, pQECEtCTD) was cultured in LB medium containing ampicillin, kanamycin, and 2% glucose at 37°C. When cell growth reached an OD_{600} of 0.7 to 0.9, isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration, 2 mM) was added to the culture. After a 30-min incubation, the cells were harvested by centrifugation and resuspended in a 10 mM imidazole NPB solution (10 mM imidazole and 0.5 M NaCl in 20 mM sodium phosphate buffer [pH 7.4]). After ultrasonication, the suspension was centrifuged and the supernatant was filtered through a 0.45-μm-pore-size membrane filter (Nalgene), followed by loading onto a HiTrap chelating column (1 ml of resin; Pharmacia). Then the column was washed with 20 ml of the above-described buffer, and H-LytF was eluted with 10 ml each of 60, 100, 150, 200, 250, 300, and 500 mM imidazole NPB solutions. Imidazole in the enzyme solutions was removed with a HiTrap desalting kit (Pharmacia).

Effect of pH on enzyme activity. For determination of the optimal pH of the cell wall hydrolase activity of H-LytF, the following buffers (20 mM) containing 100 mM KCl and *B. subtilis* cell wall (10 mg/ml) were used: citrate buffer for pHs 3.0, 4.0, 5.0, and 5.5; Good's buffer for pHs 5.5, 6.0, 6.5, 7.5, 8.5, 9.5, and 10.5; and phosphate buffer for pHs 10.5, 11.5, and 12.5. Purified H-LytF was added to the buffers to a final concentration of 10 μg per ml, followed by incubation at 37°C, and the decrease in OD_{540} was measured with a Shimadzu UV-1200 spectrometer. One unit of enzyme was defined as the amount of enzyme necessary to decrease the OD_{540} by 0.001 in 1 min.

Determination of the cleavage site of cell wall peptidoglycan. To determine the cleavage site of the H-LytF protein, *B. subtilis* cell wall peptidoglycan (3.3 mg) and the purified H-LytF protein (30 μg) were added to 10 ml of Good's buffer (20 mM MES [morpholinoethanesulfonic acid], pH 6.5) containing 100 mM KCl. After enzymatic reaction at 37°C for 0, 10, 20, or 60 min, 1.5-ml samples were boiled at 100°C for 10 min. After centrifugation, the supernatants (released fractions) were filtered through a membrane filter (0.45 μm pore size). For detection of free amino groups in the released fractions, samples (500 μl each) were mixed with 60 μl of 10% $K_2B_4O_7$ and 50 μl of 0.1 M 1-fluoro-2,4-dinitrobenzene and were then incubated for 45 min at 65°C in the dark. Then dinitrophenyl (DNP) derivatives were hydrolyzed in 4 M HCl for 12 h at 95 to 100°C. The hydrolyzed samples were dried under a vacuum and were then resuspended in 500 μl of a mixture (4:1, vol/vol) of buffer A (10% acetonitrile and 0.02 N acetic acid) and buffer B (90% acetonitrile and 0.02 N acetic acid). The hydrolyzed DNP compounds were analyzed by high-performance liquid chromatography (HPLC) on a reverse-phase column (Wakosil-II5 C_{18}; 4.0 by 250 mm; Wako, Kyoto, Japan). The release of free reducing groups during the enzymatic reaction

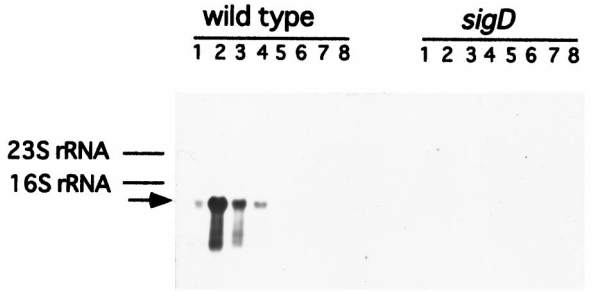

FIG. 1. Northern blot analysis of the *yhdD* region. Northern hybridization was performed with the *yhdD*-specific RNA probe as described in Materials and Methods. The lanes contained 10 μg of RNA from *B. subtilis* 168 (wild type) and 168SD1 (*sigD*) obtained at t_{-2} (lanes 1), t_{-1} (lanes 2), $t_{-0.5}$ (lanes 3), t_0 (lanes 4), $t_{1.5}$ (lanes 5), t_3 (lanes 6), $t_{4.5}$ (lanes 7), or t_6 (lanes 8). The arrow indicates a hybridizing RNA.

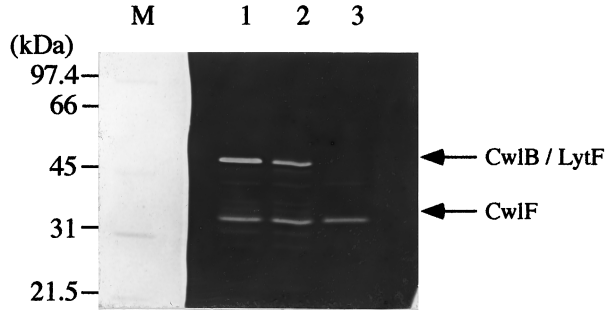

FIG. 2. Zymography of proteins from *B. subtilis* 168, EN8 (*cwlB*), and BED (*cwlB yhdD*) cells. Electrophoresis was performed in an SDS–10% polyacrylamide gel containing 0.1% (wt/vol) *B. subtilis* cell wall as a substrate. Samples were prepared and subjected to zymography as described in Materials and Methods. Equal amounts of proteins (equivalent to 10 OD_{600} units of cells) were applied to the lanes. Lane M contained the protein standards (Bio-Rad), the molecular masses of which are shown on the left. Lanes 1 to 3, extracts S of the 168, EN8, and BED strains, respectively.

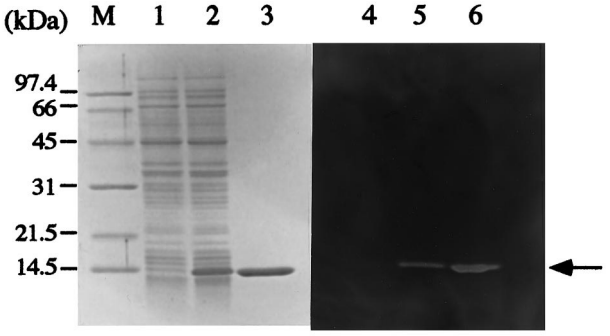

FIG. 3. SDS–12% PAGE and zymography of proteins produced by *E. coli* M15 harboring a *lacI* plasmid, pREP4, and pQECEtCTD containing H-*lytF*. Lanes M and 1 to 3, SDS-PAGE; lanes 4 to 6, zymography. M, protein standards; lanes 1 and 4, cell lysate without induction; lanes 2 and 5, cell lysate with a 30-min induction; lanes 3 and 6, purified protein after HiTrap column chromatography. Proteins equivalent to 0.2 OD_{660} unit of cells were applied to lanes 1, 2, 4, and 5. The purified protein (2 μg) was applied to lanes 3 and 6.

was assayed by the Thompson and Shockman (41) modification of the Park and Johnson method by using *N*-acetylglucosamine as the standard.

Northern blot and primer extension analyses. *B. subtilis* 168 and 168SD1 (OD_{600} of 15 to 20) cells cultured in Schaeffer medium were harvested at various times. RNA was prepared as described previously (14). Agarose-formaldehyde gel electrophoresis was performed as described by Sambrook et al. (35), and the transfer of the RNAs onto a nylon membrane was performed as described previously (14). The DNA fragment used for preparing an RNA probe was amplified by PCR with PM-FK (5′-CGGGGTACCG_{-113}*TGTGGAATTGTGAG* CG_{-97}; the pMUTIN2 sequence is italicized, the numbering is with respect to the first G of the translational start codon of *lacZ*, and the *Kpn*I site is underlined) and PM-T7 (5′-TAATACGACTCACTATAA_{-36}*GTGTATCAACAAG* $CTGG_{-53}$; the sequence complementary to the pMUTIN2 sequence is italicized, and the T7 promoter is underlined) as primers and with pM2-HDD DNA, containing the internal region of *lytF*, as a template. The amplified fragment was digested with *Hin*dIII, and then the fragments were purified by phenol-chloroform treatment, followed by precipitation with ethanol. The RNA probe was prepared with a 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 (14), using primer PEX-HDD (5′-GACCTTAATCGTTGCTGC; the 5′ and 3′ ends correspond to the complementary nucleotides at positions 93 and 76 with respect to the 5′ end of the *lytF* gene).

Microscopic observation and determination of cell density. Cells were shake cultured at 120 strokes per min in test tubes (17-mm diameter) containing 5 ml of LB medium at 37°C. The cell morphology was observed by phase-contrast microscopy. The OD_{600} was measured after strong vortexing of samples. In the case of *sigD cwlF* and *lytF cwlF* mutants, a small amount of lysozyme was added to the samples just before vortexing.

RESULTS AND DISCUSSION

The *B. subtilis* genome project has revealed the existence of many cell wall hydrolase homologs. Since there was a possibility that LytF (CwlE) corresponds to one of the homologs, we selected three candidates, i.e., an approximately 50-kDa polypeptide (YrvJ [518 amino acids], YhdD [488 amino acids], and YvcE [473 amino acids]) (17, 37). Among these candidates, complete loss of RNA expression by the *sigD* mutation was observed only for the *yhdD* gene on the Northern blot analysis with the internal region of *yhdD* as a probe (Fig. 1).

Identity of LytF (CwlE) to the *yhdD* gene product. Zymography of cell wall extract (extract S) proteins from the 168 (wild type), EN8 (*cwlB*), and BED (*cwlB lytF*) strains was carried out, and the results are shown in Fig. 2. The 50-kDa protein, having cell wall hydrolase activity, was present in smaller amounts in the EN8 strain (Fig. 2, lane 2) and was completely lacking in the BED strain (lane 3). Since CwlB is a 50-kDa protein, the activity band at 50 kDa in lane 2 corresponds to the activity of LytF, and disruption of the *yhdD* gene led to the loss of LytF activity. These results indicate that YhdD is identical to LytF.

Amino acid sequence similarity of LytF (YhdD, CwlE) with other proteins. The *lytF* (*yhdD*, *cwlE*) gene encodes a 488-amino-acid polypeptide with a molecular mass of 51,397 Da (17). LytF has three positively charged amino acids, K_2, K_3, and K_4, in the N-terminal region, followed by a hydrophobic core (from L_5 to G_{16}) and a deduced signal peptidase cleavage site ($A_{24}EA \downarrow A_{27}$; the arrow indicates the cleavage site). LytF also contains five tandem repeated regions with five polyserine regions and a C-terminal domain. The C-terminal domain, consisting of 118 amino acid residues, exhibits 67.0 and 45.2% identities over 115 amino acids with those of CwlF (13, 28) and the p60 protein (Iap) of *L. monocytogenes* (16, 43), respectively. The C-terminal region of LytF also exhibits high se-

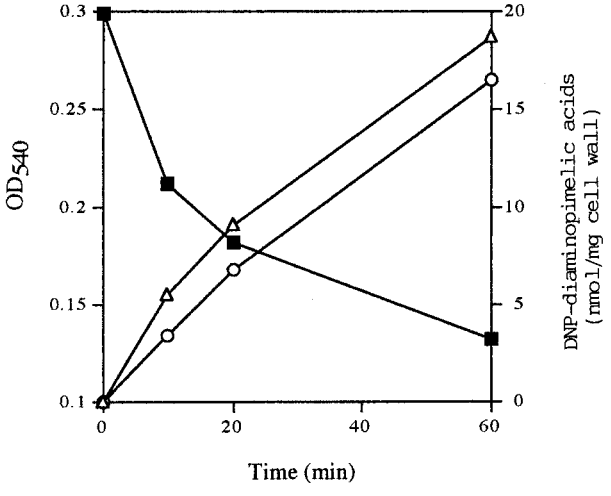

FIG. 4. Time course of the hydrolyzed compounds released from peptidoglycan with the H-LytF enzyme. The time course of the absorbance of peptidoglycan on H-LytF digestion is also shown. After 0-, 10-, 20-, and 60-min digestions of peptidoglycan, the reaction mixtures were centrifuged, and the amino groups of the released peptides and/or mucopeptides in the supernatants were labeled with 1-fluoro-2,4-dinitrobenzene. The labeled compounds were hydrolyzed with a high concentration of HCl, followed by separation by reverse-phase HPLC. Only the amounts of DNP derivatives of diaminopimelic acid were considerably increased during incubation. ■, cell wall turbidity; △, mono-dinitrophenyl diaminopimelic acid; ○, bis-dinitrophenyl diaminopimelic acid.

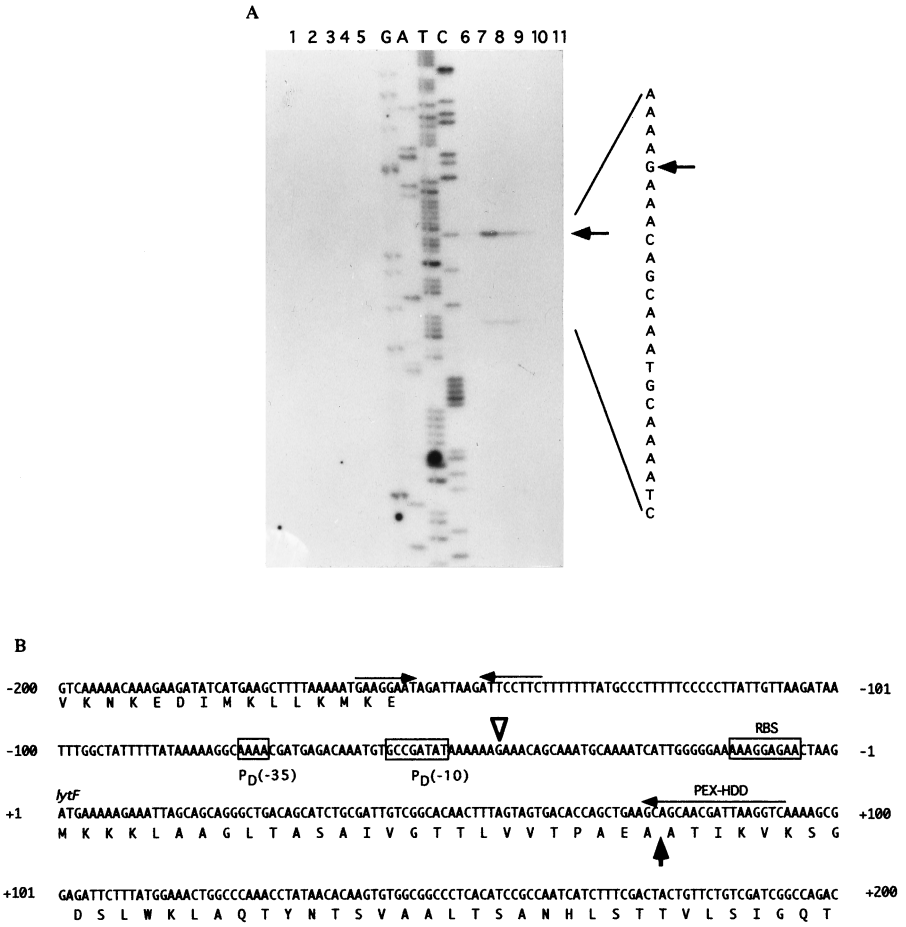

FIG. 5. (A) Determination of the 5′ end of the *lytF* transcript, by primer extension analysis (10 μg), isolated from the wild-type strain 168SD1 (*sigD*) at t_{-2} (lane 1), t_{-1} (lane 2), $t_{-0.5}$ (lane 3), t_0 (lane 4), or $t_{1.5}$ (lane 5) and from 168 (wild type) at t_{-2} (lane 6), t_{-1} (lane 7), $t_{-0.5}$ (lane 8), t_0 (lane 9), or $t_{1.5}$ (lane 10). Arrows indicate the transcriptional start site and the position of the product. The dideoxy DNA-sequencing reaction mixture with the same primer (PEX-HDD) was electrophoresed in parallel (lanes G, A, T, and C). (B) Nucleotide sequence of the putative promoter region of the *lytF* gene. The nucleotides are numbered with respect to the translational start point (+1) of *lytF*. P_D (−35) and P_D (−10) represent the −35 and −10 regions of the σ^D-like promoter. The open arrowhead indicates the transcriptional start site. PEX-HDD is the primer used for primer extension analysis. The rho-independent terminator ($\Delta G = -15.3$ kcal/mol) is indicated by opposing arrows. The thick arrow indicates the deduced signal sequence cleavage site. RBS, ribosome binding site.

quence similarity with the C-terminal regions of p60s from different *Listeria* species (4). *E. coli* NlpC and *Haemophilus influenzae* NlpC also exhibit high sequence similarities (35.8 and 33.9% identities over 123 and 115 amino acid residues, respectively) with the C-terminal domain of LytF (8, 15). Moreover, *Bacillus sphaericus* endopeptidase, EnpII, exhibits high sequence similarity (32.0% identity over 103 amino acids) with the C-terminal region of LytF (12). On the other hand, the repeated sequence in the N-terminal region of LytF exhibits its similarity with the repeated sequences in the C-terminal regions of *Lactococcus lactis* muramidase AcmA (5), *Streptococcus faecalis* autolysin (2), and *Enterococcus hirae* muramidase-2 (7). These three cell wall hydrolases contain regions showing high sequence similarities in their N termini, which encompass the active-site regions (5). The amino acid sequence of LytF indicates that it is the second example of a novel type of peptidoglycan hydrolase (probably endopeptidase) in *B. subtilis*.

Alignment of the amino acid sequences of LytF paralogs in *B. subtilis* indicated that the amino acid sequence of paralog YojL is entirely the same as those of LytF and CwlF and that the sequences of the four paralogs (YddH, YvcE, YkfC, and YwtD) are similar to the C-terminal catalytic domain of LytF (27, 29).

Production of the histidine-tagged catalytic domain of LytF in *E. coli*. When we constructed a histidine-tagged fusion with CwlF, *E. coli* cells harboring a plasmid containing the gene were dramatically lysed, and thus it was difficult to produce a significant amount of the protein (29). Moreover, the purified CwlF easily aggregated during preservation, but the truncated form, which lacked the N-terminal cell wall binding domain, did not aggregate under such conditions (29). Therefore, we prepared the catalytic domain of LytF fused with the histidine-tagged sequence (H-LytF). A considerable amount of H-LytF was produced in *E. coli* cells after a 30-min induction with IPTG (Fig. 3). The protein was purified on a nickel column, and the purified protein showed a single band in SDS-PAGE, corresponding to the cell wall hydrolyzing band observed by zymography (Fig. 3). The size of 14.5 kDa corresponds with that calculated from the amino acid sequence (M_r, 14,616). The optimal pH of the enzyme activity specific for *B. subtilis* cell wall was 6.5, and the specific activity was 1,560 U/mg of protein. Although H-LytF is a histidine-tagged truncated enzyme, this specific activity was comparable with those of CwlA

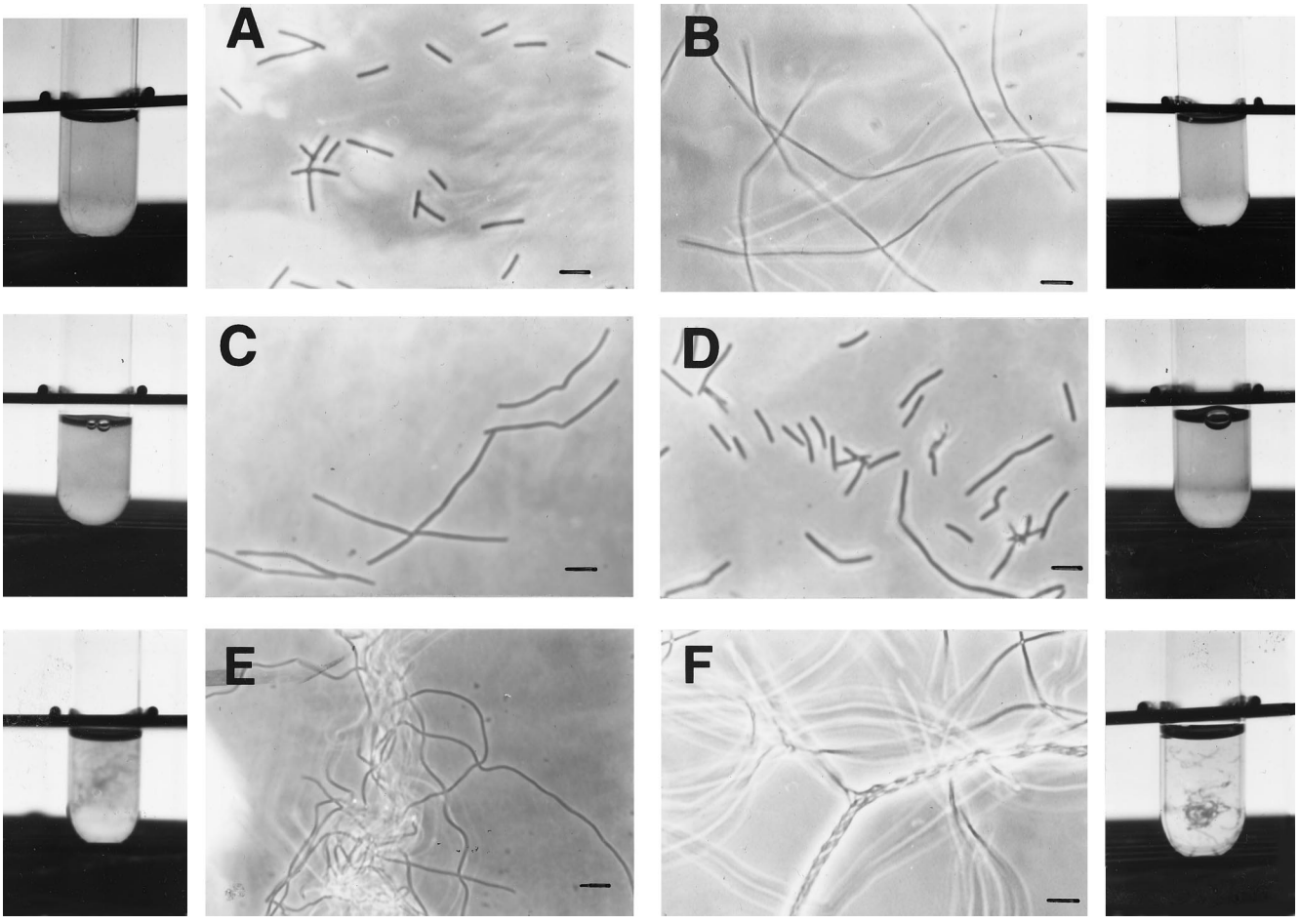

FIG. 6. Phase-contrast microscopy of *B. subtilis* 168 (wild type) (A), 168SDC (*sigD*) (B), ED (*lytF*) (C), FTD (*cwlF*) (D), FED (*lytF cwlF*) (E), and FTDSDC (*cwlF sigD*) (F) cells, as well as pictures of their corresponding test-tube cultures. The pictures of 168, 168SDC, ED, FTD, FED, and FTDSDC were taken at OD_{600}s of 0.475, 0.465, 0.550, 0.400, 0.462, and 0.457, respectively. *B. subtilis* FED (*lytF cwlF*) and FTDSDC (*cwlF sigD*) cells exhibited a superfilamentous morphology, and their cells looked like cotton waste in the test-tube cultures. Bar, 10 μm.

(2,500 U/mg of protein) (19) and CwlB (1,460 U/mg of protein) (20) but was much less than that of CwlG (26,000 U/mg of protein) (31). H-CwlF poorly digested *M. luteus* cell wall under conditions that were optimal for *B. subtilis* cell wall digestion. Among *B. subtilis* cell wall hydrolases, CwlA and CwlG (LytD) were able to digest *M. luteus* cell wall (21, 30), but CwlB (LytC) (30) did not digest *M. luteus* cell wall. Therefore, the poor activity of H-LytF for *M. luteus* cell wall is not a rare case, although the N-terminal region of LytF may affect the activity for *M. luteus* cell wall.

Determination of the peptidoglycan cleavage site of H-LytF. The purified peptidoglycan from *B. subtilis* cell wall was digested with H-LytF, but an increase in free reducing groups derived from peptidoglycan was not observed, thus indicating that the enzyme is neither an endo-*N*-acetylglucosaminidase nor an endo-*N*-acetylmuramidase. Moreover, the enzyme was not an *N*-acetylmuramoyl-L-alanine amidase (29), as determined by the method of Ghuysen et al. (10, 20). Since LytF exhibits high amino acid sequence similarity with *B. sphaericus* D-γ-glutamyl-*meso*-diaminopimelic acid endopeptidase II, free amino groups of the released compounds (supernatant fraction) derived from peptidoglycan after enzyme digestion were labeled with 1-fluoro-2,4-dinitrobenzene, followed by hydrolysis with 4 M HCl. The DNP-labeled and hydrolyzed compounds were separated by HPLC as described in Materials and Methods. After a 60-min digestion, the cell wall density was reduced by 57% and the amounts of mono-DNP-diaminopimelic acid and bis-DNP-diaminopimelic acid increased (Fig. 4). If the enzyme is assumed to be an endopeptidase which digests the D-alanine-*meso*-diaminopimelic acid crosslinkage, then only mono-DNP-diaminopimelic acid should be detected. However, both mono- and bis-DNP-diaminopimelic acid were formed, thus suggesting that the enzyme is a D-γ-glutamyl-*meso*-diaminopimelic acid endopeptidase.

Determination of the size and the 5′ end of *lytF* RNA. Northern blot analysis of RNAs from the wild type also showed that one transcription band hybridized to a probe containing the internal region of the *lytF* gene (Fig. 1). This transcript, estimated to be approximately 1.5 kb, was detected at t_{-2} to t_0, but not after $t_{1.5}$. Since *yhdD* comprises 1,464 bp, it was expressed as a monocistronic operon, and this result was supported by the existence of two deduced rho-independent terminators ($\Delta G = -15.3$ and -10.8 kcal/mol) just upstream and downstream of *yhdD* (*lytF*, *cwlE*) (17).

Primer extension analysis was performed with an oligonucleotide primer (PEX-HDD) that is complementary to the 5′ region of *lytF* (Fig. 5). A strong transcriptional signal starting at G_{-44} (the nucleotide being numbered with respect to the

translational start point [+1] of *lytF*) was observed with RNA from the wild-type cells at t_{-1} (Fig. 5A, lane 7), $t_{-0.5}$ (lane 8), and t_0 (lane 9). A weak signal starting at A_{-29} was also observed at t_{-1} (lane 7) and $t_{-0.5}$ (lane 8), but no signals were detected for the *sigD*-deficient mutant. When we used a different primer for the primer extension analysis, the weak signal was not found, thus suggesting that it is a misannealing one. From the similarities in the length and the timing of the appearance of the transcript, the strong primer extension product seemed to correspond to the 5′ end of the 1.5-kb RNA. The −35 region (AAAA) and the −10 region (GCCGATAT), with a spacing of 15 bp, were almost identical to those of the σ^D consensus sequence (TAAA for the −35 region and GCCGA TAT for the −10 region, with a spacing of 15 bp) (Fig. 5B) (11).

Cell morphology of the *lytF* and *lytF cwlF* disruptants. *B. subtilis* mutant cells which have deficiencies in the major autolysin gene (*cwlB*) and/or the glucosaminidase gene (*cwlG*) are rod shaped, while the *sigD* mutant forms filamentous cells, especially during exponential growth (20, 30, 32). Both autolysin genes are mainly transcribed by $E\sigma^D$ RNA polymerase (22, 24, 26, 31). These results suggest that an unknown gene regulated by SigD is important for cell morphology. Moreover, we reported that *cwlF* mutant cells were only twice as long as wild-type ones but that *cwlF sigD* mutant cells showed extraordinarily dense microfiber formation and looked like cotton waste in a transparent culture (13). Since *lytF* is regulated by SigD, we compared the cell morphology among six strains, including the *lytF*, *lytF cwlF*, *sigD*, and *cwlF sigD* mutants. The *lytF* mutant cells were approximately 4.6 and 3.4 times longer than the wild-type and *cwlF* mutant cells, respectively (41.1 ± 25.7 μm for ED, 8.9 ± 3.8 μm for 168, and 12.1 ± 5.8 μm for FTD) (Fig. 6). The *lytF cwlF* mutant (FED) cells showed extraordinarily dense fiber formation and looked like cotton waste, like the *cwlF sigD* (FTDSDC) cells (Fig. 6). These results indicate that the effect of the *sigD* deficiency depends mainly on the effect of the *lytF* deficiency. However, the morphological difference between ED and 168SDC was still present, because the filamentation of the ED strain was not as great as that of 168SDC (Fig. 6). Therefore, other autolysins regulated by SigD may still have minor effects on cell separation. Although LytF (CwlE) and CwlF mainly play roles in cell separation, LytF and CwlF in combination with other cell wall hydrolases are still important for cell separation in *B. subtilis*. Cell wall hydrolases AcmA, p60, and Atl are involved in the cell separation of *L. lactis*, *L. monocytogenes*, and *Staphylococcus aureus*, respectively (5, 43, 44). Atl is a bifunctional protein which has an amidase domain and a glucosaminidase domain, and it undergoes proteolytic processing into two extracellular cell wall hydrolases (amidase and glucosaminidase). These enzymes synergistically act on cell separation (40). Blackman and colleagues and Smith and colleagues reported that the *cwlB* (*lytC*) *sigD* double mutant and the *cwlB cwlG* (*lytD*) *sigD* triple mutant formed typical long chains and that the *cwlB cwlG* mutant also formed long chains (3, 39). But our results, obtained under the conditions used in this study, indicated that the *cwlB cwlG* mutant does not form long chains (Fig. 6). The difference is probably due to the culture conditions, because those researchers used very gentle shaking (35 or 45 rpm) for the culture (3, 39).

Genome sequencing of the *B. subtilis* chromosome indicated seven p60 paralogs, including LytF (CwlE) and CwlF. Therefore, research on the combinations of cell wall peptidases will definitely reveal their cellular functions. Moreover, the use of combinations of other types of cell wall hydrolases (amidase, D,D-endopeptidase, glucosaminidase, muramidase, and lytic transglycosylase) will be very valuable for elucidating their functions.

ACKNOWLEDGMENTS

This research was supported by grant JSPS-RFTF96L00105 from the Japan Society for the Promotion of Science.

We thank Yasuhiro Yamada for valuable help and suggestions on experimental analyses.

REFERENCES

1. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81:**741–746.
2. **Béliveau, C., C. Potvin, J. Trudel, A. Asselin, and G. Bellemare.** 1991. Cloning, sequencing, and expression in *Escherichia coli* of a *Streptococcus faecalis* autolysin. J. Bacteriol. **173:**5619–5623.
3. **Blackman, S. A., T. J. Smith, and S. J. Foster.** 1998. The role of autolysins during vegetative growth of *Bacillus subtilis* 168. Microbiology **144:**73–82.
4. **Bubert, A., M. Kuhn, W. Goebel, and S. Köhler.** 1992. Structural and functional properties of the p60 proteins from different *Listeria* species. J. Bacteriol. **174:**8166–8171.
5. **Buist, G., J. Kok, K. J. Leenhouts, M. Dabrowska, G. Venema, and A. J. Haandrikman.** 1995. Molecular cloning and nucleotide sequence of the gene encoding the major peptidoglycan hydrolase of *Lactococcus lactis*, a muramidase needed for cell separation. J. Bacteriol. **177:**1554–1563.
6. **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.
7. **Chu, C.-P., R. Kariyama, L. Daneo-Moore, and G. D. Shockman.** 1992. Cloning and sequence analysis of the muramidase-2 gene from *Enterococcus hirae*. J. Bacteriol. **174:**1619–1625.
8. **Fleischmann, R. D., et al.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
9. **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.
10. **Ghuysen, J.-M., D. J. Tipper, and J. L. Strominger.** 1966. Enzymes that degrade bacterial cell walls. Methods Enzymol. **8:**685–699.
11. **Haldenwang, W. G.** 1995. The sigma factors of *Bacillus subtilis*. Microbiol. Rev. **59:**1–30.
12. **Hourdou, M.-L., C. Duez, B. Joris, M.-J. Vacheron, M. Guinand, G. Michel, and J.-M. Ghuysen.** 1992. Cloning and nucleotide sequence of the gene encoding the γ-D-glutamyl-L-diamino acid endopeptidase II of *Bacillus sphaericus*. FEMS Microbiol. Lett. **91:**165–170.
13. **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.
14. **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.
15. **Kadner, R. J.** 1 November 1991, posting date. Sequences. [Online.] http://www.tokyo-center.genome.ad.jp. [5 April 1999, last date accessed.]
16. **Köhler, S., M. Leimeister-Wächter, T. Chakraborty, F. Lottspeich, and W. Goebel.** 1990. The gene coding for protein p60 of *Listeria monocytogenes* and its use as a specific probe for *Listeria monocytogenes*. Infect. Immun. **58:** 1943–1950.
17. **Kunst, F., et al.** 1997. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. Nature **390:**249–256.
18. **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.
19. **Kuroda, A., M. Imazeki, and J. Sekiguchi.** 1991. Purification and characterization of a cell wall hydrolase encoded by the *cwlA* gene of *Bacillus subtilis*. FEMS Microbiol. Lett. **81:**9–14.
20. **Kuroda, A., and J. Sekiguchi.** 1991. Molecular cloning and sequencing of a major *Bacillus subtilis* autolysin gene. J. Bacteriol. **173:**7304–7312.
21. **Kuroda, A., and J. Sekiguchi.** 1992. Characterization of the *Bacillus subtilis* CwbA protein which stimulates cell wall lytic amidases. FEMS Microbiol. Lett. **95:**109–114.
22. **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.
23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
24. **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.
25. **Leclerc, D., and A. Asselin.** 1989. Detection of bacterial cell wall hydrolases after denaturating polyacrylamide gel electrophoresis. Can. J. Microbiol. **35:**749–753.

26. **Margot, P., C. Mauël, and D. Karamata.** 1994. The gene of the *N*-acetylglucosaminidase, a *Bacillus subtilis* 168 cell wall hydrolase not involved in vegetative cell autolysis. Mol. Microbiol. **12:**535–545.
27. **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.
28. **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.
29. **Ohnishi, R., S. Ishikawa, and J. Sekiguchi.** Unpublished results.
30. **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.
31. **Rashid, M. H., M. Mori, and J. Sekiguchi.** 1995. Glucosaminidase of *Bacillus subtilis*: cloning, regulation, primary structure and biochemical characterization. Microbiology **141:**2391–2404.
32. **Rashid, M. H., N. Sato, and J. Sekiguchi.** 1995. Analysis of the minor autolysins of *Bacillus subtilis* during vegetative growth by zymography. FEMS Microbiol. Lett. **132:**131–137.
33. **Rashid, M. H., and J. Sekiguchi.** 1996. *flaD* (*sinR*) mutations affect SigD-dependent functions at multiple points in *Bacillus subtilis*. J. Bacteriol. **178:** 6640–6643.
34. **Rogers, H. J., H. R. Perkins, and J. B. Ward.** 1980. Microbial cell walls and membranes. Chapman and Hall, London, England.
35. **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.
36. **Schaeffer, P., J. Millet, and J. P. Aubert.** 1965. Catabolic repression of bacterial sporulation. Proc. Natl. Acad. Sci. USA **54:**704–711.
37. **Sekiguchi, J., S. Ishikawa, and R. Ohnishi.** Unpublished data.
38. **Shockman, 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.
39. **Smith, T. J., S. A. Blackman, and S. J. Foster.** 1996. Peptidoglycan hydrolases of *Bacillus subtilis* 168. Microb. Drug Resist. **2:**113–118.
40. **Sugai, M., H. Komatsuzawa, T. Akiyama, Y.-M. Hong, T. Oshida, Y. Miyake, T. Yamaguchi, and H. Suginaka.** 1995. Identification of endo-β-*N*-acetylglucosaminidase and *N*-acetylmuramyl-L-alanine amidase as cluster-dispersing enzymes in *Staphylococcus aureus*. J. Bacteriol. **177:**1491–1496.
41. **Thompson, J. S., and G. D. Shockman.** 1968. A modification of the Park and Johnson reducing sugar determination suitable for the assaying of insoluble materials: its application to bacterial cell walls. Anal. Biochem. **22:**260–268.
42. **Vagner, V., E. Dervyn, and S. D. Ehrlich.** 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. Microbiology **144:**3097–3104.
43. **Wuenscher, M. D., S. Köhler, A. Bubert, U. Gerike, and W. Goebel.** 1993. The *iap* gene of *Listeria monocytogenes* is essential for cell viability, and its gene product, p60, has bacteriolytic activity. J. Bacteriol. **175:**3491–3501.
44. **Yamada, S., M. Sugai, H. Komatsuzawa, S. Nakashima, T. Oshida, A. Matsumoto, and H. Suginaka.** 1996. An autolysin ring associated with cell separation of *Staphylococcus aureus*. J. Bacteriol. **178:**1565–1571.