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Regulation of a New Cell Wall Hydrolase Gene, *cwlF*, Which Affects Cell Separation in *Bacillus subtilis*

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Bacillus subtilis **produces a 35-kDa cell wall hydrolase, CwlF, during vegetative growth. The CwlF protein was extracted from *B. subtilis cwlB sigD* mutant cells and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. N-terminal amino acid sequencing revealed that its sequence is completely identical to that of the internal region of the *papQ* gene product. Disruption of the *papQ* gene in the *B. subtilis* chromosome led to the complete loss of CwlF, indicating that *papQ* is identical to *cwlF*. CwlF exhibits high sequence similarity to the p60 proteins of *Listeria* species, NlpC proteins of *Escherichia coli* and *Haemophilus influenzae*, and Enp2 protein of *Bacillus sphaericus*. The β-galactosidase activity of the *cwlF-lacZ* transcriptional fusion and Northern blot analysis of the *cwlF* gene indicated that the gene is expressed as a monocistronic operon during the exponential growth phase, and primer extension analysis suggested that the *cwlF* gene is transcribed mainly by EσA RNA polymerase and weakly by EσH RNA polymerase. While the cells of the *cwlF*-deficient mutant were about twice as long as those of the wild-type strain, the *cwlF sigD* double mutant cells exhibited extraordinary microfiber formation, in contrast to the filamentation of the *sigD* mutant. The CwlF production was not affected by the pleiotropic mutations *flaD1* and *degU32*(Hy), which endow cells with the ability of extensive filamentation.**

Bacillus subtilis produces a complement set of enzymes capable of hydrolyzing the shape-maintaining and stress-bearing peptidoglycan layer of its own cell wall (8, 43, 50). Some of these peptidoglycan hydrolases can trigger cell lysis; therefore, they can truly be called autolysins or suicide enzymes (43). 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 (3, 9, 10, 38, 43). Therefore, fine-tuning of autolysin activity through efficient and strict regulation is a must for bacterial survival (17).

Two major vegetative-phase autolysins (a 50-kDa *N*-acetylmuramoyl-L-alanine amidase [amidase], CwlB [LytC], and a 90-kDa endo-β-*N*-acetylglucosaminidase [glucosaminidase], CwlG [LytD]) were initially purified and characterized from *B. subtilis* (16, 44). The *cwlB* gene is part of an operon containing sequences encoding a putative lipoprotein and a modifier protein and containing the *cwlB* gene, in that order (25, 29). Transcription of this operon proceeds from a distal σ^A-type promoter and a proximal σ^D-type one, the latter transcript being predominant in the exponential growth phase (23, 26). The *cwlG* gene has also been cloned by two groups (33, 41), and it is transcribed mainly, as a monocistronic operon, by Eσ^D RNA polymerase (33, 41). A study on the physiological functions of CwlB and CwlG revealed that CwlB is responsible for cell lysis in the stationary phase (25) and after cold shock treatment (58) and that both proteins, but only in concert, are required for the motility function.

Several other amidase genes and their homologs have been cloned for the genus *Bacillus*. From *B. subtilis*, two prophage-borne amidase genes (*cwlA* and *xlyA*) (12, 24, 31), a sporulation-specific amidase gene (*cwlC*) (13, 22), a cortex maturation-specific and deduced amidase gene (*cwlD*) (48), and a germination-specific and deduced amidase gene (*sleB*) (35) have been cloned and studied, in addition to *cwlB* (25). Two amidase genes (*cwlM* and *cwlL*) from *Bacillus licheniformis*, a cell wall hydrolase (probably an amidase) gene from *Bacillus* species, and an amidase gene (*sleB*) from *Bacillus cereus* have been cloned and studied (27, 32, 36, 37, 39). Recently, the *cwlB cwlC* double mutant was found to be resistant to mother cell lysis during the late stage of sporulation (51). Evidence that many of these amidases are composed of a cell wall-specific and binding domain and a catalytic domain has accumulated (27). On the basis of the amino acid sequence similarity in their catalytic domains, these amidases can be classified into three groups. Class I includes CwlA, CwlL, XlyA, and *Bacillus* sp. amidase, class II includes CwlB, CwlC, CwlD, and CwlM, and class III includes the SleB proteins of *B. subtilis* and *B. cereus*. The cell wall-specific and binding domains of these amidases contain several (usually two or three) tandem repeated sequences. Interestingly, three tandem repeated sequences have also been observed in the noncatalytic cell wall-binding proteins CwbA (LytB) and WapA (14, 23).

The genome sequencing project on *B. subtilis* has revealed many cell wall hydrolase gene homologs (47). One of the homologs is the *papQ* gene, whose product was submitted to protein databases, as a phosphatase-associated and cell wall turnover-related protein precursor, by Whalen and Piggot (55). The *papQ* gene encodes a 334-amino-acid polypeptide having a molecular mass of 35,455 Da.

In this study, we identified *papQ* as a new cell wall hydrolase gene, *cwlF*, during the vegetative growth phase of *B. subtilis*, characterized the gene expression, and determined the cell morphology of the *cwlF* and *cwlF sigD* mutants.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
B. subtilis		
168	*trpC2*	D. Ehrlich
1A165	*trpC2 degU32*(Hy)	BGSC[a]
AC327	*purB his-1 smo-1*	1
AC334	*purB his-1 smo-1 flaD1*(*sinR*)	26
327SD1	*purB his-1 smo-1 sigD*::*tet*	42
AN8SD1	*purB his-1 smo-1 sigD*::*tet cwlB*::*cat*	42
EFD	*trpC2 papQ*(*cwlF*)::pM2cF	This study
327FD	*purB his-1 smo-1 papQ*(*cwlF*)::pM2cF	This study
SD1F	*purB his-1 smo-1 sigD*::*tet papQ*(*cwlF*):: pM2cF	This study
ABDF	*purB his-1 smo-1 sigD*::*tet cwlB*::*cat papQ*(*cwlF*)::pM2cF	This study
327UH	*his-1 smo-1 degU32*(Hy)	53
E. coli		
JM109	*recA1* Δ(*lac-proAB*) *endA1 gyrA96 thi-1 hsdR17 relA1 supE44* [F′:*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	Takara
C600	*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21*	Laboratory stock
Plasmids		
pMUTin2	*lacZ lacI bla erm*	D. Ehrlich
pM2cF	*lacZ lacI bla erm* Δ*papQ*(*cwlF*)	This study
pGEM-3Zf(+)	*lacZ bla*	Promega
pGEMΔES	*lacZ bla*	This study
pGEMΔES-cwlF	*lacZ bla*	This study

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

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains of *B. subtilis* and *Escherichia coli* and the plasmids used in this study are described in Table 1. *B. subtilis* was grown on nutrient agar medium (Difco) at 30°C for 10 to 12 h and then inoculated into DSM (Schaeffer) medium (46), followed by a shake culture at 37°C. If necessary, tetracycline, chloramphenicol, and erythromycin were added to the medium to final concentrations of 15, 8, and 0.3 μg/ml, respectively. *E. coli* was grown in Luria-Bertani (LB) medium (45) at 37°C. If necessary, ampicillin was added to a final concentration of 50 or 100 μg/ml.

Preparation of the CwlF protein. *B. subtilis* cells were harvested at various times, followed by washing with T buffer (25 mM Tris-HCl, pH 7.2, containing 1 mM phenylmethylsulfonyl fluoride). Then the cells were suspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (28), followed by boiling for 10 min. The suspensions were centrifuged at 11,000 × *g* for 5 min, and the supernatants (extract S [42]) were used as samples for SDS-PAGE and zymography.

Preparation of cell wall. Cell wall of *B. subtilis* 168S was prepared essentially as described previously (10, 24).

SDS-PAGE and zymography. SDS-PAGE of proteins was performed in 10 or 12% (wt/vol) polyacrylamide gels as described by Laemmli (28). Zymography was performed essentially as described by Leclerc and Asselin (30), using SDS-polyacrylamide gels containing 0.1% (wt/vol) *B. subtilis* cell wall as a substrate.

N-terminal amino acid sequence. After SDS-PAGE, peptides in the gel were transferred to a polyvinylidene difluoride membrane (Millipore) and then the gel was stained with Coomassie brilliant blue as described previously (25). The N-terminal amino acid sequence of the CwlF protein was determined with an automatic protein sequencer (model LF-3000; Beckman).

Plasmid construction. To remove extra cloning sites, pGEM-3Zf(+) was digested with *Eco*RI and *Sma*I, blunt ended with mung bean nuclease, and then self-ligated. The resultant plasmid was designated pGEMΔES. To construct a *B. subtilis cwlF* mutant, an internal fragment in the *cwlF* gene was amplified by PCR with two primers, cFHF forward primer (5′-GCCGAAGCTTC_{15}*ATTACA GCTACGACAGC*$_{32}$; the internal sequence of the *cwlF* region is italicized, the numbers are with respect to the first A of the translational start codon of *cwlF*, and the *Hin*dIII site is underlined) and cFBR reverse primer (5′-GCGCGGAT CCA_{250}*GGAAGAAGAACTGCTGC*$_{233}$; the sequence complementary to the internal region of *cwlF* is italicized and the *Bam*HI site is underlined), and *B. subtilis* 168 DNA as a template. The PCR fragment was digested with *Hin*dIII and *Bam*HI. Plasmids pMUTin2 and pGEMΔES were digested with *Hin*dIII and *Bam*HI and then ligated into the digested PCR fragment. Transformation of *E. coli* C600 and JM109 was performed with the former and latter ligated solutions, respectively. The resulting pM2cF DNA was used for the transformation of *B. subtilis* strains, and the resulting pGEMΔES-cwlF DNA was used for Northern blot analysis. Insertion of the PCR fragment into pMUTin2 was confirmed by PCR with the primer PM-FK (5′-CGGGGTACCG_{-113}*TGTGGAATTGTGA GCG*$_{-97}$; the pMUTin2 sequence is italicized, the numbers are with respect to the first G of the translational start codon of *lacZ*, and the *Kpn*I site is underlined) and primer PM-RX2 (5′-CCGCTCGAGG_{64}*ATTAAGTTGGGTAA CGC*$_{47}$; the numbers are with respect to the complementary base of the first G of the translational start codon of *lacZ*, and the *Xho*I site is underlined).

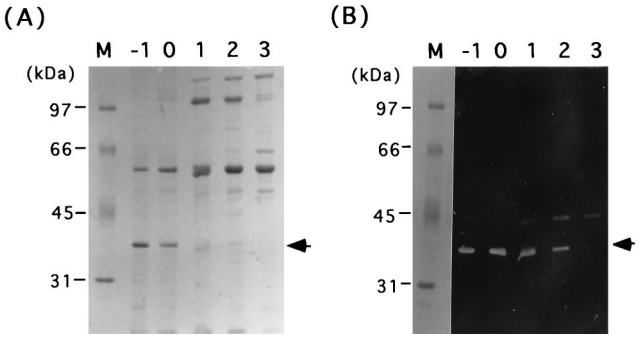

FIG. 1. Time course of production of CwlF. SDS-PAGE (A) and zymography (B) of protein extracts of *B. subtilis* AN8SD1 (*sigD cwlF*) cultured in DSM medium at 37°C are shown. Electrophoresis was performed in SDS–10% polyacrylamide gels, and the zymographic gel (B) contained 0.1% (wt/vol) *B. subtilis* cell wall as the substrate. Extracts S were prepared, and protein amounts equivalent to 4 OD_{600} units of cell growth were applied to each lane. Lane M contained protein standards (Bio-Rad), the molecular masses of which are shown on the left. Lanes −1 to 3 correspond to t_{-1}, t_0, t_1, t_2, and t_3, respectively. The arrowheads indicate the CwlF protein.

Mutant construction. A *cwlF*-deficient mutant, EFD, was constructed by transformation of *B. subtilis* 168 with pM2cF. Disruption of the *cwlF* gene by means of Campbell-type recombination was confirmed by Southern hybridization analysis (45) with an RNA probe. To construct isogenic disruptants, DNA from the EFD strain was used for the transformation of AC327, 327SD1, and AN8SD1, and transformants were selected on LB agar plates containing erythromycin. Disruption of the *cwlF* gene in the resultant disruptants, 327FD, SD1F, and ABDF, was confirmed by Southern hybridization analysis.

Transformation of *E. coli* and *B. subtilis*. *E. coli* transformation was performed as described by Sambrook et al. (45), and *B. subtilis* transformation was performed by the competent-cell method (2).

β-Galactosidase assay. The β-galactosidase assay was performed basically as described by Shimotsu and Henner (49). One unit of β-galactosidase activity was defined as the amount of enzyme necessary to release 1 nmol of 2-nitrophenol from *o*-nitrophenyl-β-D-galactopyranoside (ONPG) in 1 min.

Northern blot and primer extension analyses. *B. subtilis* AC327 cells (optical density at 600 nm [OD_{600}] of 15) 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) (54). After centrifugation at 11,000 × *g* for 1 min at 4°C, the pellet was suspended in 1 ml of SET buffer containing lysozyme (final concentration, 6 mg/ml) (26). After incubation for 10 min at 0°C, the suspension was centrifuged at 11,000 × *g* for 1 min at 4°C. The pellet was used

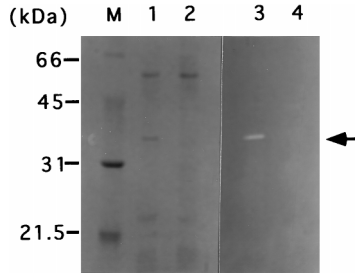

FIG. 2. SDS-PAGE and zymography of *B. subtilis* ABDF (*sigD cwlB cwlF*) and AN8SD1 (*sigD cwlB*). Electrophoresis was performed in an SDS–12% polyacrylamide gel. Proteins were stained with Coomassie brilliant blue (lanes 1 and 2) or subjected to zymography (lanes 3 and 4). Samples were prepared as described in the legend to Fig. 1. Lane M contained protein standards (Bio-Rad), the molecular masses of which are shown on the left. Lanes 1 and 3, extract S of AN8SD1; lanes 2 and 4, extract S of ABDF. The arrowhead indicates the CwlF protein.

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CwlF_BACSU    1    MKKQIITATTAVVLGSTLFAGAASAQSIKVKKGDTLWDLSRKYDTTIS
p60_LISMO     1   MNMKKATIAATAGIAVTAFAAPTIASASTVVVEAGDTLWGIAQSKGTTVD

CwlF_BACSU   49   KIKSENHLRSDIIYVGQTLSINGKSTSSKSSSSSSSSSTYKVKSGDSL--
p60_LISMO    51   AIKKANNLTTDKIVPGQKLQVNNEVAAAEKTEKSVSATWLNVRSGAGVDN

CwlF_BACSU   97   --------------------W-KISKKYGMT--IN--------------
p60_LISMO   101   SIITSIKGGTKVTVETTESNGWHKITYNDGKTGFVNGKYLTDKAVSTPVA

CwlF_BACSU  109   ---ELKKLNGLKSDLLRVGQVLKLKGSTSSSS-------SSSSKVSSSST
p60_LISMO   151   PTQEVKKETTTQQAAPAAETKTEVKQTTQATTPAPKVAETKETPVVDQNA

CwlF_BACSU  149   STYKVKSGDSLSKIASKYGTTVSKLKSLNGLKSDVIYVNQVLKVKGTSTS
p60_LISMO   201   TTHAVKSGDTIWALSVKYGVSVQDIMSWNNLSSSIYVGQKLAIKQTANT

CwlF_BACSU  199   SSKPASSSSSSSSKTSSTS----------------------------------
p60_LISMO   251   ATPKAEVKTEAPAAEKQAAPVVKENTNTNTATTEKKETATQQQTAPKAPT

CwlF_BACSU  215   ------------------------------------------------
p60_LISMO   301   EAAKPAPAPSTNTNANKTNTNTNTNTNTNNTNTNTPSKNTNTNSNTNTNT

CwlF_BACSU  216   ---------------LNVSKLVSDAKALVGTPYKWGGTTTSGFDCSGF-
p60_LISMO   351   NSNTNANQGSSNNNSNSSASAIIAEAQKHLGKAYSWGGNGPTTFDCSGY-
NLPC_ECOLI   31                -SITVIAGLNDQLQSWHGTPYRYGGMTRRGVDCSGF-
NLPC_HAEIN   64                -ALMGDKALASVYNEWVGTRYRMGGTTKRGIDCSAF-
ENP2_BACSH  147                -EHAFRENVVQTALSYIATPYRWGGKSPLGIDCSGL C
                                              *  **       ***

CwlF_BACSU  251   -IWYVLNKQ-TSVGRT-S--TAGYWSSMK---S-IASPSV--GDFVFFTT
p60_LISMO   400   -TKYVFAKAGISLPRT-S--GAQYASTTRISESQ-AKP----GDLVFFD-
NLPC_ECOLI   66   -VVVTMRDR-FDLQLP-R--ETKQQASIG---TQIDKDELLPGDLVFFKT
NLPC_HAEIN   99   -MQTTFSEV-FGIELPRS--TAEQRHLGR---K-INKSELKKGDLVFFRK
ENP2_BACSH  183   SMAYLLNG--VIIFRD-ARIVEGFPIKEI---T-IDRMQK--GDLLFF--
                                                    **  **

CwlF_BACSU  290   YKSGPS--HMGIYIGNNSFIHA--G-S-DGVQISSLNNSYWKPRYLGAKRF
p60_LISMO   440   YGSGIS--HVGIYVGNGQMINA--Q-D-NGVKYDNIHGSGWGKYLVGFGRV
NLPC_ECOLI  108   -GSGQNGLHVGIYDTNNQFIHA--STS-KGVMRSSLDNVYWQKNFWQARRI
NLPC_HAEIN  141   N----N--HVGVYIGNNQFMHA--S-TGQGVTISSLDEKYWARTYTQSRRI
ENP2_BACSH  222   ----PG--HVALYLGQTLYVHASLG-G-NEVNVNSLDEQH--PLYRQDLAT
                        *   *         *       *

NLPC_HAEIN  183   M
ENP2_BACSH  263   TITAIGSLF
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FIG. 3. Alignment of the amino acid sequences of cell wall hydrolases *B. subtilis* CwlF (CwlF_BACSU; 334 amino acids) (55), *L. monocytogenes* p60 (p60_LISMO; 484 amino acids) (21), and *B. sphaericus* Enp2 (ENP2_BACSH; 271 amino acids) (18) and the deduced lipoproteins *E. coli* NlpC (NLPC_ECOLI; 154 amino acids) (20) and *H. influenzae* NlpC (NLPC_HAEIN; 183 amino acids) (11). Amino acid identities are indicated by two types of shading, and amino acids identical among the five proteins are indicated by asterisks. Amino acids are numbered from the N termini of the proteins. Dashes indicate the introduction of gaps in the alignment, and arrows and double overlines indicate tandem repeated regions and polyserine regions in CwlF, respectively. The arrowhead indicates a deduced signal peptidase cleavage site.

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. (45). 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 (Takara) as primers and pGEMΔES-cwlF DNA, containing the internal region of *cwlF*, as a template. The amplified fragment was digested with *Hin*dIII, and then fragments were purified by phenol and chloroform treatments 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 (48), using the PEX-cF2 primer (5′-AGATCCC ATAACGTGTCG; the 5′ and 3′ ends correspond to the complementary nucleotides at positions 116 and 99 with respect to the 5′ end of the *cwlF* gene).

Microscopic observation and determination of cell density. Cells were shake cultured in a test tube (17-mm diameter) containing 5 ml of LB medium at 37°C. After 4, 6, and 8 h, 5-μl samples were mixed with 5 μl of 2% agarose on glass slides, 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 the *sigD cwlF* mutant cells after 6 h of incubation, a small amount of lysozyme was added to the samples just before vortexing.

RESULTS AND DISCUSSION

Production of a vegetative cell wall hydrolase, CwlF. Cell wall extracts of *B. subtilis* AC327 in the exponential growth phase in modified Spizizen medium gave two strong cell wall-hydrolyzing bands during zymography after electrophoresis on an SDS-polyacrylamide gel containing *B. subtilis* cell wall (42). One of the bands corresponded to the major autolysin, CwlB (50 kDa), which overlapped with that of a new cell wall hydrolase, CwlE. The new cell wall hydrolase, CwlF, has a molecular mass of 35 kDa, as judged by SDS-PAGE. A σ^D-deficient strain lost the ability to produce CwlE but not CwlF (42). Since it was expected that the *cwlB sigD*-deficient mutant would exhibit a reduction in the total amount of cell wall binding proteins other than CwlF, extract S of a *sigD cwlB*-deficient mutant, AN8SD1, was applied to an SDS–10% polyacrylamide gel containing 0.1% (wt/vol) *B. subtilis* cell wall. Figure 1 shows SDS-PAGE of the cell wall proteins. The 35-kDa protein band in panel A, corresponding to the cell wall-hydrolyzing band in panel B, was well separated from those of other proteins (Fig. 1A). Therefore, we prepared a sample at t_{-1} (1 h before the onset of sporulation) which was transferred to a polyvinylidene difluoride membrane. Then the N-terminal amino acid sequence of CwlF was determined to be QSIKVKKGDTLWDL SRKYDT.

Identity of CwlF to the *papQ* gene product. Comparison of the N-terminal amino acid sequence with those of proteins in a nonredundant protein database revealed that the sequence is completely identical to the internal 20-amino-acid sequence starting at position 26 (with respect to the N-terminal amino

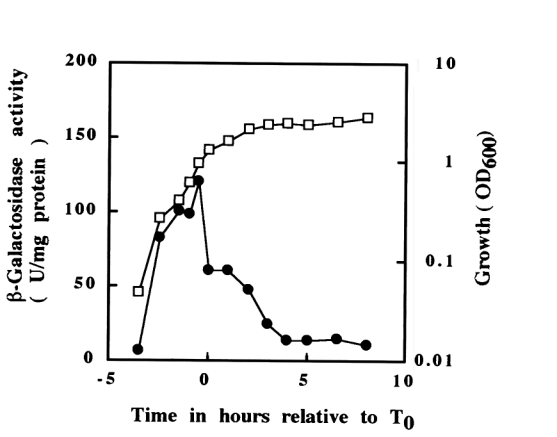

FIG. 4. (A) β-Galactosidase activity of the *cwlF-lacZ* transcriptional fusion strain 327FD. Open symbols indicate growth; closed symbols indicate β-galactosidase activity. t_0 is defined as the onset of sporulation. (B) Northern blot analysis with a *cwlF* RNA probe. Each lane contained 10 μg of total RNA isolated from *B. subtilis* AC327 at t_{-1}, t_0, t_1, t_2, t_3, $t_{4.5}$, and t_6 (lanes −1 to 6, respectively). The 23S and 16S rRNAs are 2.8 and 1.4 kb, respectively. The calculated size of the detected transcript is shown on the left.

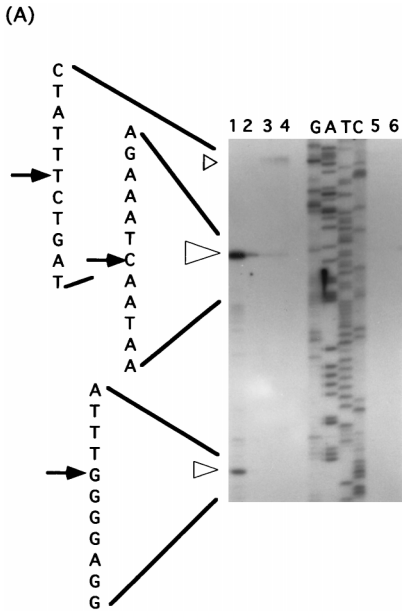

FIG. 5. (A) Determination of the 5′ end of the *cwlF* transcript, by primer extension analysis (25 μg), isolated from the wild-type strain, AC327, at t_{-1} (lane 1), t_0 (lane 2), t_1 (lane 3), t_2 (lane 4), t_3 (lane 5), and t_6 (lane 6). Open arrowheads indicate the three apparent transcriptional start sites. The dideoxy DNA sequencing reaction mixtures with the same primer (PEX-cF2) were electrophoresed in parallel (lanes G, A, T, and C). The positions of the products are indicated by arrows, and the sizes of arrowheads show the proportions of the activities of the three transcriptional start sites. (B) Nucleotide sequence of the putative promoter region of the *cwlF* gene. The nucleotides are numbered with respect to the translational start point (+1) of *cwlF*. P_A-35 and P_A-10 represent the −35 and −10 regions of a σ^A-like promoter, and P_H-35 and P_H-10 represent the −35 and −10 regions of a σ^H promoter. Arrowheads indicate the transcriptional start sites, and the sizes of the arrowheads indicate the proportions as described above. Dots indicate the N-terminal amino acid sequence of CwlF prepared from the culture. PEX-cF2 was the primer used for primer extension analysis. RBS, ribosome binding site. ρ-independent terminators are indicated by opposing arrows.

acid) of PapQ. The *papQ* gene encodes a 334-amino-acid polypeptide with a molecular mass of 35,455 Da (55). PapQ has two positively charged amino acids, K_2 and K_3 at the N terminus, followed by a hydrophobic core (from A_{11} to A_{23}) and a deduced signal peptidase cleavage site ($A_{23}SA \downarrow Q_{26}$ [the arrow indicates the cleavage site]). These results strongly suggest that the CwlF protein is identical to the PapQ protein. Therefore, the 236-bp internal region of the *papQ* gene was amplified by PCR with primers cFHF and cFBR and 168 DNA and then inserted into pMUTin2. The resultant plasmid, pM2cF, was introduced into strain 168, resulting in the EFD strain, and then the isogenic strains, AC327, 327SD1, and AN8SD1, were transformed with DNA of the EFD strain. Figure 2 shows SDS-PAGE and zymography of extract S proteins from the *papQ*-proficient AN8SD1 strain and *papQ*-deficient ABDF strain. The 35-kDa protein, having cell wall hydrolase activity, was completely lacking in the ABDF strain (Fig. 2, lanes 2 and 4). Moreover, the introduction of a plasmid containing the entire *papQ* gene into *E. coli* led to the appearance of a 35-kDa cell wall-hydrolytic band in zymography (19). These results definitely indicate that CwlF is identical to PapQ.

Amino acid sequence similarity of CwlF (PapQ) with other proteins. The CwlF (PapQ) protein comprises a 25-amino-acid signal peptide region, three tandem repeated regions with three polyserine regions, and a C-terminal domain (Fig. 3). The C-terminal domain consists of 119 amino acid residues and exhibits 40.3% identity over 119 amino acids with that of p60 protein (Iap) of *Listeria monocytogenes* (21). Moreover, there is significant similarity in their N-terminal regions (25.1% identity over 227 amino acids). The p60 protein is associated with virulence in the mouse model of infection and possesses murein hydrolase activity (56). The C-terminal region of the CwlF protein also exhibits a high degree of sequence similarity with the C-terminal regions of p60 proteins from the different *Listeria* species (5). *E. coli* NlpC, comprising 154 amino acid residues, and *Haemophilus influenzae* NlpC, comprising 183 amino acid residues, also exhibit high degrees of sequence similarity (40.7 and 38.3% over 108 and 115 amino acid residues, respectively) with the C-terminal domain of CwlF (Fig. 3) (11, 20). NlpCs contain lipoprotein motif. Moreover, *Bacillus sphaericus* endopeptidase, Enp2, comprising 271 amino acids, exhibits a high degree of sequence similarity (31.3% over 112 amino acids) with the C terminus of CwlF (18). On the other hand, the repeated sequence in the N-terminal region of CwlF exhibits similarity with the repeated ones in the C-terminal regions of *Lactococcus lactis* muramidase AcmA (6), *Streptococcus faecalis* autolysin (4), and *Enterococcus hirae* muramidase-2 (7). These three cell wall hydrolases contain regions with high degrees of sequence similarity in their N termini, which encompass the active-site regions (6). Therefore, CwlF is not an AcmA-type muramidase. The amino acid sequence of CwlF indicates that it is a novel type of cell wall hydrolase in *B. subtilis*.

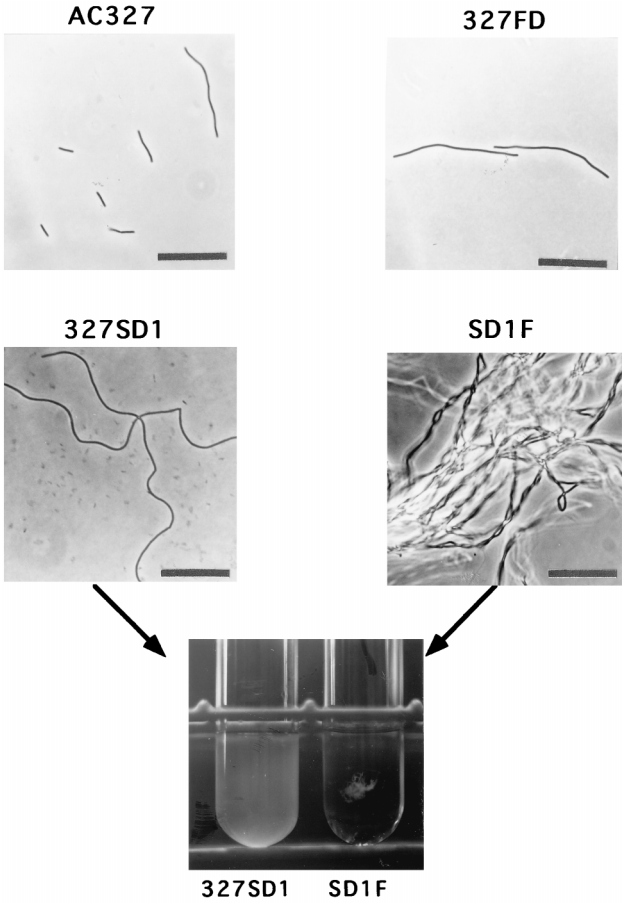

FIG. 6. Phase-contrast microscopy of AC327 (wild-type), 327FD (*cwlF*), 327SD1 (*sigD*), and SD1F (*cwlF sigD*) cells and a picture of the test tube cultures of 327SD1 and SD1F. The pictures of AC327, 327FD, 327SD1, and SD1F were taken at OD_{600}s of 0.263, 0.117, 0.146, and 0.205, respectively. Bar, 25 μm. To measure the OD_{600} of the SD1F strain, a small amount of lysozyme was added to the samples just before vigorous vortexing.

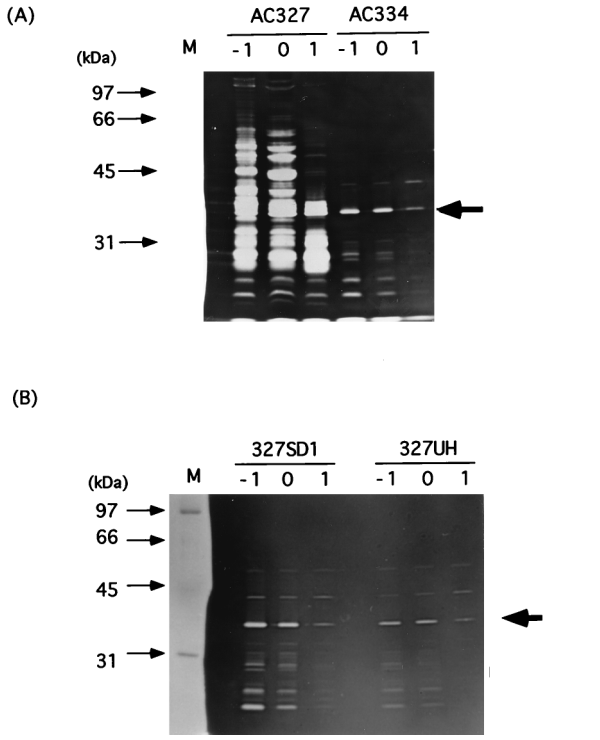

FIG. 7. Comparison of CwlF production by AC334 (*flaD1*), 327SD1 (*sigD*), 327UH [*degU32*(Hy)], and a parent strain, AC327 (wild type), by zymography. Extracts S at t_{-1} (lanes −1), t_0 (lanes 0), and t_1 (lanes 1) were prepared, and a protein amount equivalent to 3.6 OD_{600} units of cell growth was applied to each lane. Lane M contained protein standards (Bio-Rad), the molecular masses of which are shown on the left. The arrows indicate the CwlF protein.

Regulation of the *cwlF* gene. To study the expression of the *cwlF* gene in vivo, a *cwlF-lacZ* transcriptional fusion gene was constructed in the AC327 strain as described in Materials and Methods. Figure 4A shows the time courses of growth and expression of the *cwlF-lacZ* transcriptional fusion gene of the 327FD strain. The fusion gene expression started from the early growth phase, reaching a maximum at t_{-1}, and then sharply decreased. The parent strain, *B. subtilis* AC327, exhibited β-galactosidase activities of less than 10 U/mg of protein during the exponential phase and less than 15 U/mg of protein during the sporulation phase (19). The Northern blot analysis in Fig. 5B shows that one transcript hybridized to a probe containing the internal region of the *cwlF* gene. This transcript, estimated to be 1.1 kb, was detected at t_{-1} to t_1 but not after t_2. Since the intensity of the signal was highest at t_{-1}, *cwlF* is expressed as a monocistronic operon and may be transcribed by $E\sigma^A$ RNA polymerase.

Determination of the 5′ end of *cwlF* RNA. The *cwlF* gene is located near the *phoA* region, and the gene order is *phoA-cwlF* (*papQ*)*-citR*. *phoA* and *citR* are transcribed and translated in the same direction, but *cwlF* is transcribed and translated in a different direction (55). Two deduced ρ-independent terminators ($\Delta G = -10.2$ and -10.8 kcal/mol) are located between *phoA* and *cwlF*, and one terminator ($\Delta G = -24.3$ kcal/mol) is located between *cwlF* and *citR*. From the sequence information and the results of Northern blot analysis, it seemed likely that the 5′ end of *cwlF* RNA would be located upstream of *cwlF* and between *phoA* and *cwlF*. Primer extension analysis was performed with an oligonucleotide primer (PEX-cF2) that is complementary to the 5′ region of *cwlF* (bases 116 to 99) (Fig. 5A). A strong transcriptional signal starting at C_{-62} (the nucleotide is numbered with respect to the translational start point [+1] of *cwlF*) was observed with RNA from cells at t_{-1} (Fig. 5A, lane 1), t_0 (lane 2), and t_1 (lane 3). A signal with medium intensity, starting at G_{-12}, was observed at t_{-1}. Interestingly, a very weak but still significant signal, starting at T_{-93}, was observed at t_1 and t_2. From the similarities in length and in the timing of the appearance of transcripts, the strong primer extension product seemed to correspond to the 5′ end of the 1.1-kb RNA. The −35 region (TTCTGA) and −10 region (TATAAT), with a spacing of 14 bp, were similar to those of the σ^A consensus sequence (TTGACA for the −35 region and TATAAT for the −10 region, with a spacing of 17 bp; the underlined nucleotides are highly conserved) (Fig. 5B) (15, 34). The transcript, starting at G_{-12}, is estimated to be 1.05 kb in length, if the same ρ-independent terminator located between *cwlF* and *citR* is used. The −35 region (AAAGAGATA) and −10 region (AGAGT), with a spacing of 10 bp, were very similar to those of the σ^H consensus sequence (RWAGGA XXT for the −35 region and HGAAT for the −10 region, with a spacing of 14 bp; R = A or G; W = A, G, or C; X = A or T; and H = A or C) (15). The transcription of dual promoters was also found for those of the major autolysin gene, *cwlB* (σ^D

and σ^A) (26, 29), and a cortex maturation gene, *cwlD* (σ^E and σ^G) (48).

Cell morphology of the *cwlF* and *cwlF sigD* 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 (25, 40, 42). Both autolysin genes were mainly transcribed by $E\sigma^D$ RNA polymerase, and the *sigD* mutation led to less than 13% of the wild-type *cwlB* expression (26) and less than 8% of the wild-type *cwlG* expression (41). These results suggest that an unknown gene regulated by SigD is important for cell morphology. Although the *cwlF* gene is not regulated by SigD, we compared the morphology of the *cwlF* mutant 327FD with that of the wild type, AC327. The *cwlF* mutant cells were about twice as long as the wild-type cells (10.7 ± 8.7 μm and 6.5 ± 1.9 μm, respectively) (Fig. 6). However, in the case of the p60 protein of *L. monocytogenes*, a reduction in the amount of p60 leads to the formation of long cell chains (5, 56). Since these morphological differences may depend on the complement set of cell wall hydrolases in *B. subtilis*, we constructed a *cwlB cwlF* mutant and a *sigD cwlF* mutant. While the former double mutant showed a cell morphology similar to that of the *cwlF* mutant (19), the latter one showed extraordinary, dense microfiber formation (Fig. 6). The *sigD* mutant grew as a turbid suspension in a test tube culture, but SD1F grew like cotton waste in a transparent culture (Fig. 6). Recent studies have identified cell wall hydrolases (AcmA, p60, and Atl) involved in cell separation in *L. lactis*, *L. monocytogenes*, and *Staphylococcus aureus*, respectively (6, 56, 57). Atl is a bifunctional protein which has an amidase domain and a glucosaminidase domain and undergoes proteolytic processing to generate two extracellular cell wall hydrolases (amidase and glucosaminidase). These enzymes synergistically act on cell separation (52). Therefore, a combination of cell wall hydrolases may be important for cell separation in *B. subtilis*.

CwlF production is not affected by the pleiotropic genes *flaD1* and *degU32*(Hy). Since it is known that the pleiotropic mutations *flaD1* and *degU32*(Hy) lead to extensive filamentation (10, 53), we measured CwlF productivity in organisms carrying these mutations. Figure 7 shows zymography of extracts S of the wild-type (AC327) strain and the *sigD* (327SD1), *flaD1* (AC334), and *degU32*(Hy) (327UH) mutants. Compared with the wild-type cells, the mutants exhibited an extensive reduction in cell wall hydrolases. However, the 35-kDa CwlF protein was not greatly affected by these mutations.

This study indicated that CwlF is a new cell-separating enzyme. The gene is suggested to be transcribed mainly by $E\sigma^A$ RNA polymerase and weakly by $E\sigma^H$ RNA polymerase. The extraordinary microfiber formation by organisms carrying the *sigD cwlF* double mutation suggests that a SigD-dependent factor(s) and CwlF cooperatively play an important role in cell separation during the vegetative growth phase. Many *cwlF* homologs were recently found in the *B. subtilis* genome (47), and it is predicted that the *cwlF* group will become a big one among cell wall hydrolase genes. Research on the combination of cell wall hydrolases should reveal their cellular functions. The substrate specificity of CwlF has not been determined, and thus our research is now directed toward the purification and characterization of this enzyme.

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