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

SHU ISHIKAWA, YOSHIKO HARA, RYO OHNISHI, AND JUNICHI SEKIGUCHI*
Department of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, 3-15-1 Tokida, Ueda-shi, Nagano 386, Japan

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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 EcrA RNA polymerase and weakly by EcrH 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-acetyl-muramoyl-l-alanine amidase [amidase], CwlB [LytC], and a 90-kDa endo-β-N-acetylglucosaminidase [glucosaminidase], CwG [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 σ70-type promoter and a proximal σ70-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 Eσ70 RNA polymerase (33, 41). A study on the physiological functions of CwlB and CwG 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. (Preliminary data were presented at the 9th International Conference on Bacilli [Lausanne, Switzerland, 15 to 19 July 1997].)
**TABLE 1. Strains and plasmids used in this study**

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*B. subtilis* strains, and the resulting pGEMES-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′ CGGGGATCCCAAGCTTTAGC 3′), 327FD, 327SD1D, and ABDF, was confirmed by Southern hybridization analysis.

**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 (40), 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 100 μg/ml.

**Preparation of 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 rpm for 10 min at 4°C, the pellet was suspended in 1 ml of SET buffer containing lysozyme (1 mg/ml) and a shake culture at 37°C. If necessary, the antibiotic was added to a final concentration of 100 μg/ml.

**Preparation of cwlF protein.** *B. subtilis* cells were grown essentially as described previously (16, 24).

**SDS-PAGE and zymography.** SDS-PAGE of proteins was performed in 10 or 12% (w/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) dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (28), followed by boiling for 10 min. The suspensions were centrifuged at 11,000 rpm for 10 min at 4°C, the pellet was suspended in 1 ml of SET buffer containing lysozyme (1 mg/ml) and a shake culture at 37°C. If necessary, the antibiotic was added to a final concentration of 100 μg/ml.

**Preparation of cell wall.** Cell wall of *B. subtilis* 168S was prepared essentially as described previously (16, 24).

**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 pGEM protein was determined with an automatic protein sequencer (model LF-3000; Beckman). The N-terminal amino acid sequence of the CwlF protein was determined with an automatic protein sequencer (model LF-3000; Beckman).

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

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**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 Shimotot 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-nitrophenylβ-D-galactopyranoside (ONPG) in 1 min.

**Northern blot and primer extension analyses.** *B. subtilis* AC327 cells (optical density at 600 nm [OD600] 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 MgCl2 and 20 mM Na2S2O3) (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 37°C, the suspension was centrifuged at 11,000 × g for 1 min at 4°C. The pellet was used for Northern blot and primer extension analyses.

**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% (w/vol) *B. subtilis* cell wall as the substrate. Extracts S were prepared, and protein amounts equivalent to 4 OD600 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 t1, t2, and t3, respectively. The arrowheads indicate the CwlF protein.
After 4, 6, and 8 h, 5-ALAcultured in a test tube (17-mm diameter) containing 5 ml of LB medium at 37°C. The OD600 was measured after strong vortexing of samples. In the case of the slides, and then the cell morphology was observed by phase-contrast microscopy.

The arrowhead indicates a deduced signal peptidase cleavage site. Indicated tandem repeated regions and polyserine regions in CwlF, respectively. Amino acids are numbered from the N termini of the proteins. Dashes indicate 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 new cell wall hydrolase, CwlF, has a molecular weight of 50 kDa, which overlapped with that of a new cell wall hydrolase, CwlE. The new cell wall hydrolase, CwlF, has a molecular weight of 35 kDa, as judged by SDS-PAGE. A σ°-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.

Identity of CwlF to the papG gene product. Comparison of the N-terminal amino acid sequence with those of proteins in a nonredundant protein database revealed that the sequence was 62% identical to the starting internal 20-amino-acid sequence at position 26 (with respect to the N-terminal amino acid)

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°-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.

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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_23SA_2Q_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.
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 t₁ but not after t₂. Since the intensity of the signal was highest at t = 1, cwlF is expressed as a monocistronic operon and may be transcribed by Eo- 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 (55). Two deduced ρ-independent terminators (∆G = -10.2 and -10.8 kcal/mol) are located between phoA and cwlF, and one terminator (∆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), t₀ (lane 2), and t₁ (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 t₀ and t₁. 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 σ54 consensus sequence (TTGACA for the −35 region; H consensus sequence (RWAGGA XXXT 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 (σ70
and σ^{E} (26, 29), and a cortex maturation gene, cwlD (σ^{F} and σ^{E}) (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^β 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 the wild-type cells (10.7 ± 6 × 10^6 cfu/mL). The wild-type strain (AC327) and the mutants were examined in Escherichia coli of a Streptococcus faecalis autolysin. J. Bacteriol. 173:5619–5623.


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