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Characterization of new L,D-endopeptidase gene product CwlK (previous YcdD) that hydrolyzes peptidoglycan in *Bacillus subtilis*

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26   **Abstract**   *Bacillus subtilis* has various cell wall hydrolases, however, the functions and

27   hydrolase activities of some enzymes are still unknown. *Bacillus subtilis* CwlK (YcdD)

28   exhibits high sequence similarity with the peptidoglycan hydrolytic L,D-endopeptidase

29   (PLY500) of *Listeria monocytogenes* phage and CwlK has the VanY motif which is a

30   D-alanyl-D-alanine carboxypeptidase (Pfam: http://www.sanger.ac.uk/Software/Pfam/).

31   The β-galactosidase activity observed on *cwlK-lacZ* fusion indicated that the *cwlK* gene

32   was expressed during the vegetative growth phase, and Western blotting suggested that

1

1 CwlK seems to be localized in the membrane. Truncated CwlK fused with a

2 histidine-tag (h-ΔCwlK) was produced in *E. coli* and purified on a nickel column. The

3 h-ΔCwlK protein hydrolyzed the peptidoglycan of *B. subtilis*, and the optimal pH,

4 temperature and NaCl concentration for h-ΔCwlK were pH 6.5, 37ºC, and 0 M,

5 respectively. Interestingly, h-ΔCwlK could hydrolyze the linkage of

6 L-alanine-D-glutamic acid in the stem of the peptidoglycan, however, this enzyme could

7 not hydrolyze the linkage of D-alanine-D-alanine, suggesting that CwlK is an

8 L,D-endopeptidase not a D,D-carboxypeptidase. CwlK could not hydrolyze

9 polyglutamate from *Bacillus natto* or peptidoglycan of *Staphylococcus aureus.* This is

10 the first report describing the characterization of an L,D-endopeptidase in *B. subtilis* and

11 also the first report in bacteria of the characterization of a PLY500 family protein

12 encoded in chromosomal DNA.

13

Introduction

1
2 Gram-positive bacteria produce many peptidoglycan hydrolases. In *Bacillus subtilis,*
3 these enzymes are important in various cellular processes during vegetative growth,
4 sporulation and germination, and more than 30 candidate peptidoglycan hydrolases are
5 proposed on the basis of amino acid sequence similarity (Smith et al. 2000). In
6 particular, during the vegetative growth phase, they play roles in cell wall turnover,
7 motility, cell separation, and autolysis (Foster and Popham 2002; Smith et al. 2000).
8 However, we do not know why the roles of multiple hydrolases overlap. Atrih et al.
9 described that vegetative peptidoglycan has the modification such as amidation and
10 de-*N*-acetylation (Atrih A et al. 1999). However, we do not know why *B. subtilis* needs
11 the modification of the peptidoglycan. Recently, it is known that vancomycin sometimes
12 does not work against bacteria which have the modified peptidoglycan. Thus, the
13 modification of the peptidoglycan is very important for bacteria. We believe that it is
14 possible that identification of the role and enzymatic activity of cell wall hydrolases can
15 help to dissolve these questions.

16 The primary structure of a typical peptidoglycan of *B. subtilis* is shown in Fig. 1.
17 Several hydrolases produced during the vegetative growth phase, such as LytC (CwlB),

Fig. 1

18 LytD (CwlG), LytG (YubE), LytE (CwlF), LytF (CwlE), CwlO (YvcE), and CwlS
19 (YojL), have been characterized in *B. subtilis* (Blackman et al. 1998; Foster and Popham
20 2002; Fukushima et al. 2006; Horsburgh et al. 2003b; Ishikawa et al. 1998; Kuroda and
21 Sekiguchi 1991; Lazarevic et al. 1992; Margot et al. 1994; Margot et al. 1998; Margot et
22 al. 1999; Ohnishi et al. 1999; Rashid et al. 1995; Shida and Sekiguchi 2005; Yamaguchi
23 et al. 2004). LytD and LytG have been identified as *N*-acetylglucosaminidases
24 (Horsburgh et al. 2003b; Margot et al. 1994; Rashid et al. 1995), LytC as an
25 *N*-acetylmuramoyl-L-alanine amidase (Kuroda and Sekiguchi 1991), and LytF, CwlO,
26 and CwlS as D,L-endopeptidases hydrolyzing the D-γ-glutamyl-*meso*-diaminopimelic
27 acid linkage in the peptidoglycan of *B. subtilis* (Fukushima et al. 2006; Margot et al.
28 1999; Ohnishi et al. 1999; Yamaguchi et al. 2004). Moreover, PgdS (YwtD) is a
29 polyglutamic acid-degrading enzyme produced during the vegetative growth phase and
30 it belongs to the "D,L-endopeptidase" family (Suzuki and Tahara 2003). However, the
31 characterization of several hydrolases i.e., muramidase and lytic transglycosylase
32 (cleaving the *N*-acetylmuramic acid-*N*-acetylglucosamine linkage), L,D-endopeptidase

1 (cleaving the L-alanine-D-glutamic acid linkage) and D,D-endopeptidase (cleaving the

2 cross-linked D-alanine-*meso*-diaminopimeric acid linkage) (see Fig. 1 and Foster and

3 Popham 2002), was not reported until recently in *B. subtilis*.

4   Horsburgh *et al.* reported that LytH (YunA) is associated with modification of the

5 spore cortex and muropeptide analysis of the cortex of a *lytH* mutant suggested that

6 LytH is an L,D-endopeptidase (Horsburgh et al. 2003a). However, there is a possibility

7 that LytH is a carboxypeptidase cleaving the D-alanine-*meso*-diaminopimeric

8 acid-D-alanine linkage in the spore cortex (Horsburgh et al. 2003a).

9   Based on sequence similarity, CwlK is proposed to belong to an L,D-endopeptidase

10 family including the L,D-endopeptidase (Ply500) of *Listeria monocytogenes* phage

11 (Smith et al. 2000). Moreover, this protein also has a VanY motif which corresponds to

12 a D-alanyl-D-alanine carboxypeptidase (Pfam: http://www.sanger.ac.uk/Software/Pfam/).

13 Although CwlK is predicted to be an L,D-endopeptidase, it shows no sequence similarity

14 to LytH.

15   Since there has been no enzymatic characterization of the L,D-endopeptidase in *B.*

16 *subtilis* and information on the L,D-endopeptidase family is also very poor, it would be

17 useful to determine the enzymatic properies of L,D-endopeptidase. In this study, we

18 identified *cwlK* (*ycdD*) as a new peptidoglycan hydrolase gene that is expressed during

19 the vegetative growth phase. Moreover, we determined the cleavage sites of CwlK *in*

20 *vitro* in order to clear the enzymatic properties. As a result, CwlK was an

21 L,D-endopeptidase that cleaves the linkage of the L-alanine-D-glutamic acid of *B.*

22 *subtilis* cell wall. Furthermore, it is probable that CwlK is localized in the membrane as

23 a lipoprotein.

1 **Materials and Methods**

2

3 Strains, plasmids, and growth and transformation conditions

4

5 The strains of *B. subtilis* and *Escherichia coli* used in this study are listed in Table 1. *B.*

6 *subtilis* 168 was the parent strain and mutants having the 168 background were used

7 throughout this study. *B. subtilis* strains were cultured in Luria-Bertani (LB) medium

8 (Sambrook et al. 1989) or DSM (Schaeffer) medium as a sporulation medium (Schaeffer

9 et al. 1965) at 37°C. If necessary, tetracycline, erythromycin, kanamycin, and

10 chloramphenicol were added to final concentrations of 5, 0.3, 5 or 25, and 3 or 5 µg/ml,

11 respectively. *E. coli* was grown in LB medium or 2xYT medium (16 g of Bacto

12 Tryptone [Difco], 10 g of yeast extract, and 5 g of NaCl per liter; pH 7.3) at 37°C. If

13 necessary, ampicillin and kanamycin were added to final concentrations of 100 and 25

14 µg/ml, respectively. *E. coli* transformation was performed as described by previously

15 (Sambrook et al. 1989), and *B. subtilis* transformation was performed by the competent

16 cell method (Anagnostopoulos and Spizizen 1961).

17

18 Construction of *cwlK* null (YCDDd) and *cwlK sigD*-double (cdDSD) mutants

19

20 For construction of a *cwlK* mutant, a part of *cwlK* was amplified by PCR with the

21 ycdD-HF (gccgaagcttGGCATGAATGGCATTCTC, the lowercase letters and

22 underlining indicating a tag sequence and a restriction enzyme site, respectively) and

23 ycdD-BX (gcgcggatccCCTCTTGCATAGGTGACA) primers, and *B. subtilis* 168 DNA

24 as a template. The amplified fragment was digested with *Hin*dIII and *Bam*HI, and then

25 ligated to the corresponding sites of pMUTIN4, resulting in pM4ycdD (Fig. 2). The

26 plasmid (pM4ycdD) from *E. coli* C600 was used for transformation of *B. subtilis* 168

27 (wild-type) to obtain the YCDDd strain (*cwlK*::*erm*) by single crossing-over

28 recombination. For construction of a *cwlK sigD*-double mutant, cdDSD (*cwlK*::*erm*

29 *sigD*::*cat*), the chromosomal DNA of 168SDC (*sigD*::*cat*) was extracted and used for

30 the transformation of the YCDDd strain. Proper integration was checked throughout this

31 study with appropriate primers.

32

1 Construction of a *cwlK* mutant (YCDDp) with an isopropyl-β-D-thiogalactopyranoside

2 (IPTG) inducible promoter

3

4 For construction of a conditional *cwlK* null mutant, a fragment containing the predicted

5 Shine-Dalgarno (SD) sequence and a part of *cwlK* were amplified by PCR with the

6 ycdD-HS (gccgaagcttGCACATCTTGAGAAAGTGA) and cdD-R1

7 (gcgcggatccCATCTTGTTCCTTAAAGGA) primers, and *B. subtilis* 168 DNA as a

8 template. The amplified fragment was digested with *Hin*dIII and *Bam*HI, and then

9 ligated to the corresponding sites of pMUTIN4, resulting in pM4SDcdD (Fig. 2). The

10 plasmid (pM4SDcdD) from *E. coli* C600 was used for transformation of *B. subtilis* 168

11 to obtain the YCDDp strain (*cwlK*::[P*spac*-*cwlK erm*]) by single crossing-over

12 recombination.

13

14 Construction of the cdD3FL strain containing *cwlK* fused with a 3xFLAG-tag

15

16 To construct a strain of *B. subtilis* containing *cwlK* fused with a 3xFLAG-tag

17 (*cwlK*-3xFLAG), the C-terminal region of *cwlK* was amplified by PCR with the cdD-N1

18 (gccgaagcttGAATGCCGATGCACTCAA) and cdD-C1

19 (gcgcggatccGTTAGGAATCATCTCCAAG) primers, and *B. subtilis* 168 DNA as a

20 template. The amplified fragment was digested with *Hin*dIII and *Bam*HI, and then

21 ligated to the corresponding sites of pCA3xFLAG, resulting in pCA3FLcdD (Fig. 2).

22 The plasmid (pCA3FLcdD) from *E. coli* C600 was used for transformation of a protease

23 mutant, *B. subtilis* WE1 (*wprA epr*), to obtain the cdD3FL strain (*cwlK*::*cwlK*-3xFLAG

24 *wprA epr*) by single crossing-over recombination.

25

26 Construction of the CwlK3FLp strain containing P*spac*-*cwlK*-3xFLAG

27 To construct a strain of *B. subtilis* containing *cwlK* fused with a 3xFLAG-tag and the

28 P*spac* promoter (P*spac*-*cwlK*-3xFLAG), cdD3FL strain (*cwlK*::*cwlK*-3xFLAG *wprA epr*)

29 was transformed with chromosomal DNA of YCDDp strain (*cwlK*::[P*spac*-*cwlK erm*]) by

30 double crossing-over recombination. The obtained transformant, CwlK3FLp

31 (*cwlK*::[P*spac*-*cwlK*-3xFLAG] *wprA epr*), was checked by means of PCR and antibiotic

32 resistance.

1

2 Construction of plasmid pQEΔcdD for over-producing a protein fused with a

3 histidine-tag

4

5 To construct a strain over-expressing CwlK, *E. coli* BL21 (pREP4) harboring a plasmid

6 for over-producing histidine-tagged CwlK was constructed. At first truncated *cwlK* was

7 amplified by PCR with the YCDD-sg (gccgggatccCATGAATGGCATTCTCAAAA) and

8 YCDD-HR (gcgcaagctTCTAGTTAGGAATCATCTCC) primers, and *B. subtilis* 168

9 DNA as a template. The PCR fragment was digested with *Bam*HI and *Hin*dIII, and then

10 ligated to the corresponding sites of pGEM3Zf(+), resulting in pGEMΔcdD. Then, the

11 truncated *cwlK* fragment was obtained by digesting pGEMΔcdD with *Bam*HI and

12 *Hin*dIII, and then ligated to the corresponding sites of pQE-30, resulting in pQEΔcdD

13 (Fig. 2). After plasmid pQEΔcdD had been used for transformation of *E. coli* BL21

14 (pREP4), the *E. coli* BL21 (pREP4) harboring pQEΔcdD was used for over-production

15 of h-ΔCwlK [truncated form of CwlK (YcdD); 26 a.a. to 167 a.a. of CwlK with respect

16 to the N-terminal amino acid residue] with a 6x His-tag fused at its N-terminal. All

17 constructed plasmids were confirmed by sequencing with a DNA sequencer (Applied

18 Biosystems, model 373A or 310).

19

20 Assaying of β-galactosidase activity

21

22 The assay was performed as described previously (Shimotsu and Henner 1986). One

23 unit of β-galactosidase activity was defined as the amount of enzyme necessary to

24 release 1 nmol of 2-nitrophenol from *o*-nitrophenyl-β-D-galactopyranoside in 1 min.

25

26 Preparation of *B. subtilis* cell wall, *Staphylococcus aureus* peptidoglycan and

27 poly-γ-glutamic acids

28

29 Cell wall derived from *B. subtilis* 168 was prepared as described previously (Ohnishi et

30 al. 1999; Fein and Rogers 1976). *S. aureus* peptidoglycan (SIGMA-ALDRICH) and

31 poly-γ-glutamic acids from *B. subtilis* (Wako, M.W.4,000,000~6,000,000) were chosen

1   for this study.

2

3   SDS-PAGE, zymography and Western blotting

4

5   SDS-PAGE was performed in a polyacrylamide gel as described previously (Sambrook

6   et al. 1989). Zymography was performed as described by previously (Leclerc and

7   Asselin 1989) with a SDS-polyacrylamide gel containing 0.5 mg/ml of purified *B.*

8   *subtilis* cell wall as a substrate for cell wall hydrolases. Renaturation was performed at

9   37°C with a renaturation buffer (25 mM Tris-HCl [pH 7.2] and 1% [vol/vol] Triton

10   X-100) as described previously (Fukushima et al. 2006). For Western blotting, proteins

11   on a SDS-polyacrylamide gel were transferred to a PVDF membrane (Amersham

12   Bioscience) as described previously (Yamamoto et al. 2003). For immunoblotting and

13   immunodetection, the ECL plus Western blotting detection system (Amersham

14   Biosciences), mouse anti-FLAG M2 monoclonal antibodies (Sigma), as the primary

15   antibodies, and horseradish peroxidase-linked whole sheep antibodies (Amersham

16   Biosciences), as the secondary antibodies, were used as described previously

17   (Fukushima et al. 2006; Yamamoto et al. 2003).

18

19   Purification of h-ΔCwlK, which contains the entire CwlK (YcdD) without the signal

20   peptide

21

22   Truncated CwlK fused with a histidine-tag at its N-terminal (h-ΔCwlK) was

23   over-expressed in *E. coli* and then purified as follows. *E. coli* BL21 cells harboring

24   pREP4 and pQEΔcdD were incubated at 37°C in LB medium (200 ml) containing 100

25   and 25 μg/ml of ampicillin and kanamycin, respectively. At OD_{600} of 0.8, 1 mM IPTG

26   was added to the culture. After 40-min incubation, the cells were harvested by

27   centrifugation and then disrupted by ultrasonication in 10 mM imidazole NPB buffer

28   (10 mM imidazole, 1 M NaCl and 20 mM sodium phosphate [pH 7.4]). Purification of

29   h-ΔCwlK was performed as described previously (Yamaguchi et al. 2004) on a HiTrap

30   chelating column (1 ml of resin; Amersham Biosciences), and fractions were eluted with

31   a stepwise gradient of 100-300 mM imidazole solutions (100-300 mM imidazole, 1 M

32   NaCl and 20 mM sodium phosphate [pH 7.4]). The eluate was dialyzed against dialysis

1 buffer (25 mM NaCl and 20 mM Tris-HCl [pH 8.0]).

2

3 Determination of the optimum pH, temperature and NaCl concentration for h-ΔCwlK

4

5 Determination of the optimum pH of h-ΔCwlK was performed as described by Ohnishi

6 *et al.* (1999) and Fukushima *et al.* (2006). The following buffers (50 mM) and 0.33

7 mg/ml of *B. subtilis* 168 cell wall were used at 37°C: citrate buffer for pHs 5.0, 5.5 and

8 6.0; and Good's buffer for pHs 6.0, 6.5, 7.0, 7.5, 8.0, 9.0 and 10.0. To determine the

9 optimum temperature, 50 mM MOPS (3-morpholinopropanesulfonic acid)-NaOH buffer

10 (pH 6.5) without NaCl and 0.33 mg/ml of cell wall were used. For determination of the

11 optimum NaCl concentration, the reaction was performed at 37°C with 50 mM

12 MOPS-NaOH buffer (pH 6.5) containing 0.33 mg/ml of cell wall. For all experiments,

13 purified h-ΔCwlK was added to the cell wall mixture to a final concentration of 5 μg/ml.

14 The absorbance of the cell wall at OD_{540} was measured with a spectrophotometer

15 (model V-560, JASCO). One unit of hydrolase activity was defined as the amount of

16 enzyme necessary to decrease the absorbance at 540 nm by 0.001 in 1 min (Fukushima

17 et al. 2006; Ohnishi et al. 1999).

18

19 Determination of the cleavage sites of cell wall peptidoglycan

20

21 Purified cell wall of *B. subtilis* 168 (0.33 mg/ml) and purified h-ΔCwlK (5 μg/ml) were

22 mixed in 50 mM MOPS (pH 7.0) buffer, and then the mixture was incubated at 37°C. At

23 0, 15, and 60 min, the absorbance of the mixture at 540 nm was measured and samples

24 were taken, and then the samples were boiled for 10 min to completely stop the reaction

25 (it being confirmed that h-ΔCwlK was inactivated on boiling for 10 min). Lysozyme

26 (100 μg /ml) was added to each sample without centrifugation of the sample and the

27 mixtures were kept at 37°C for 12 h in order to dissolve the degraded cell wall in the

28 solution. The sample was centrifuged to remove insoluble fraction such as denatured

29 CwlK by boiling, the supernatant (150 μl), 50 μl of 10 % $K_2B_4O_7$ (pH 9.0), 295 μl of

30 distilled water (DW), and 5 μl of 1 M 1-fluoro-2,4-dinitrobenzene (FDNB) were mixed,

31 and the mixture was incubated at 65°C for 30 min in the dark. To digest glycosidic and

32 peptide bonds, the samples were hydrolyzed with about 4 M HCl for 12 h at 95°C, and

1   then the samples were dried up under vacuum. Finally, the samples were resuspended in

2   200 µl of a 10 % acetonitrile/0.025 % trifluoro acetic acid (TFA) solution. For

3   identification of DNP-amino acids, 50 µl of each sample was separated by reverse phase

4   (RP)-HPLC on Wakosil-II 5C18 (4.0 mm X 250 mm; Wako) (flow rate, 0.5 ml/min;

5   monitoring wave length, 365 nm; Shimadzu LC-10A series). Elution buffers A and B

6   comprised 0.025% TFA and 0.025% TFA/60% CH_3CN, respectively. Elution was

7   performed with a linear gradient of buffer B (from 0 to 100%) in 60 min at 40°C

8   (column heater). The separated peaks were identified as described previosly (Fukushima

9   et al. 2005). After RP-HPLC analysis, the separated peak materials were lyophilized

10   overnight. The dried samples were dissolved in 50% acetonitrile and then subjected to

11   electrospray ionization-mass spectrometry (ESI-MS; Agilent 1100 series LC/MSD Trap

12   VP).

13   For preparation of dinitrophenol (DNP), DNP-L-Ala, DNP-D-Ala, DNP-D-Glu,

14   mono-DNP-A_2pm (diaminopimelic acid), and bis-DNP-A_2pm as standard materials,

15   DW and 0.01 mM L-Ala, D-Ala, D-Glu, and A_2pm were reacted with FDNB as

16   described above, and then all samples were separated and purified by RP-HPLC as

17   described above. Finally, separated samples were identified as described previously

18   (Fukushima et al. 2005).

19

20   Preparation of cell surface proteins and supernatant proteins

21

22   *B. subtilis* strains were incubated in LB medium at 37°C, and sampling was performed

23   during the exponential and early stationary growth phases. After centrifugation of a

24   sample, the supernatant (for supernatant proteins) and pellet (for cell surface proteins)

25   were separated. The collected supernatant (for supernatant proteins) was passed through

26   a filter (0.45 µm diameter), and then precipitated with 2% TCA as described previously

27   (Yamaguchi et al. 2004). The precipitated sample was washed with 70% ethanol and

28   then used as the supernatant proteins. On the other hand, the collected pellet (for cell

29   surface proteins) was washed with 25 mM Tris-HCl (pH 7.2) twice and then suspended

30   in 3 M LiCl containing 25 mM Tris-HCl (pH 7.2), followed by incubation at 20 min on

31   ice as described previously (Yamamoto et al. 2003) in order to remove cell surface

32   proteins from the cell wall. After centrifugation of the sample, proteins in the

1 supernatant were precipitated with 2% TCA. The precipitated sample was washed with

2 70% ethanol and then used as the cell surface proteins. Supernatant and cell surface

3 proteins were analyzed by SDS-PAGE and Western blotting.

4

5 Preparation of whole extracted proteins and supernatant proteins

6

7 *B. subtilis* strains were incubated in LB medium at 37°C, and sampling was performed

8 during the early stationary growth phase. After centrifugation of a sample, the

9 supernatant (for supernatant proteins) and pellet (for whole extracted proteins) were

10 separated. The supernatant proteins were prepared as above in Materials and Methods.

11 For whole extracted proteins the pellet was suspended in a lysozyme solution (2 mg/ml

12 lysozyme/ 50 mM Tris-HCl [pH 8.0]/ 0.1 M EDTA [pH 8.0]). The sample was treated at

13 37°C for 30 min, and SDS-PAGE sample buffer was added to the sample. Proteins from

14 supernatants and whole cells were analyzed by Western blotting.

15

16 Hydrolase activity of h-ΔCwlK toward poly-γ-glutamic acids and *Staphylococcus*

17 *aureus* peptidoglycan

18

19 Determination of the hydrolase activity of poly-γ-glutamic acid was performed

20 according to the method of Urushibata *et al.* (2002). A mixture of 10 μg/ml of

21 poly-γ-glutamic acids and 10 μg/ml of h-ΔCwlK in 50 mM MOPS (pH 6.5) was

22 incubated at 37°C for 3 hours, and then whether or not the poly-γ-glutamic acids had

23 been digested was examined by SDS-PAGE. Moreover, in order to confirm that the

24 sample contained free amino groups of glutamic acids due to hydrolysis of

25 poly-γ-glutamic acids, the sample was labeled with FDNB by the dinitrophenyl method

26 and the labeled amino acids were separated by RP-HPLC as described in the above

27 section.

28 For the hydrolase activity toward *Staphylococcus aureus* peptidoglycan, a mixture of

29 5 μg/ml of h-ΔCwlK and 0.33 mg/ml of *S. aureus* peptidoglycan in 50 mM MOPS (pH

30 6.5) was incubated at 37°C. The absorbance of the cell wall at OD_{540} was measured with

31 a spectrophotometer (model V-560, JASCO).

1

1   **Results and discussion**

2

3   Peptidoglycan is a main cell wall component and maintains cell shape and osmotic

4   resistance even toward growing cells. Since peptidoglycan hydrolases are associated

5   with digestion of peptidoglycans and also various cellular functions, it is important to

6   know the functions of entire peptidoglycan hydrolases in one strain. *B. subtilis* is one of

7   the best bacteria in this aspect because many peptidoglycan hydrolases have been

8   widely investigated. However, some of the peptidoglycan hydrolases are still

9   uncharacterized, especially L,D-endopeptidase cleaving the L-alanine-D-glutamic acid

10   linkage in peptidoglycan. To address this importance, we characterized a gene product

11   (YcdD) that is a homolog of Ply500, L-alanoyl-D-glutamate peptidase, in *Enterococcus*

12   *faecium.*

13

14   The *cwlK* (*ycdD*) gene

15

16   The *cwlK* (*ycdD*) gene is located at positions 303,859 to 303,359 bp in the *B. subtilis*

17   genome and it is 501 bases in size. Fig. 2 shows a gene map around *cwlK*. *rapJ*

18   (function unknown, putative response regulator of aspartate phosphatase) and *ycdC*

19   (function unknown) are upstream and downstream of *cwlK*, respectively. Since the

20   orientation of *rapJ* and *ycdC* is opposite to that of *cwlK* (Fig. 2), it is predicted that

21   *cwlK* is transcribed as a monocistronic operon, the genes around *cwlK* thus not being

22   affected by *cwlK*. The *cwlK* gene encodes a polypeptide consisting of 167 amino acid

23   residues and its N-terminal region contains a signal sequence based on the SignalP

24   (http://www.cbs.dtu.dk/services/SignalP/) algorithm, and the signal sequence is

25   predicted to be $MNLPAKTFVILCILFLLDLCFSYIRH_{26}\downarrow E_{27}$ (the arrow indicates the

26   cleavage point and the numbers are with respect to the N-terminal amino acid residue).

27   Tjalsma *et al.* (2000) also described that the signal sequence of CwlK is assumed to be

28   $MNLPAKTFVILCILFLLDL_{19}\downarrow C_{20}$ (the arrow indicates the cleavage point and the

29   numbers are with respect to the N-terminal amino acid residue) cleavage point because

30   CwlK has the sequence ($LDLC_{20}$ [the numbers are with respect to the N-terminal amino

31   acid residue]) which is similar to the lipobox (L-X-X-C) amino sequence.

1

2 Cell morphology of YCDDd (*cwlK* mutant) and YCDDp (*cwlK* conditional

3 over-expression strain)

4

5 The wild-type *B. subtilis* is rod-shaped, and exists as short chains in the vegetative

6 growth phase and as short rods in the stationary phase. Several previous reports

7 indicated that autolysins in *B. subtilis* can induce lysis, and play roles in cell separation,

8 cell elongation, motility, cell wall turnover, etc. (Smith et al. 2000). At first, to confirm

9 the effect of *cwlK* disruption, a mutant of it, YCDDd (*cwlK*::*erm*), was constructed. Cell

10 growth of the YCDDd strain was similar to that of the wild-type (168) strain (Fig. 3).

11 The cell morphology of YCDDd, as investigated under a microscope, was also similar

12 to that of the wild-type strain (data not shown). Furthermore, the sporulation,

13 germination, and outgrowth by the YCDDd strain were investigated as described

14 previously (Fukushima et al. 2002, 2003, 2004). As a result, the phenotypes of YCDDd

15 were found not to be different from those of the wild-type strain (data not shown).

16 To confirm the effect of over-expression of CwlK, the YCDDp (*cwlK*::[P$_{spac}$-*cwlK*

17 *erm*]) strain was constructed. Moreover, in order to confirm that the IPTG inducible

18 promoter can work, new strain, CwlK3FLp (*cwlK*::[P$_{spac}$-*cwlK*-3xFLAG] *wprA epr*)

19 which can over-express the CwlK-3xFLAG protein by IPTG induction, was also

20 constructed. As a result, when CwlK-3xFLAG was over-expressed by IPTG, a strong

21 band derived from the protein could be detected by Western blotting in Fig. 6B. Thus,

22 this result suggests that the IPTG inducible promoter can work by IPTG. When CwlK

23 was over-expressed with 1 mM IPTG, the growth rate of YCDDp was the same as that

24 of the wild-type (Fig. 3; data not shown). Moreover, the phenotypes of YCDDp were

25 similar to those of the wild-type (data not shown). These results indicate that CwlK does

26 not affect the cell shape, but the cellular function of CwlK remains obscure.

27

28 Transcriptional analysis of *cwlK*

29

30 To determine the transcriptional period of *cwlK* in the life cycle of *B. subtilis*, the

31 ß-galactosidase assay was performed with YCDDd, which has the transcriptional fused

32 gene *cwlK-lacZ* (Fig. 3). For this assay, LB medium (rich medium) and DSM

Fig. 3

Fig. 6

14

1 (sporulation medium containing less nutrients compared with LB medium) were used

2 because some genes were expressed only LB medium or DSM medium (refer to the

3 BSORF database http://bacillus.genome.jp/). The ß-galactosidase activity of YCDDd

4 could be detected during the vegetative growth phase in LB medium (Fig. 3, closed

5 circles), although it could not be detected in DSM (data not shown). Therefore, it was

6 indicated that *cwlK* is transcribed during the vegetative growth phase in LB medium.

7 It is known that many genes are transcribed by $E\sigma^A$ (σ^A, house-keeping sigma factor)

8 and/or $E\sigma^D$ (σ^D, sigma factor mainly associated with flagellar motility and chemotaxis

9 during the mid- and late vegetative growth phases) RNA polymerases during the

10 vegetative growth phase in *B. subtilis* (Helmann and Moran 2002; Serizawa et al. 2004).

11 In particular, several autolysins such as *lytC* (*cwlB*), *lytD* (*cwlG*), and *lytF* (*cwlE*) are

12 transcribed by $E\sigma^D$ RNA polymerase (Kuroda and Sekiguchi 1993; Lazarevic et al.

13 1992; Margot et al. 1994; Ohnishi et al. 1999; Rashid et al. 1995). Thus, it is predicted

14 that the transcription of *cwlK* may depend on $E\sigma^A$ and/or $E\sigma^D$ RNA polymerases

15 because *cwlK* is transcribed during the vegetative growth phase (Fig. 3). Thus, it was

16 examined whether this gene is dependent on or independent of σ^D by means of

17 ß-galactosidase assaying with a *sigD*-deficient *cwlK-lacZ* transcriptional fusion strain,

18 cdDSD (*cwlK*::*erm sigD*::*cat*). As a result, the level of ß-galactosidase activity of

19 cdDSD (Fig. 3, closed squares) was found to be similar to that of YCDDd (Fig. 3,

20 closed circles). This result indicates that the transcription of *cwlK* is independent of

21 *sigD*.

22

23 Characterization of CwlK

24

25 It is predicted that CwlK is a cell wall hydrolase because it has an L,D-endopeptidase

26 (Smith et al. 2000) and/or a D,D-carboxypeptidase domain (Pfam:

27 http://www.sanger.ac.uk/Software/Pfam/). In order to confirm that CwlK is a cell wall

28 hydrolase, the entire CwlK without its N-terminal signal sequence (aa 26 to aa 167) was

29 fused with a six-histidine tag at its N-terminal (h-ΔCwlK), and the fused protein was

30 produced in *E. coli*, followed by purification on a HiTrap chelating column. Figure 4

31 shows the results of SDS-PAGE and zymography with h-ΔCwlK. The purified

Fig. 4

1   h-ΔCwlK gave a single band on SDS-PAGE (Fig. 4, lane 3) corresponding to the cell

2   wall-hydrolysing band observed on zymography (Fig. 4, lane 4). Since the molecular

3   mass of h-ΔCwlK is 17.6 kDa, this result also supports the idea that the band containing

4   cell wall-hydrolysing activity represents h-ΔCwlK.

5   In order to determine the characteristics of the C-terminal domain of CwlK

6   (h-ΔCwlK), the optimum pH, temperature and NaCl strength for h-ΔCwlK as to cell

7   wall lytic activity were examined. As a result, the cell wall lytic activity of h-ΔCwlK

8   was found to be maximum at pH 6.5 under the conditions of 37ºC without NaCl.

9   Moreover, the optimum temperature and NaCl strength for cell wall lytic activity were

10   37ºC (conditions: 50 mM MOPS-NaOH [pH 6.5] without NaCl) and 0 mM (conditions:

11   50 mM MOPS-NaOH [pH 6.5] at 37ºC), respectively. The h-ΔCwlK protein is a cell

12   wall lytic enzyme exhibiting a specific activity of 1,086 U/mg under the optimum

13   conditions (pH 6.5, 37ºC and 0 M NaCl). In the case of D,L-endopeptidases hydrolyzing

14   a stem peptide, CwlS exhibits the specific activity of 1,500 U/mg under the conditions

15   of pH 7.0, 40ºC, and 0.025 mM NaCl (Fukushima et al. 2006). LytF (CwlE) exhibits an

16   optimal pH at 6.5 and specific activity of 1,560 U/mg (Ohnishi et al. 1999).

17

18   Determination of the cleavage site in peptidoglycan for h-ΔCwlK

19

20   Since it is clear that h-ΔCwlK has cell wall lytic activity, the site of cleavage in cell wall

21   by h-ΔCwlK was determined by the dinitrophenyl method (DNP method). Cell wall of

22   *B. subtilis* 168 was digested with h-ΔCwlK for 0, 15 min and 60 min, and samples were

23   boiled in order to inactivate h-ΔCwlK and the degraded cell wall in the solution was

24   dissolved with lysozyme (muramidase). The sample was centrifuged to remove

25   insoluble materials, and then each supernatant obtained on centrifugation was labeled

26   with FDNB and hydrolysed with HCl. Finally, the samples of DNP-labeled amino acids

27   were separated by RP-HPLC (Fig. 5). The cell wall densities at A_{580} after 0, 15, and 60

28   min digestion were 0.296, 0.216, and 0.178, respectively. Figure 5 shows the results for

29   the samples of DNP-labeled amino acids. Peak 3 (retention time, 41.1 min) was greatly

30   increased after digestion of the cell wall with h-ΔCwlK. The retention time of peak 3

31   was the same as that of a standard sample of DNP-D-Glu (41.1 min). Moreover, because

32   ESI-MS analysis of the peak 3 material in the negative mode revealed a fragment ion at

Fig. 5

1   *m/z* 312.4 and this value corresponds to the [M-H]⁻ of DNP-D-Glu (*M*r, 313.1), peak 3 is

2   derived from DNP-D-Glu. The amounts of DNP-D-Glu were 0, 89, and 111 nmol/mg

3   cell wall after 0, 15 and 60 min incubation, respectively.

4   In contrast, the intensity of peak 1 (retention time, 29.7 min) was strong at 0 min (Fig.

5   5), and the peak corresponded to mono-DNP-A$_2$pm, because *m/z* 355.2 of the fragment

6   on ESI-MS corresponded to [M-H]⁻ of mono-DNP-A$_2$pm (*M*r, 356.1), and the retention

7   time of peak 1 was almost the same as that of a standard sample of mono-DNP-A$_2$pm

8   (29.1 min). Peptidoglycan contains a stem peptide without crosslinkage in addition to a

9   crosslinked stem peptide (Foster and Popham 2002). Therefore, a free amino group

10 derived from *meso*-A$_2$pm without a crosslinkage was modified with FDNB to produce

11 mono-DNP-A$_2$pm. This is the reason why the intensity of peak 1 was strong at 0 min.

12 After 15 and 60 min digestion with h-ΔCwlK, peak 1 was slightly increased but the

13 increase was very small. These results indicate that h-ΔCwlK is an L-alanoyl-D-glutamic

14 acid endopeptidase.

15   Peaks 4 and 5 at 42.5 and 45.8 min, respectively, were due to DNP (*M*r, 184.0),

16 because the standard sample of DNP was also eluted at 42.2 and 45.5 min on RP-HPLC,

17 and ESI-MS analysis of the materials in the negative mode gave the same fragment ion

18 at m/z 183.7. The structure of the peak 2 material has not been determined by ESI-MS.

19 Since the standard samples of DNP-L-Ala and DNP-D-Ala were eluted at the same time

20 (48.1 min) and the retention time of the standard sample of bis-DNP-A$_2$pm was 58.2

21 min, it is clear that this peak is not that of DNP-L-Ala, DNP-D-Glu, mono-DNP-A$_2$pm,

22 bis-DNP-A$_2$pm or DNP-D-Ala. From these results, it is also indicated that h-ΔCwlK is

23 an L-alanoyl- D-glutamic acid endopeptidase.

24

25 Determination of cell wall hydrolase activity of h-ΔCwlK toward substrates,

26 poly-γ-glutamic acids from *Bacillus subtilis* and peptidoglycan from *Staphylococcus*

27 *aureus*

28

29 To identify whether that CwlK can specifically work as a LD-endopeptidase or not,

30 poly-γ-glutamic acids consisting of D- and L-glutamic acids was used as a substrate for

31 CwlK because several bacteria such as *B. anthracis* (Zwartouw and Smith 1956) and *B.*

subtilis (*natto*) (Hara et al. 1982) have poly-γ-glutamic acids as capsules and CwlK can hydrolyse the L-Ala-D-Glu bond in *B. subtilis* cell wall. Interestingly, YwtD, which was recently predicted to be a DL-endopeptidase (Smith et al. 2000), was identified as an enzyme involved in γ-glutamic acid degradation (Suzuki and Tahara 2003). Thus, it may be possible that CwlK can hydrolyse poly-γ-glutamic acids. The experiment on poly-γ-glutamic acid hydrolysis was carried out with SDS-PAGE and the dinitrophenyl method as described in Materials and Methods. But poly-γ-glutamic acid was not hydrolyzed by h-ΔCwlK under the optimum conditions for the hydrolysis of *B. subtilis* cell wall even with different concentrations of poly-γ-glutamic acid (1.2, 9.6 and 36 mg/ml; data not shown). Although poly-γ-glutamic acid contains the L-Glu-D-Glu linkage with a gamma glutamyl bond, CwlK exhibits specific activity to the L-Ala-D-Glu linkage with an ordinary alpha bond in peptidoglycan.

We also examined whether peptidoglycan of *Staphylococcus aureus* could become a substrate for h-ΔCwlK, as described in Materials and Methods because this peptidoglycan has L-Ala-D-iGln-L-Lys-D-Ala stem peptide instead of L-Ala-D-Glu-*meso*-A_2pm-D-Ala stem peptide. However, h-ΔCwlK did not show any hydrolytic activity toward *Staphylococcus aureus* peptidoglycan. Since peptidoglycan of *Staphylococcus aureus* contains an L-Ala-D-iGln-L-Lys-D-Ala stem peptide and the L-Lys residue is crosslinked to a glycine pentapeptide, it is interesting that h-ΔCwlK did not cleave the L-Ala-D-iGln linkage. In other words, CwlK can only digest the L-Ala-D-Glu linkage in peptidoglycan.

SDS-PAGE and Western blotting of cell surface proteins and supernatant proteins of the cdD3FL (*cwlK*::*cwlK*-3xFLAG *wprA epr*) and CwlK3FLp (*cwlK*::[P$_{spac}$-*cwlK*-3xFLAG] *wprA epr*) strains

Determination of the localization of cell wall lytic enzymes is important to know their functions in cells. For example, LytF (CwlE) and LytE (CwlF) associated with cell separation are localized at the cell poles and cell division sites (Yamamoto et al. 2003), and LytC (CwlB), a major cell wall autolysin, is localized entirely in the cell wall (Yamamoto et al. 2003). Moreover, recently it was shown that CwlO is secreted into the

culture supernatant (Yamaguchi et al. 2004). Then, it is thought the localization of a cell wall lytic enzyme may be related to its function. Since from the SignalP algorithm it is predicted that CwlK seems to have a secretory signal peptide, it is possible that CwlK is secreted and localized outside of the cell. On the other hand, because it is also predicted that this protein seems to have a lipoprotein signal peptide described by Tjalsma *et al.* (2000), CwlK may be localized in membrane as a lipoprotein. In order to clarify the role of CwlK, we determined the localization of CwlK with YCDDd (*cwlK*::*erm*) and the wild-type strain during the vegetative growth phase by SDS-PAGE and zymography. However, no difference between these two was found in the profiles on SDS-PAGE and zymography (data not shown).

Because it is thought that the transcriptional level of *cwlK* is very low (Fig. 3), and some cell wall hydrolases are degraded by several proteases (Antelmann et al. 2002; Yamamoto et al. 2003), it is better to use Western blotting for the detection of CwlK. A protease-deficient mutant, cdD3FL (*cwlK*::*cwlK*-3xFLAG *wprA epr*), which has the *cwlK*-3xFLAG translational gene instead of original *cwlK*, was constructed for Western blotting. Figure 6A shows the results of Western blotting with extracted supernatant proteins and cell surface proteins from the same amount of cells (0.1 OD_{600}) of the cdD3FL strain. On Western blotting with anti-FLAG antibodies for detecting CwlK-3xFLAG, a band was detected for the supernatant fractions at OD_{600} of 0.6 (corresponding to the mid-vegetative growth phase) and OD_{600} of 1.5 (late-vegetative growth phase) (Fig. 6A, lanes 1 and 3). The detected band (about 20 kDa) corresponded to the calculated size of the truncated CwlK-3xFLAG (the putative signal peptide of CwlK being removed [20.0 kDa]). Thus, it is possible that CwlK is secreted or CwlK is | Fig. 6 shaved from membrane by some protease. At least, it is clear that CwlK cannot be localized on the cell wall because we could not detect any CwlK-3xFLAG in the cell surface fraction (Fig. 6A lanes 2 and 4).

To clear the localization of CwlK, new strain, CwlK3FLp (*cwlK*::[P*$_{spac}$*-*cwlK*-3xFLAG] *wprA epr*), which can express the *cwlK*-3xFLAG translational gene with IPTG, was constructed. Figure 6B shows the results of Western blotting with extracted supernatant proteins and whole cell proteins from the same amount of cells (0.01 OD_{600}) (This amount is 10 times lower than the amount of cells in Fig. 6A). As a result, when CwlK-3xFLAG was over-expressed by IPTG in CwlK3FLp

1   strain, a strong band could be mainly detected for whole cell proteins (Fig. 6B lane 2),

2   however few CwlK-3xFLAG was secreted in the culture (Fig. 6B lane 1). Moreover,

3   when the expression level of CwlK-3xFLAG was regulated by no addition of IPTG,

4   weakly expressed CwlK-3xFLAG was detected for whole cell proteins only (Fig. 6B

5   lane 4). Thus, from these results, it is probable that CwlK is localized in membrane and

6   CwlK is a lipoprotein. In other word, the detected CwlK in the culture may be a result

7   of shaving from membrane.

8   Atrih et al. described that vegetative peptidoglycan in *B. subtilis* does not contain only

9   L-Ala stem peptide (Atrih A et al. 1999). This result indicates that LD-endopeptidase

10   which digests the L-Ala-D-Glu linkage does not work during vegetative phase. However,

11   because Atrih et al. used nutrient broth (similar to DSM medium) for identification of

12   the component of vegetative peptidoglycan (Atrih A et al. 1999) and *cwlK* could not be

13   expressed in DSM (data not shown), it is possible that vegetative peptidoglycan may

14   contain L-Ala stem peptide when *B. subtilis* grows in LB medium. As another possibility,

15   CwlK may be able to work on the modification of peptidoglycan under some

16   conditions.

17

18   Amino acid sequence of CwlK

19   The amino acid sequence similarity of CwlK determined with the BLASTP program

20   in NCBI revealed that there is no paralog but many orthologs in bacteria including

21   *Bacillus, Oceanobacillus, Geobacillus, Exiguobacterium, Listeria,* and *Clostridium* in

22   the Firmicutes, *Pelodictyon* and *Prosthecochloris* in the Bacteroidetes/Chlorobi group,

23   and *Syntrophus, Xanthomonas, Bradyrhizobium, Chromobacterium, Dechloromonas,*

24   *Agrobacterium, Acinetobacter, Geobacter, Ralstonia, Burkholderia, Myxococcus* and

25   *Yersinia* in the Proteobacteria (bacteria with E-values of less than 0.001). Some phages

26   in *Listeria, Enterobacteria,* and *Vibrio* also contain homologues of CwlK. The

27   C-terminal region (nos. 43-164 with respect to the N-terminal amino acid) has been

28   assigned as an L-alanoyl-D-glutamate peptidase (Smith et al. 2000) and/or a

29   D-alanine-D-alanine carboxypeptidase (Pfam: http://www.sanger.ac.uk/Software/Pfam/).

30   In spite of the occurrence of a large number of homologous proteins, characterization of

31   the proteins has been quite limited. Phage proteins Ply500 and Ply118 of *Listeria,* and

32   VanY of *Enterococcus faecium* have been reported to be L-alanoyl-D-glutamate

1 peptidases (Loessner et al. 1995, 2002) and a D-alanine-D-alanine carboxypeptidase

2 (Reynolds et al. 2001), respectively.

3   From Fig. 5, it is clear that CwlK is an L-alanoyl-D-glutamate peptidase. Figure 7

4 shows alignment of the amino acid sequences of L-alanoyl-D-glutamate peptidases,

5 CwlK, Ply500 and Ply118. CwlK is very similar to Ply proteins, especially Ply500 (Fig.

6 7). Recently, the protein structure of Ply500 was determined (Protein Data Bank, Fig. 7

7 http://www.rcsb.org/pdb/Welcome.do; identification number: 1XP2). Loessner *et al.*

8 showed that Ply118 and Ply500 are able to hydrolyze the cell walls of three *Bacillus*

9 species (Loessner et al. 1995). Because the cell walls of *L. monocytogenes* and *B.*

10 *subtilis* have the A1γ-variation as to the peptidoglycan type (Schleifer and Kandler

11 1972), it may be possible that CwlK hydrolyzes the cell wall of *L. monocytogenes*.

12   It is also clear that CwlK is not a D-alanine-D-alanine carboxypeptidase because we

13 could not detect any DNP-D-Ala derived from the hydrolyzed cell wall (by CwlK)

14 labeled with FDNB (Fig. 5). We tried to align CwlK, Ply500 and Ply118 with VanY.

15 However, the similarities were not very high. Actually, the VanY structure, as shown in

16 the Protein Data Bank (identification number: 1QWY), seems not to be similar to that of

17 Ply500. Thus, it is indicated that CwlK is not a member of the VanY family, as stated in

18 the Pfam database.

19   We also tried to align CwlK and *B. subtilis* LytH, which was identified as an

20 L-alanoyl-D-glutamate peptidase (Horsburgh et al. 2003a). However, the similarity was

21 very low. LytH can act on spore peptidoglycan, whose structure is somehow different

22 from vegetative peptidoglycan (Foster and Popham 2002). It may be possible that CwlK

23 and LytH recognize different structures of peptidoglycan (cortex).

24   Finally, Ply118 and Ply500 are proteins of *Listeria monocytogenes* bacteriophages.

25 On the other hand, CwlK in *B. subtilis* is a bacterial protein, and it is actually expressed

26 (Fig. 3) and is localized in the membrane (Fig. 6B). Interestingly, CwlK seems to be

27 localized in the membrane as a lipoprotein, however, Ply118 and Ply500 seem to be

28 transported across the membrane with holing proteins (Loessner et al. 1995). Moreover,

29 more than 30 candidate peptidoglycan hydrolases are proposed in *B. subtilis* (Smith et al.

30 2000) and only 3 cell wall hydrolases, CwlK (YcdD), YddH (similar to lytic

31 transglycosylase and DL-endopeptidase) and YqiI (L-alanine amidase), are candidates of

32 lipoproteins. Thus, CwlK seems to be a very unique enzyme.

This is the first report describing the characterization of an L,D-endopeptidase in *B. subtilis* and also the first report of a member of the "PLY500" family of L,D-endopeptidases in *B. subtilis*. Since the L,D-endopeptidases reported previously were phage-encoded proteins, this is also the first report in bacteria of the characterization of a PLY500 family protein encoded in chromosomal DNA.

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22

1 **(Figure legends)**

2 Fig. 1. Structure of typical *B. subtilis* peptidoglycan of vegetative cells. The arrows

3 indicate hydrolytic bonds attacked by cell wall hydrolases: 1,

4 *N*-acetylmuramoyl-L-alanine amidase; 2, LD-endopeptidase; 3, DL-endopeptidase; 4,

5 carboxypeptidase; 5, DD-endopeptidase; 6, muramidase and lytic transglycosylase; 7,

6 *N*-acetylglucosaminidase. MurNAc, *N*-acetyl-D-muramic acid; GlcNAc,

7 *N*-acetylglucosamine.

8

9 Fig. 2. A map of *cwlK* (*ycdD*) and the neighboring genes in *B. subtilis*. *rapJ* (putative

10 response regulator aspartate phosphatase) and *ycdC* (function unknown) are upstream

11 and downstream of *cwlK*, respectively. The DNA fragments inserted into plasmids are

12 shown by lines (with the plasmid names) and the original vectors are indicated on the

13 right. The base and a.a. (amino acid) numbers are respect to the first A and the first

14 amino acid (methionine) of the translational start codon of *cwlK*, respectively. The black

15 box (1 aa-19 aa) is the putative lipoprotein signal peptide described by Tjalsma *et al.*

16 (2000). 167 aa means the amino acid number of the C-terminus of CwlK.

17

18 Fig. 3. Cell growth (open symbols) and β-galactosidase activity (closed symbols) of the

19 *cwlK-lacZ* transcriptional fusion (*cwlK*-minus) strains in LB medium. Circles, squares

20 and triangles indicate *B. subtilis* YCDDd (*cwlK*::*erm*), cdDSD (*cwlK*::*erm sigD*::*cat*),

21 and 168 (wild-type), respectively.

22

23 Fig. 4. SDS-14% polyacrylamide gel electrophoresis and zymography (14%

24 polyacrylamide gel) of h-ΔCwlK. h-ΔCwlK was over-expressed in *E. coli* BL21 cells

25 harboring pREP4 (*lacI kan*) with 1 mM IPTG. Lanes 1 and 2, proteins extracted from

26 whole cells (OD$_{600}$, 0.2) without and with 1 mM IPTG addition for 40 min, respectively;

27 lane 3, h-ΔCwlK (2 μg) purified on a HiTrap chelating column; lane 4, zymography of

28 purified h-ΔCwlK (2 μg) after 3 hours incubation at 37ºC in 50 mM MOPS-NaOH (pH

29 7.0) containing 1% Triton X-100. The arrow indicates the position of purified h-ΔCwlK.

30

31 Fig. 5. Determination of the sites of cleavage of peptidoglycan by CwlK. The cell wall

32 of *B. subtilis* 168 was treated without (A) or with h-ΔCwlK for 15 min (B) and 60 min

1 (C). After free amino groups in the samples had been labeled with FDNB, they were

2 hydrolyzed with HCl and then separated by RP-HPLC. Peak 1, mono-DNP-A_2pm; peak

3 2, unknown peak (it could not be identified by ESI-MS); peak 3, DNP-D-Glu; peaks 4

4 and 5, DNP. The values indicate elution times in minutes. mAU, milliabsorbance unit.

5

6 Fig. 6. Determination of the localization of CwlK-3xFLAG by Western blotting. (A)

7 The cdD3FL (*cwlK*::*cwlK*-3xFLAG *wprA epr*) strain was incubated in LB medium at

8 37ºC. At 0.6 (mid-vegetative phase) and 1.5 (late vegetative phase) OD_{600}, the cells and

9 supernatants were harvested. Preparations of supernatant proteins (lanes 1 and 3) and

10 cell surface proteins extracted from cells with LiCl (lanes 2 and 4) were carried out as

11 described by Yamaguchi *et al.* (2004) and Yamamoto *et al.* (2003). Proteins prepared

12 from the same amount of cells (0.1 OD_{600}) were applied to a SDS-gel for Western

13 blotting. (B) All strains were incubated in LB medium at 37ºC, and the cells and

14 supernatants were harvested at 1.7-1.9 of OD_{600}. Proteins from the supernatants (lanes 1,

15 3, and 5) and proteins extracted from the whole cells (lanes 2, 4, and 6) [the same

16 amount of cells (0.01 OD_{600})] were applied to a SDS-gel for Western blotting. Lane 1

17 and 2, CwlK3FLp (*cwlK*::[P*spac*-*cwlK*-3xFLAG] *wprA epr*) with 1 mM IPTG

18 (over-expression of CwlK-3xFLAG); lane 3 and 4, CwlK3FLp without IPTG (few

19 expression of CwlK-3xFLAG); lane 5 and 6, WE1 (*wprA epr*) (negative control). The

20 position of 20 kDa is derived from standard protein (MagicMark XP, Invitrogen) and

21 the arrow indicates the position of CwlK-3xFLAG.

22

23 Fig. 7. Alignment of the amino acid sequences of CwlK and LD-endopeptidases, Ply500

24 and Ply118. The numbers are with respect to the N-terminal amino acid of each protein.

25 Shading indicates conserved amino acid residues. CwlK_BACSU, *Bacillus subtilis*

26 CwlK; AEPE_BPA50, Ply500 in *Listeria monocytogenes* bacteriophage A500;

27 AEPE_BPA18, Ply118 in *Listeria monocytogenes* bacteriophage A118.

28

1   **Table 1** Strains and plasmids used in this study

| Strain or plasmid	Genotype	Source or Reference
B. subtilis strain		
168	*trpC2*	D. Ehrlich
YCDDd	*trpC2 cwlK* (*ycdD*)::*erm*	pM4ycdD→168
168SDC	*trpC2 sigD*::*cat*	Serizawa et al. 2004
cdDSD	*trpC2 cwlK*::*erm sigD*::*cat*	168SDC ch.[a]→YCDDd
YCDDp	*trpC2 cwlK*::[P*spac*-*cwlK erm*]	pM4SDcdD→168
WE1	*trpC2 wprA*::*kan epr*::*tet*	Yamamoto et al. 2003
cdD3FL	*trpC2 wprA*::*kan epr*::*tet* *cwlK*::*cwlK*-3xFLAG	pCA3FLcdD→WE1
CwlK3FLp	*trpC2 wprA*::*kan epr*::*tet* *cwlK*::[P*spac*-*cwlK*-3xFLAG *cat erm*]	YCDDp→cdD3FL
E. coli strain		
JM109	*recA*1 Δ(*lac-proAB*) *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *relA*1 *SupE*44 [F' *traD*36 *proAB*+ *lacI*q *lacZ* ΔM15]	Takara
C600	*supE44 hsdR17 thi-1 thr-1 leuB6 lacY1 tonA21*	Laboratory stock
BL21	*ompT hsdS gal*	Laboratory stock
Plasmids		
pMUTIN4	*bla erm lacZ lacI*	D. Ehrlich
pCA3xFLAG	*bla cat* 3xFLAG	N. Ogasawara and K. Kobayashi
pGEM3Zf(+)	*bla* Δ*lacZ*	Promega
pQE-30	*bla*	QIAGEN
pREP4	*lacI kan*	QIAGEN
pM4ycdD	pMUTIN4::Δ*cwlK* (*ycdD*)	This study
pM4SDcdD	pMUTIN4::Δ*cwlK* (*ycdD*) with SD sequence of *cwlK*	This study
pCA3FLcdD	pCA3xFLAG::Δ*cwlK*	This study
pGEMΔcdD	pGEM3Zf(+)::Δ*cwlK*	This study
pQEΔcdD	pQE-30::Δ*cwlK*	This study

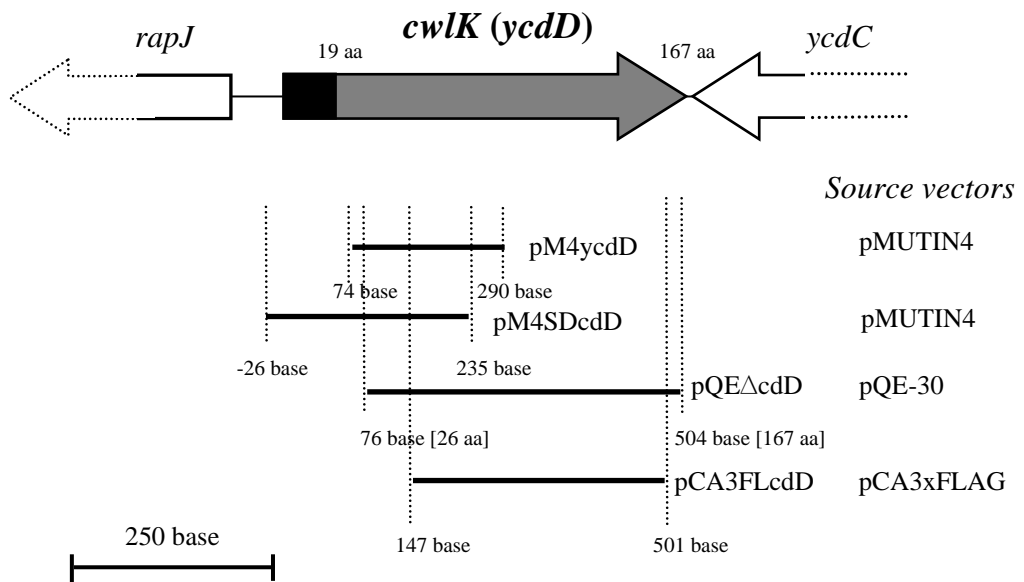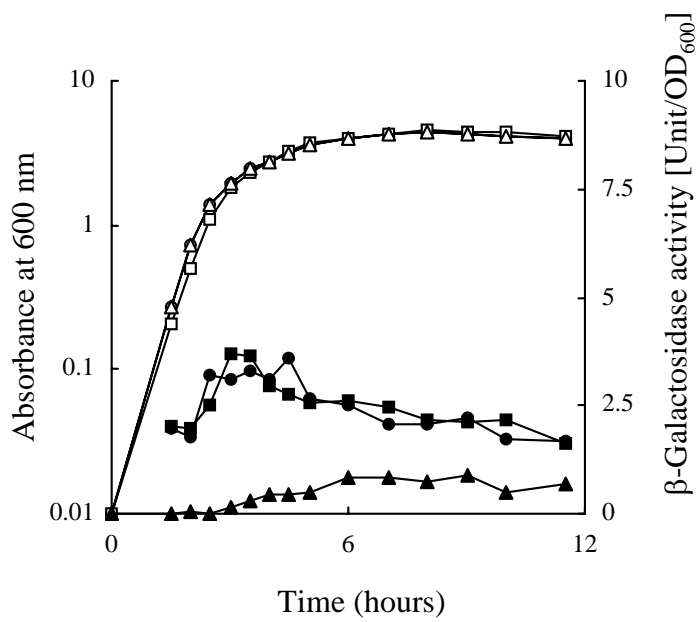2   [a]ch., chromosomal DNA.

3
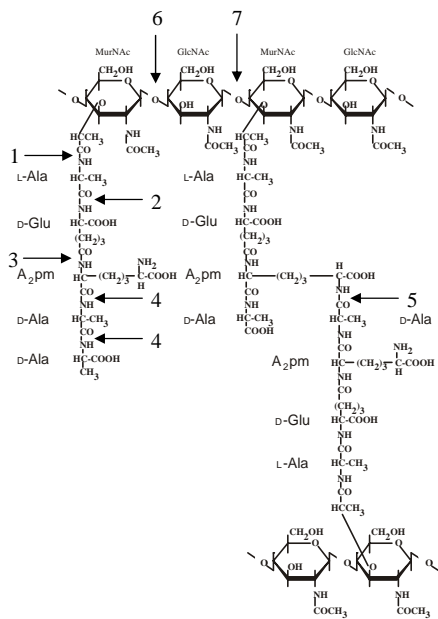
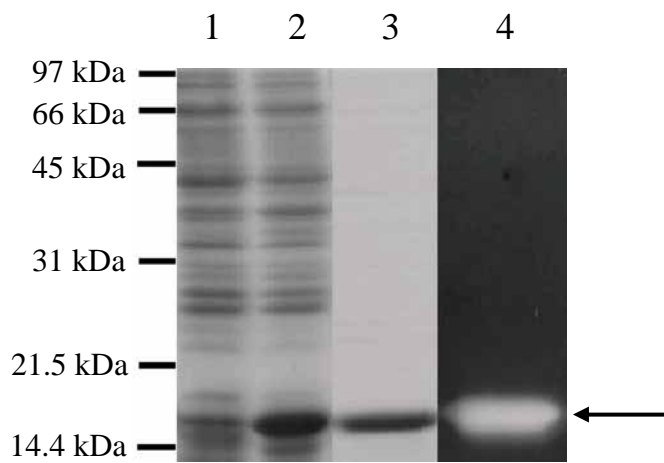Fig. 1. Fukushima *et al.*

Fig. 2. Fukushima *et al.*

Fig. 3. Fukushima *et al.*

Fig. 4. Fukushima *et al.*

(A)

(B)

(C)

Fig. 5. Fukushima *et al.*

(A)

0.6 of OD$_{600}$

1	2

(kDa)

21.5

1.5 of OD$_{600}$

3	4

(kDa)

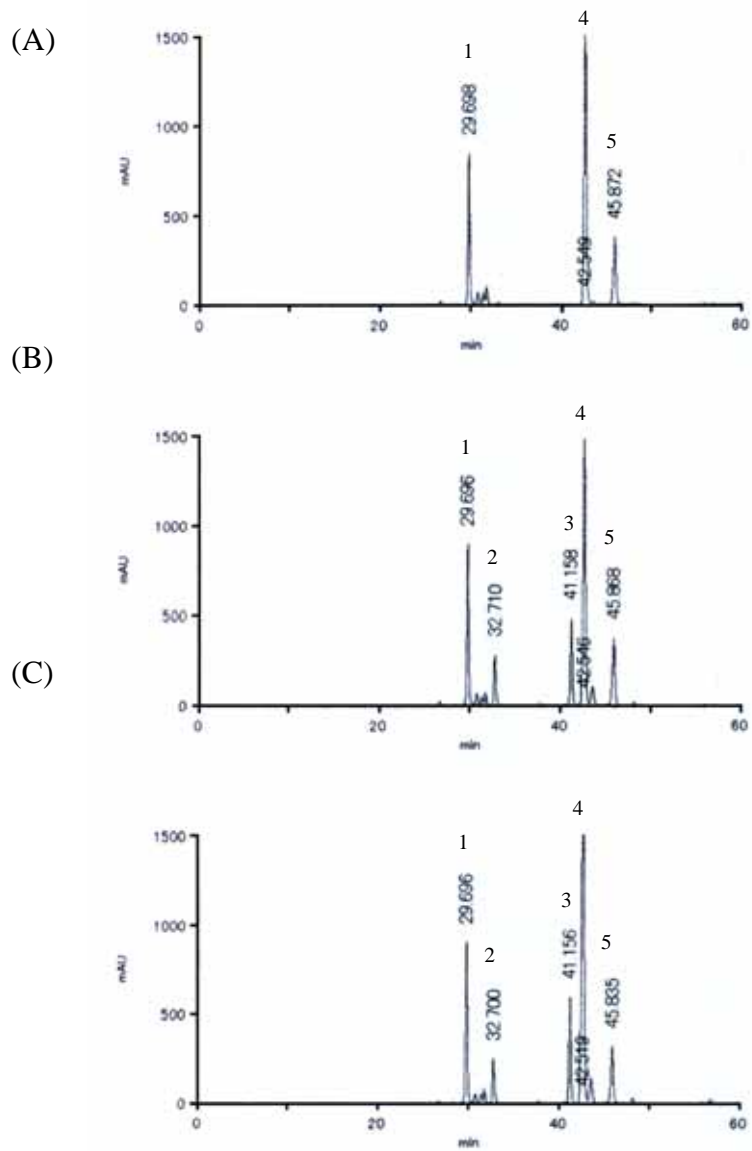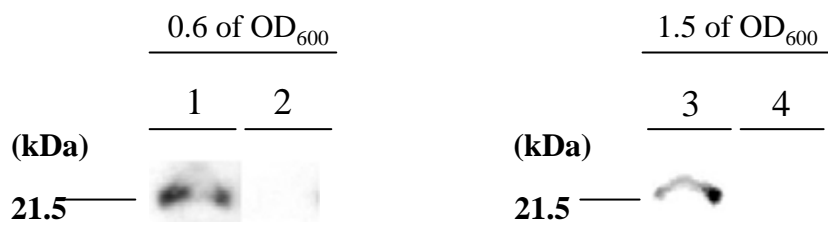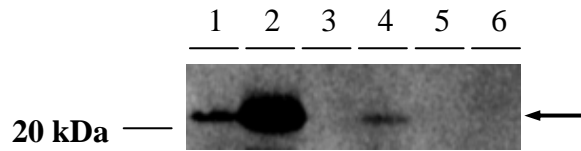**21.5**

(B)

1	2	3	4	5	6

20 kDa

Fig. 6. Fukushima *et al.*

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CwlK_BACSU   MNLPAKTFVILCILFLLDLCFSYIRHE WHSQNALQDMPVPSD LHPIVKQNADALKAAAAN       60
AEPE_BPA50   --------------------MALTEA WLIEKANRKLNAG-GMYK ITSDKTRNVIKKMAK           38
AEPE_BPA18   -------------------------MTSYYYS RSLANVNKLADNTKAAARKLLDWSES            33

CwlK_BACSU   KGIDVVITEGFRSFKEQDELYKQGRTKKGNIVTYARGGESYHNYGLAIDFALQKKDG-SI        119
AEPE_BPA50   EGIYLCVAQGYRSTAEQNALYAQGRTKPGAIVTNAKGGQSNHNYGVAVDLCLYTNDGKDV         98
AEPE_BPA18   NGIEVLIYETIRTKEQQAANVNSG---------ASQTMR SYHLVGQALDFVMA—-KGKTV         82

CwlK_BACSU   IWDMEYDGNQNGK SDWLEVVEIAKTLGFEWGGDWK RFKDYPHLE MIPN------------       167
AEPE_BPA50   IWESTT------- SRWKKVVAAMKAEGFKWGGDWK SFKDYPH FELCDAVS GEKIPAATQN      151
AEPE_BPA18   DWGAYRS------DKG KKFVAKAKSLGFEWGGDW SGFVDNPHLQFNYKGY GTDTFGKGAS       136
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Fig. 7. Fukushima *et al.*