The synthetic lethality of *lytE cwlO* in *Bacillus subtilis* is caused by lack of D,L-endopeptidase activity at the lateral cell wall

Masayuki Hashimoto,[1] Seika Ooiwa,[2] and Junichi Sekiguchi[2,*]

[1] International Young Researchers Empowerment Center, [2] Department of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, Ueda, Japan

Running title: Synthetic lethality of *lytE cwlO* in *B. subtilis*

Section: Genetics and Molecular Biology

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

Tel: +81 268 21 5344; Fax: +81 268 21 5344; E-mail: jsekigu@shinshu-u.ac.jp

(**Abstract)**

Bacterial peptidoglycan acts as an exoskeleton to protect the bacterial cell. Although peptidoglycan biosynthesis by penicillin-binding proteins is well studied, few studies have described peptidoglycan disassembly, which is necessary for a dynamic structure that allows cell growth. In *Bacillus subtilis*, more than 35 genes encoding cell wall lytic enzymes have been identified; however, only two D,L-endopeptidases (*lytE* and *cwlO*) are involved in cell proliferation. In this study, we demonstrated that the D,L-endopeptidase activity at the lateral cell wall is essential for cell proliferation. Inactivation of LytE and CwlO by point mutation of the catalytic residues caused cell growth defects. However, the forced expression of LytF or CwlS, which are paralogs of LytE, did not suppress *lytE cwlO* synthetic lethality. Subcellular localization studies of these D,L-endopeptidases showed LytF and CwlS at the septa and poles, CwlO at the cylindrical part of the cell, and LytE at the septa and poles as well as the cylindrical part. Furthermore, construction of N-terminal and C-terminal domain-swapped enzymes of LytE, LytF, CwlS, and CwlO revealed that localization was dependent on the N-terminal domains. Only the chimeric proteins that were enzymatically active and localized to the sidewall were able to suppress the synthetic lethality, suggesting that lack of D,L-endopeptidase activity at the cylindrical part of the cell leads to a growth defect. The functions of LytE and CwlO in cell morphogenesis were discussed.

(Introduction)

Autolysins are bacterial cell wall lytic enzymes found in all bacteria that possess peptidoglycan. In the *Bacillus subtilis* genome, more than 35 definite or probable autolysin genes have been identified and shown to be involved in cell morphogenesis, cannibalism, sporulation, and germination. (22, 25). The bacterial peptidoglycan sacculus requires a dynamic structure for cell elongation and separation; therefore, a balance between peptidoglycan synthesis and disassembly is essential for cell proliferation. Although a number of autolysins are thought to be involved in peptidoglycan disassembly, none have been found to be essential for cell growth, perhaps due to their functional redundancy. However, it was recently reported that disruption of both *lytE* and *cwlO* in *B. subtilis* is lethal (4). To date this is the sole report of an autolysin mutant of *B. subtilis* with a serious growth defect. Bisicchia *et al.* also demonstrated that *cwlO* depletion in a *lytE* disrupted background strain impairs cell elongation (4).

LytE and CwlO are D,L-endopeptidases that hydrolyze the linkage of D-γ-glutamyl-*meso*-diaminopimelic acid in peptidoglycan (13, 27). The *B. subtilis* genome contains seven D,L-endopeptidase genes. The mature forms of LytE, LytF, and CwlS all contain N-terminal LysM repeats, although the number of LysM domains differs, and C-terminal D,L-endopeptidase domains belonging to the NlpC/P60 family. Although phenotypes of single-gene knockout mutants were indistinguishable from that of wild type, multiple gene disruptions led to a chained-cell morphology (10, 13, 19), suggesting that these proteins are involved in cell separation. In contrast, CwlO contains a domain with unknown function at the N-terminus and a D,L-endopeptidase domain at the C-terminus. The phenotype of the *cwlO* mutant was also indistinguishable from wild

type, but the *lytE cwlO* double disruption leads to synthetic lethality (4, 27). Two D,L-endopeptidase genes (*pgdS* and *cwlT*) are not likely to be involved in cell morphology, because the *pgdS* gene encodes a poly-γ-glutamic acid degradase, and the *cwlT* gene is part of an integrative and conjugative element (11, 23). The other gene is a function-unknown *ykfC*. Results of these previous studies indicate that LytE, LytF and CwlS are cell separation enzymes, and LytE and CwlO are associated with cell growth. Thus, although their catalytic domains show high amino acid sequence similarity, these enzymes play different physiological roles in cell morphology. To elucidate the roles of LytE and CwlO in cell morphogenesis, we investigated the main factors causing synthetic lethality in *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1 and Table S1 in the Supplementary material, respectively. *B. subtilis* 168 was used as the parent strain throughout this study. The details of the strains and plasmids constructs used in this study are presented in the Supplementary material. All constructed strains were confirmed by PCR.

General methods. The *B. subtilis* and *Escherichia coli* strains were grown at 37˚C in Luria Broth (LB) (21). When required, antibiotics and chemical inducers were added in the following concentrations: ampicillin, 100 μg/ml; tetracycline, 5 μg/ml; kanamycin, 25 μg/ml; spectinomycin, 50 μg/ml; erythromycin, 0.3 μg/ml chloramphenicol, 5 μg/ml; isopropyl β-D-1-thiogalactopyranoside (IPTG), 1 mM; and xylose, 1%.

DNA manipulation and *E. coli* transformation were performed using standard methods (21). *B. subtilis* transformation was performed by conventional transformation

procedures (1).

Sample preparation for immunofluorescence microscopy (IFM). Cells harvested from an overnight culture in LB medium were diluted 50-fold in 5 ml fresh LB medium. The cells were grown to the late exponential growth phase (optical density at 600 nm [OD_{600nm}] = 2.0), and then the precultured cells were inoculated into fresh LB medium to give an initial absorbance of OD_{600nm} = 0.001. Cells corresponding to 0.3 of the OD_{600nm} unit for WECLytE6FL (LytE-6×FLAG), OH015 (CWB_{LytE}-6×FLAG), WECO6FL (CwlO-6×FLAG), OH013 (overexpressed CwlO-6×FLAG), or OH018 (overexpressed NTD_{CwlO}-6×FLAG) were collected when each culture reached OD_{600nm} = 0.1. As described below, LytE-6×FLAG and CwlO-6×FLAG were functional for *B. subtilis* cell proliferation. Likewise, 0.3 of the OD_{600nm} unit cells were collected for WECLytF6FL (LytF-6×FLAG) and OH014 (CWB_{LytF}-6×FLAG) when the cultures reached OD_{600nm} = 0.6. Similarly, 0.3 of the OD_{600nm} unit cells were collected for WECS6FL (CwlS-6×FLAG) and OH016 (CWB_{CwlS}-6×FLAG) when each culture reached OD_{600nm} = 2.0. To determine the subcellular localization of the domain-swapped chimeric enzymes, cells were collected when the cultures reached OD_{600nm} = 0.3 (for chimeric proteins transcribed from the *lytE* promoter) or OD_{600nm} = 0.1 (for those transcribed from the *cwlO* promoter). Cell samples were prepared for IFM as described previously (30).

Fluorescence microscopy. Fluorescence microscopy was performed as described previously (29) with an Olympus BX61 microscope equipped with a BX-UCB control unit, a UPPlan Apo Fluorite phase-contrast objective (×100 magnification; numerical aperture, 1.3), and a standard rhodamine filter set for visualizing Cy3. Exposure times

were 0.1 s for phase-contrast microscopy and 0.1s (gain 2) for Cy3. The cells were photographed with a charge-coupled device camera (CoolSNAP HQ; Nippon Roper) driven by MetaMorph software (version 4.6; Universal Imaging). For Cy3 imaging, out-of-focus light was removed using the two-dimensional deconvolution utility of the AutoDeblur software. All images were processed with Adobe Photoshop software.

Western blot analysis and zymography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 14% (w/v) polyacrylamide gels as described previously (15). For western blot analysis, the 6×FLAG-fused proteins were separated by 14% SDS-PAGE gels. After electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes (Invitrogen) in a transfer buffer (25 mM Tris, 192 mM glycine, 20% [v/v] methanol, 0.1% SDS) using a semidry blotting system (Bio-Rad). Immunoblot detection was carried out as described in the instruction manual for the ECL Plus Western Blotting Detection System (Invitrogen) using a mouse anti-FLAG M2 monoclonal antibody (Sigma) and horseradish peroxidase-labeled anti-mouse IgG antibody. Zymography was performed as described previously using 14% SDS-PAGE gels containing 0.5 mg/ml *B. subtilis* cell wall extract (17). The cell wall derived from *B. subtilis* 168 was prepared as described previously (8, 19). Renaturation was performed at 37°C in a renaturation solution (25 mM Tris-HCl [pH 7.2], 1% [v/v] Triton X-100) as described previously (10).

RESULTS

D,L-Endopeptidase activity of LytE or CwlO is essential for cell proliferation. The catalytic domains of LytE and CwlO belong to the NlpC/P60 family, which

hydrolyzes the γ- D -glutamyl-*meso*-diaminopimelic acid linkage or *N*-acetylmuramoyl-L-alanine linkage. In this superfamily of papain-like enzymes, a conserved cysteine residue was predicted to be a catalytic residue on amino acid sequence alignment (2, 16). Recently, the three-dimensional structures of NlpC/P60 enzymes were reported (Spr from *E. coli*, ABA23003 from *Anabaena variabilis*, and ACC79413 from *Nostoc punctiforme*) (3, 26). In these enzymes, the conserved cysteine residues are located at a predicted active site and are structurally conserved. To determine whether the conserved cysteine residues are involved in the catalytic activity of D,L-endopeptidases, we constructed point mutations in LytE and CwlO, replacing the conserved cysteine residue with a serine residue ($LytE_{C247S}$ and $CwlO_{C377S}$). To evaluate the lytic activities of these mutated enzymes, the intact or mutated catalytic domains of LytE and CwlO were expressed in *E. coli*, and zymography was carried out with the cell lysates using *B. subtilis* cell wall as a substrate (see Fig. S1B in the Supplementary material). The intact catalytic domains of LytE and CwlO exhibited cell wall-degrading activity, but mutants in which the cysteine residue had been replaced appeared to be inactive. This finding suggests that the conserved cysteine residue is important for the catalytic activity of NlpC/P60 enzymes.

Next, we examined whether the D,L-endopeptidase activities of LytE and CwlO are involved in the synthetic lethality of the *lytE cwlO* double mutants (Fig. 1A and B). OH004 (*lytE-6×flag* P_{xyl}-*cwlO*) grew normally without xylose induction of CwlO, indicating that LytE-6×FLAG was functional. In contrast, the growth of OH005 ($lytE_{C247S}$-*6×flag* P_{xyl}-*cwlO*) was normal in the presence of xylose, but was arrested in the absence of xylose. Similarly, CwlO-6×FLAG was functional, but OH007 ($cwlO_{C377S}$-*6×flag* P_{spac}-*lytE*) showed growth arrest without LytE induction by IPTG.

These results indicate that the D,L-endopeptidase activity of either LytE or CwlO is essential for cell proliferation.

As described above, LytE, LytF, and CwlS exhibit similar domain structures. However, *lytE* expression is regulated by σ^A and σ^H, *cwlO* expression is regulated by σ^A, and *lytF* and *cwlS* are regulated by σ^D and σ^H, respectively (5, 13, 19, 27). The σ^D and σ^H regulons are induced later than the σ^A regulon. Therefore, although LytF and CwlS can suppress the synthetic lethality, the LytE CwlO double-depleted cells may be dead before LytF or CwlS can be expressed. Consequently, OH009 (Δ*lytE* P_{xyl}-*cwlO* P_{spac}-*lytF*) and OH012 (Δ*lytE* P_{xyl}-*cwlO* P_{spac}-*cwlS*) were constructed to determine whether induction of LytF or CwlS could suppress the synthetic lethality. These strains were cultured in the presence of 1 mM IPTG to induce LytF or CwlS and in the presence or absence of 1% xylose to induce CwlO (Fig. 1C, D). Both strains grew normally when CwlO was expressed; however, growth was arrested by CwlO depletion, even though LytF or CwlS was expressed. The hydrolytic activities of induced LytF and CwlS were confirmed by zymography with *B. subtilis* cell wall as a substrate (see Fig. S2 in the Supplementary material). We found that LytF and CwlS are not able to suppress the LytE CwlO-depleted synthetic lethality, even though their domain structures are similar to that of LytE.

Subcellular localization of *B. subtilis* D,L-endopeptidases. The C-terminal D,L-endopeptidase domains of LytE, LytF, CwlS, and CwlO show strong sequence similarity. In contrast, the N-terminal domains of LytE, LytF, and CwlS contain different numbers of the LysM repeats, and the N-terminus of CwlO contains a COG3883 domain. Although the D,L-endopeptidase activity of either LytE or CwlO is essential for cell proliferation, forced expression of LytF or CwlS did not suppress the *lytE cwlO*

synthetic lethality. These results suggest that the N-terminal domains are important for the function of the D,L-endopeptidases. Previously, we reported that *B. subtilis* WE1, a strain with defects in extracellular proteases WprE and Epr, accumulates D,L-endopeptidases on the cell surface (29). Therefore, we evaluated the subcellular localization of FLAG-tagged LytE, LytF, CwlS, and CwlO (full-length proteins and N-terminal domains) by IFM with *wprE epr*-deleted WEC background strains. Because these D,L-endopeptidases are regulated by different σ factors, we also evaluated the localization of these enzymes during different growth phases. Full-length LytE and CwlO and their N-terminal domains (CWB_{LytE} and NTD_{CwlO}, respectively) were observed during early exponential growth phase (OD_{600nm} = 0.1), full-length LytF and its N-terminal domain (CWB_{LytF}) were observed in mid-exponential growth phase (OD_{600nm} = 0.6), and full-length CwlS and its N-terminal domain (CWB_{CwlS}) were observed in early stationary phase (OD_{600nm} = 2.0). The results showed that LytE is localized at the cell septa, poles, and sidewall (Fig. 2A). LytF-6×FLAG and CwlS-6×FLAG were localized at the cell septa and poles, but neither was detected at the lateral cell wall (Fig. 2C and E). CwlO-6×FLAG expressed from the intact promoter was weakly detected at the lateral cell wall but not at the septa or poles (Fig. 2G). To better assess CwlO localization, we then used a CwlO-6×FLAG-overexpressing strain (Fig. 2H), which increased cell surface CwlO-6×FLAG expression to 2.4 times that of normal, as determined by western blot analysis (data not shown). The overexpressed CwlO-6×FLAG was more clearly visualized at the sidewall but not detected at the cell septa or poles. To determine whether the localization of these D,L-endopeptidases depends on the N-terminal domain, we investigated the subcellular localization of the N-terminal domains under the same conditions used for the full-length proteins (Fig. 2B,

D, F, and I). The localization pattern of each N-terminal domain was identical to that of the corresponding full-length protein, indicating that these D,L-endopeptidases localized on the cell surface through their N-terminal domains.

Characterization of domain-swapped D,L-endopeptidases. IFM analysis demonstrated that LytF and CwlS (involved in cell separation) localize to the septa and poles, CwlO (involved in cell elongation) localizes to the lateral cell wall, and LytE (involved both in cell separation and elongation) localizes to the septa, poles, and lateral cell wall. These results suggest that the functions of these D,L-endopeptidases depend on their subcellular localization. To test this hypothesis, we generated domain-swapped D,L-endopeptidases and examined their ability to suppress the *lytE cwlO* synthetic lethality.

Domain-swapped D,L-endopeptidases (other than $N_{LytF}C_{LytE}$) were generated by C-terminal domain substitution at the original genetic loci of the N-terminal domains. For example, $N_{LytE}C_{CwlS}$ was constructed by substituting the C-terminal domain of LytE with that of CwlS at the *lytE* locus. Thus, the chimeric genes were transcribed from the promoters of the gene encoding the N-terminal domain. However, $N_{LytF}C_{LytE}$ was constructed by substituting the N-terminal domain of LytE with that of LytF at the *lytE* locus; the chimeric gene was transcribed from the *lytE* promoter. All chimeric proteins were fused to a 6×FLAG tag at the C-terminus to evaluate their expression and localization. Expression was confirmed by western blot analysis, and the chimeric proteins were detected at positions corresponding to the predicted molecular sizes (Fig. 3A). Enzyme activity was assessed by zymography using the *B. subtilis* cell wall as a substrate (Fig. 3B). The results show that the chimeric enzymes containing the CwlO N-terminal domain did not retain cell wall-degrading activity. The C-terminal

D,L-endopeptidase regions of $N_{CwlO}C_{LytF}$ and $N_{CwlO}C_{CwlS}$ are the same as those of $N_{LytE}C_{LytF}$ and $N_{LytE}C_{CwlS}$, respectively. Since $N_{LytE}C_{LytF}$ and $N_{LytE}C_{CwlS}$ exhibited cell wall-degrading activity, it was assumed that the C-terminal D,L-endopeptidase domains of $N_{CwlO}C_{LytF}$ and $N_{CwlO}C_{CwlS}$ would exhibit enzyme activity as well; however, it is possible that the N-terminal region of CwlO interfered with the C-terminal D,L-endopeptidase domain activity in $N_{CwlO}C_{LytF}$ and $N_{CwlO}C_{CwlS}$. Next, the subcellular localization of these domain-swapped D,L-endopeptidases was visualized by IFM (Fig. 4). The chimeric proteins containing the LytE N-terminal domain ($N_{LytE}C_{LytF}$ and $N_{LytE}C_{CwlS}$) localized to the cell septa, poles, and lateral cell wall, similar to the localization of LytE-6×FLAG and CWB_{LytE}-6×FLAG. However, $N_{LytF}C_{LytE}$ localized only to the cell septa and poles, like LytF-6×FLAG and CWB_{LytF}-6×FLAG. Only weak fluorescence of the chimeric enzymes containing the N-terminal domain of CwlO ($N_{CwlO}C_{LytF}$ and $N_{CwlO}C_{CwlS}$) was detected. However, enhancing the signal intensity of IFM images revealed that these chimeric enzymes were localized to the sidewall, similar to full-length CwlO and its N-terminal domain. These results demonstrate that the N-terminal domains of D,L-endopeptidases determine their subcellular localization. Finally, we assessed whether these domain-swapped D,L-endopeptidases were able to suppress the *lytE cwlO* synthetic lethality (Fig. 4). The transcription of *cwlO* was induced by xylose in strains expressing LytE or LytF N-terminal domain-containing chimeric enzymes ($N_{LytE}C_{LytF}$, $N_{LytE}C_{CwlS}$, or $N_{LytF}C_{LytE}$), whereas *lytE* gene transcription was induced by IPTG in strains expressing the CwlO N-terminal domain-containing chimeric enzymes ($N_{CwlO}C_{LytF}$ and $N_{CwlO}C_{CwlS}$). After exposure to the appropriate inducer, an aliquot of each culture was washed to remove the inducer, and the cells were inoculated into fresh medium with or without the inducer. OH019 (*lytE*::$N_{LytE}C_{LytF}$

P_{xyl}-*cwlO*) and OH020 (*lytE*::$N_{LytE}C_{CwlS}$ P_{xyl}-*cwlO*) were found to partially suppress the *lytE cwlO* synthetic lethality without xylose induction of *cwlO*. As described above, these chimeric proteins were enzymatically active and detected at the cell septa, poles, and sidewall. However, strains expressing chimeric proteins containing the CwlO N-terminal domain (OH023 [*cwlO*::$N_{CwlO}C_{LytF}$ P_{spac}-*lytE*] and OH024 [*cwlO*::$N_{CwlO}C_{CwlS}$ P_{spac}-*lytE*]), which were not enzymatically active, were localized at the lateral cell wall, but not able to grow without IPTG induction of *lytE*. Furthermore, lack of xylose caused the growth arrest of OH022 (*lytE*::$N_{LytF}C_{LytE}$ P_{xyl}-*cwlO*). This strain expressed $N_{LytF}C_{LytE}$, which retained enzymatic activity but was not localized at the cellular sidewall.

Taken together, our findings show that only strains expressing at least one active D,L-endopeptidase localized at the lateral cell wall were able to proliferate. Therefore, we conclude that localization of D,L-endopeptidase activity at the lateral cell wall is essential for cell proliferation.

DISCUSSION

Peptidoglycan forms a network on the outer surface of bacterial cells. The dynamic structure of the peptidoglycan sacculus allows cell growth; therefore, maintaining the balance of peptidoglycan synthesis and disassembly is important. To the best of our knowledge, the synthetic lethality of *lytE cwlO* in *B. subtilis* is the only report of an autolysin mutant with a serious growth defect (4). In this study, we found that subcellular localization of these enzymes is determined by their N-terminal domains, and synthetic lethality is caused by the lack of D,L-endopeptidase activity at the lateral cell wall. The D,L-endopeptidases required for cell separation (LytE, LytF, and CwlS) were detected at the septa and poles, and the enzymes involved in cell elongation (LytE

and CwlO) were detected at the cylindrical part of the cell. These results strongly suggest that the function of these autolysins depends on their subcellular localization. Our findings are consistent with a previous study reporting that a *lytF cwlO* double mutant and a *lytE lytF cwlS* triple mutant were not defective in cell growth (10, 27).

LytE and CwlO may participate in loosening the peptidoglycan sacculus of *B. subtilis* during growth. The cell wall of *B. subtilis* is comprised of multi-layered thick peptidoglycan. Electron microscopy images show that the thick peptidoglycan consists of three distinct parts (18). Results of pulse-labeling studies revealed a delay between the incorporation of new material into the cell wall and its eventual appearance in the culture (12, 20). These results suggest that the inner zone of the thick peptidoglycan contains the newly synthesized layers, and the outer zone consists of old peptidoglycan (i.e., inside-to-outside peptidoglycan sacculus formation) (12, 18, 20). Peptidoglycan-synthesizing enzymes are anchored to cytoskeleton proteins (MreB homologs and FtsZ), and localize to the outside surface of the cytoplasmic membrane (6). Thus, the peptidoglycan-synthesizing enzymes are accessible to the inner zone of peptidoglycan. Degradation of the outer zone loosens the cell wall, enabling construction of a new peptidoglycan layer inside the preexisting peptidoglycan sacculus (22). Since *lytE cwlO* double disruption leads to synthetic lethality and impaired cell elongation, these autolysins are strong candidates for participation in the peptidoglycan dynamics. Consistent with this hypothesis, our results show that the cell elongation defect due to the *lytE cwlO* disruption is caused by the absence of D,L-endopeptidase activity at the lateral cell wall. However, results of a pulse-labeling experiment show that the rate of *N*-acetylglucosamine incorporation is not the same for *lytE* and *cwlO* mutants, demonstrating that LytE behavior differs from that of CwlO (4). LytE and CwlO differ in

their subcellular localizations and specific activities (28). In addition, CwlO was rapidly degraded and released into culture medium, whereas most of LytE adsorbed to cell surface (27). Taken together, these findings demonstrate that although these two enzymes possess similar D,L-endopeptidase domains, they appear to have different functions in cell growth.

A previous study reported that LytE-3×FLAG transcribed from the *lytE* original promoter was observed at the septa and poles (29). However, slightly overexpressed LytE fused to a green fluorescent protein localized in a helical manner along the cylindrical wall of growing cells in addition to the poles and septa (7). In the present study, we observed the localization of 6xFLAG-tagged LytE transcribed from the original *lytE* promoter by IFM (Fig. 2A). The fluorescence intensity of the 6×FLAG fusion protein is more intense than that of the 3×FLAG fusion protein, which may be the reason we were able to detect LytE-6×FLAG at the sidewall. The work of Carballido-López *et al.* strongly suggests that LytE-GFP is localized at the sidewall in a helical manner, similar to the localization pattern of MreB homologs. CwlO-6x FLAG also localized to the lateral cell wall, but was not detected at the cell poles or septa (Fig. 2G). Although the fluorescence of the 6xFLAG-tagged CwlO was weak, staggered spots around the sidewall suggested a helical localization pattern. We then investigated whether MreB homologs are involved in the lateral localization of CwlO; however, the mutation of MreB homologs did not alter CwlO localization (data not shown).

Subcellular localization of the N-terminal domains of the four D,L-endopeptidases was similar to that of the corresponding full-length protein, suggesting that localization was determined by their N-terminal domains. This finding was supported by the localization of chimeric enzymes, which was similar to that of their N-terminal domains.

The localization of the LytF N-terminal domain at the cell poles and septa was previously reported (30). As expected, the localization of LytE and CwlS was dependent on their N-terminal domains, which contained LysM repeats like that of LytF. Yamamoto *et al.* also reported a helical localization of LytF-6×FLAG at the sidewall after partial removal of wall teichoic acid (30), suggesting that the cylindrical localization of N-terminal domains of LytE and CwlS are regulated by wall teichoic acid. Carballido-López *et al.* reported that LytE localization at the sidewall is dependent on MreBH, indicating that MreBH may regulate wall teichoic acid localization (7). It was reported that, the helical localization of the major wall teichoic acid synthesis proteins was not altered in three *mreB* homolog single mutants (9). However, we note that these cells were cultured with 20 mM $MgCl_2$, which suppresses *mreB* homolog deficiency (14).

The CwlO N-terminus contains a COG3883 domain, which is an uncharacterized conserved domain in bacteria. According to Teng *et al.*, a secreted antigen (SagA) from *Enterococcus faecium* containing a COG3883 domain showed broad-spectrum binding to extracellular matrix proteins such as fibrinogen, collagen type I, collagen type IV, fibronectin, and laminin (24). However, full-length CwlO and its N-terminal domain did not bind some of the matrix proteins evaluated in this study (data not shown). The SagA protein migrated more slowly on cell wall-containing PAGE than on SDS-PAGE, suggesting an interaction between SagA and the cell wall (24); however, the purified CwlO protein did not bind to the cell wall *in vitro* (27). In the present study, we demonstrated the involvement of the CwlO N-terminal domain in cell surface localization. Taken together, these results suggest that CwlO interacts directly, but weakly, with the cell wall or a cell surface protein.

In this study, we found that the subcellular localization of LytE, LytF, CwlS, and CwlO is dependent on their N-terminal domains, and that D,L-endopeptidase activity at the lateral cell wall is essential for cell proliferation. These results strongly suggest that LytE and CwlO are involved in cell elongation and support the inside-to-outside model for peptidoglycan sacculus formation. A more detailed study is necessary to clarify the role of D,L-endopeptidases in peptidoglycan dynamics and characterize the localization mechanisms of these proteins.

ACKNOWLEDGMENTS

We would like to thank the members of our group, particularly Hiroki Yamamoto and Tatsuya Fukushima, for the helpful advice and discussion. We also thank N. Hariyama and Y. Miyake for technical assistance with strain construction and microscopy analysis. This work was supported by Grants-in-Aid for Scientific Research (B) (19380047) and (A) (22248008), the New Energy and Industrial Department Organization (NEDO), the Global COE programs (JS), and the Program for Dissemination of Tenure-Track System funded by the Ministry of Education and Science, Japan (MH).

REFERENCES

1. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81:**741-746.
2. **Anantharaman, V., and L. Aravind.** 2003. Evolutionary history, structural features and biochemical diversity of the NlpC/P60 superfamily of enzymes. Genome Biol. **4**: R11.

3. **Aramini, J. M., P. Rossi, Y. J. Huang, L. Zhao, M. Jiang, M. Maglaqui, R. Xiao, J. Locke, R. Nair, B. Rost, T. B. Acton, M. Inouye, and G. T. Montelione.** 2008. Solution NMR structure of the NlpC/P60 domain of lipoprotein Spr from *Escherichia coli*: Structural evidence for a novel cysteine peptidase catalytic triad. Biochemistry. **47:**9715-9717.

4. **Bisicchia, P., D. Noone, E. Lioliou, A. Howell, S. Quigley, T. Jensen, H. Jarmer, and K. M. Devine.** 2007. The essential YycFG two-component system controls cell wall metabolism in *Bacillus subtilis*. Mol. Microbiol. **65:**180-200.

5. **Britton, R. A., P. Eichenberger, J. E. Gonzalez-Pastor, P. Fawcett, R. Monson, R. Losick, and A. D. Grossman.** 2002. Genome wide analysis of the stationary phase sigma factor (sigma-H) regulon of *Bacillus subtilis*. J. Bacteriol. **184:**4881-4890.

6. **Cabeen, M. T., and C. Jacobs-Wagner.** 2005. Bacterial cell shape. Nat. Rev. Microbiol. **3:**601-610.

7. **Carballido-Lopez, R., A. Formstone, Y. Li, S. D. Ehrlich, P. Noirot, and J. Errington.** 2006. Actin homolog MreBH governs cell morphogenesis by localization of the cell wall hydrolase LytE. Dev. Cell. **11:**399-409.

8. **Fein, J. E., and H. J. Rogers.** 1976. Autolytic enzyme deficient mutants of *Bacillus subtilis* 168. J. Bacteriol. **127:**1427-1442.

9. **Formstone, A., R. Carballido-Lopez, P. Noirot, J. Errington, and D. J. Scheffers.** 2008. Localization and interactions of teichoic acid synthetic enzymes in *Bacillus subtilis*. J. Bacteriol. **190:**1812-1821.

10. **Fukushima, T., A. Afkham, S. Kurosawa, T. Tanabe, H. Yamamoto, and J. Sekiguchi.** 2006. A new D,L-endopeptidase gene product, YojL (renamed CwlS), plays a role in cell separation with LytE and LytF in *Bacillus subtilis*. J. Bacteriol. **188:**5541-5550.

11. **Fukushima, T., T. Kitajima, H. Yamaguchi, Q. Ouyang, K. Furuhata, H. Yamamoto, T. Shida, and J. Sekiguchi.** 2008. Identification and characterization of novel cell wall hydrolase CwlT - A two-domain autolysin exhibiting *N*-acetylmuramidase and dl-endopeptidase activities. J. Biol. Chem. **283:**11117-11125.

12. **Graham, L. L., and T. J. Beveridge.** 1994. Structural differentiation of the *Bacillus subtilis* 168 cell wall. J. Bacteriol. **176:**1413-1421.

13. **Ishikawa, S., Y. Hara, R. Ohnishi, and J. Sekiguchi.** 1998. Regulation of a new cell wall hydrolase gene, *cwlF*, which affects cell separation in *Bacillus subtilis*. J. Bacteriol. **180:**2549-2555.

14. **Kawai, Y., K. Asai, and J. Errington.** 2009. Partial functional redundancy of MreB isoforms, MreB, Mbl and MreBH, in cell morphogenesis of *Bacillus subtilis*. Mol. Microbiol. **73:**719-731.

15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of head of bacteriophage T4. Nature. **227:**680-685.

16. **Layec, S., B. Decaris, and N. Leblond-Bourget.** 2008. Characterization of proteins belonging to the CHAP related superfamily within the firmicutes. J. Mol. Microbiol. Biotechnol. **14:**31-40.

17. **Leclerc, D., and A. Asselin.** 1989. Detection of bacterial cell wall hydrolases after denaturing polyacrylamide gel electrophoresis. Can. J. Microbiol. **35:**749-753.

18. **Merad, T., A. R. Archibald, I. C. Hancock, C. R. Harwood, and J. A. Hobot.** 1989. Cell wall assembly in *Bacillus subtilis* visualization of old and new wall material by electron microscopic examination of samples stained selectively for teichoic acid and teichuronic acid. J. Gen. Microbiol. **135:**645-655.

19. **Ohnishi, R., S. Ishikawa, and J. Sekiguchi.** 1999. Peptidoglycan hydrolase LytF plays a role in cell separation with Cw1F during vegetative growth of *Bacillus subtilis*. J. Bacteriol. **181:**3178-3184.

20. **Pooley, H. M.** 1976. Layered distribution, according to age, within cell wall of *Bacillus subtilis*. J. Bacteriol. **125:**1139-1147.

21. **Sambrook, J., E. F. Fritch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

22. **Smith, T. J., S. A. Blackman, and S. J. Foster.** 2000. Autolysins of *Bacillus subtilis*: multiple enzymes with multiple functions. Microbiology. **146:**249-262.

23. **Suzuki, T., and Y. Tahara.** 2003. Characterization of the *Bacillus subtilis ywtD* gene, whose product is involved in gamma-polyglutamic acid degradation. J. Bacteriol. **185:**2379-2382.

24. **Teng, F., M. Kawalec, G. M. Weinstock, W. Hryniewicz, and B. E. Murray.** 2003. An *Enterococcus faecium* secreted antigen, SagA, exhibits broad

spectrum binding to extracellular matrix proteins and a appears essential for *E. faecium* growth. Infect. Immun. **71:**5033-5041.

25. **Vollmer, W., B. Joris, P. Charlier, and S. Foster.** 2008. Bacterial peptidoglycan (murein) hydrolases. FEMS Microbiol. Rev. **32:**259-286.

26. **Xu, Q. P., S. Sudek, D. McMullan, M. D. Miller, B. Geierstanger, D. H. Jones, S. S. Krishna, G. Spraggon, B. Bursalay, P. Abdubek, C. Acosta, E. Ambing, T. Astakhova, H. L. Axelrod, D. Carlton, J. Caruthers, H. J. Chiu, T. Clayton, M. C. Deller, L. Duan, Y. Elias, M. A. Elsliger, J. Feuerhelm, S. K. Grzechnik, J. Hale, G. W. Han, J. Haugen, L. Jaroszewski, K. K. Jin, H. E. Klock, M. W. Knuth, P. Kozbial, A. Kumar, D. Marciano, A. T. Morse, E. Nigoghossian, L. Okach, S. Oommachen, J. Paulsen, R. Reyes, C. L. Rife, C. V. Trout, H. van den Bedem, D. Weekes, A. White, G. Wolf, C. Zubieta, K. O. Hodgson, J. Wooley, A. M. Deacon, A. Godzik, S. A. Lesley, and I. A. Wilson.** 2009. Structural basis of murein peptide specificity of a gamma-D-glutamyl-L-diamino acid endopeptidase. Structure. **17:**303-313.

27. **Yamaguchi, H., K. Furuhata, T. Fukushima, H. Yamamoto, and J. Sekiguchi.** 2004. Characterization of a new *Bacillus subtilis* peptidoglycan hydrolase gene, *yvcE* (named *cwlO*), and the enzymatic properties of its encoded protein. J. Biosci. Bioeng. **98:**174-181.

28. **Yamamoto, H., M. Hashimoto, Y. Higashitsuji, H. Harada, N. Hariyama, L. Takahashi, T. Iwashita, S. Ooiwa, and J. Sekiguchi.** 2008. Post-translational control of vegetative cell separation enzymes through a direct interaction with specific inhibitor IseA in *Bacillus subtilis*. Mol. Microbiol. **70:**168-182.

29. **Yamamoto, H., S. Kurosawa, and J. Sekiguchi.** 2003. Localization of the vegetative cell wall hydrolases LytC, LytE, and LytF on the *Bacillus subtilis* cell surface and stability of these enzymes to cell wall bound or extracellular proteases. J. Bacteriol. **185:**6666-6677.

30. **Yamamoto, H., Y. Miyake, M. Hisaoka, S. I. Kurosawa, and J. Sekiguchi.** 2008. The major and minor wall teichoic acids prevent the sidewall localization of vegetative DL-endopeptidase LytF in *Bacillus subtilis*. Mol. Microbiol. **70:**297-310.

Figure legends

FIG. 1. D,L-endopeptidase activity of LytE and CwlO is important for cell proliferation, and LytF or CwlS induction could not suppress *lytE cwlO* synthetic lethality. Strains were precultured with the appropriate inducer until late exponential phase (OD_{600nm}=2.0). An aliquot of each culture was washed and inoculated into fresh medium with or without the inducer to OD_{600nm}=0.01. The × symbol in panels A to D indicates the wild type 168 strain. (A) Growth of OH005 (*lytE*$_{C247S}$-6×*flag* P$_{xyl}$-*cwlO*; open circles) and OH004 (*lytE*-6×*flag* P$_{xyl}$-*cwlO*; closed circles). Xylose (1%) was added to the preculture, but CwlO expression was not induced by xylose in the main culture. (B) Growth of OH007 (*cwlO*$_{C377S}$-6×*flag* P$_{spac}$-*lytE*; open circles) and OH006 (*cwlO*-6×*flag* P$_{spac}$-*lytE*; closed circles). IPTG (1 mM) was added to the preculture, but LytE expression was not induced by IPTG in the main culture. (C) Growth of OH009 (Δ*lytE* P$_{xyl}$-*cwlO* P$_{spac}$-*lytF*). The strain was cultured with 1 mM IPTG to induce LytF expression, and with 1% xylose to induce CwlO induction (closed circles) or without xylose (open circles). (D) Growth of OH012 (Δ*lytE* P$_{xyl}$-*cwlO* P$_{spac}$-*cwlS*). The strain was cultured with 1 mM IPTG to induce CwlS expression, and with 1% xylose to induce CwlO expression (closed circles) or without xylose (open circles).

FIG. 2. Subcellular localization of full-length D,L-endopeptidases and their N-terminal domains. Phase-contrast and immunofluorescence microscopy analysis of FLAG-tagged proteins. The OD_{600nm} values at the sampling times were 0.1 for LytE and CwlO and their N-terminal domains (CWB_{LytE} and NTD_{CwlO}, respectively), 0.6 for LytF and its N-terminal domain (CWB_{LytF}), and 2.0 for CwlS and its N-terminal domain

(CWB_{CwlS}). A, WECLytE6FL (LytE-6×FLAG); B, OH015 (CWB_{LytE}-6×FLAG); C, WECLytF6FL (LytF-6×FLAG); D, OH014 (CWB_{LytF}-6×FLAG); E, WECS6FL (CwlS-6×FLAG); F, OH016 (CWB_{CwlS}-6×FLAG); G, WECO6FL (CwlO-6×FLAG); H, OH013 (overexpressed CwlO-6×FLAG); and I, OH018 (overexpressed NTD_{CwlO}-6×FLAG). Scale bars = 5 μm.

FIG. 3. Expression and activity of domain-swapped D,L-endopeptidases. Strains were exposed to 1% xylose or 1 mM IPTG for 2 hours to induce P_{xyl}-*cwlO* and P_{spac}-*lytE* expression, respectively. 1, OH019 ($N_{LytE}C_{LytF}$ P_{xyl}-*cwlO*, 41 kDa); 2, OH020 ($N_{LytE}C_{CwlS}$ P_{xyl}-*cwlO*, 40 kDa); 3, OH022 ($N_{LytF}C_{LytE}$ P_{xyl}-*cwlO*, 53 kDa); 4, OH023 ($N_{CwlO}C_{LytF}$ P_{spac}-*lytE*, 55 kDa); and 5, OH024 ($N_{CwlO}C_{CwlS}$ P_{spac}-*lytE*, 56 kDa). (A) Domain-swapped D,L-endopeptidases were evaluated by western blot analysis with an anti-FLAG antibody. Degraded products of the chimeric enzymes appear on lanes 4 and 5. (B) Zymography of the chimeric enzymes using *B. subtilis* cell wall as a substrate. Asterisks indicate clear zones produced by the chimeric enzymes.

FIG. 4. Subcellular localization of domain-swapped D, L-endopeptidases and suppression of the *lytE cwlO* synthetic lethality by these proteins. For microscopic imaging, OH019 (*lytE* ::$N_{LytE}C_{LytF}$ P_{xyl}-*cwlO*), OH020 (*lytE* ::$N_{LytE}C_{CwlS}$ P_{xyl}-*cwlO*), and OH022 (*lytE* ::$N_{LytF}C_{LytE}$ P_{xyl}-*cwlO*) were cultured with 1% xylose to induce CwlO, and OH023 (*cwlO* ::$N_{CwlO}C_{LytF}$ P_{spac}-*lytE*) and OH024 (*cwlO* ::$N_{CwlO}C_{CwlS}$ P_{spac}-*lytE*) were cultured with 1 mM IPTG to induce LytE. For suppression assays, the strains were grown under the same conditions as those described in Fig. 1. They were cultured with xylose (closed circles) or without xylose (open circles) for P_{xyl}-*cwlO* and IPTG for

P_{spac}-*lytE*. The × symbol indicates the wild type 168 strain. Scale bars = 5 μm.

TABLE 1. Bacterial strains used in this study.

Strains	Relevant genotype	Source or reference [a]
***E. coli* strains**		
JM109	*recA1 endA1 gyrA96 thi-1 hsdR17 relA1 supE44* Δ (*lac-proAB*) /F' [*traD36 proAB lacI*^{q} *lacZ* ΔM15]	Takara
C600	*supE44 hsdR17 thi-1 thr-1 IeuB6 lacY1 tonA21*	Laboratory stock
M15/pREP4	*lac ara gal mtl* F$^-$ *recA*$^+$ *uvr*$^+$ / lacI kan	Qiagen
B. subtilis		
168	*trpC2*	S. D. Ehrlich
FTD	*trpC2 lytE*::*tet*	30
OH001	*trpC2 cwlO*:: pXyl-cwlO (P_{xyl}-*cwlO*)	pXyl-cwlO -> 168
OH002	*trpC2 lytE*::*tet cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*)	OH001 -> 168FTD
OH003	*trpC2 lytE*::pM4LYTE	pM4LYTE -> 168
OH004	*trpC2 lytE*::*lytE-6×flag cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*)	pCA6FLCF -> OH001
OH005	*trpC2 lytE*::*lytE*$_{C247S}$*-6×flag cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*)	pCALE$_{C247S}$ -> OH001
OH006	*trpC2 cwlO*::*cwlO-6×flag lytE*::pM4LYTE (P_{spac}-*lytE*)	Supplementary data
OH007	*trpC2 cwlO*::*cwlO*$_{C377S}$*-6×flag lytE*::pM4LYTE (P_{spac}-*lytE*)	Supplementary data
OH008	*trpC2 lytF*::pM4LYTF	pM4LYTF -> 168
OH009	*trpC2 lytE*::*tet cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*) *lytF*::pM4LYTF (P_{spac}-*lytF*)	OH008 -> OH002
BKD	*trpC2 lytC*::*kan*	27
OH010	*trpC2 lytE*::*tet cwlO*::pXyl-cwlO *lytF*::pM4LYTF *lytC*::*kan*	168BKD -> OH009
OH011	*trpC2 cwlS*::pM4SDΔojL	pM4SDΔojL -> 168
OH012	*trpC2 lytE*::*tet cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*) *cwlS*::pM4SDΔojL (P_{spac}-*cwlS*)	OH011 -> OH002
WEC	*trpC2* Δ*wprA* Δ*epr*	30
WECLytF6FL[b]	*trpC2* Δ*wprA* Δ*epr lytF*::pCA6FLCE	30
WECLytE6FL[b]	*trpC2* Δ*wprA* Δ*epr lytE*::pCA6FLCF	30
WECS6FL	*trpC2* Δ*wprA* Δ*epr cwlS*::pCA6FLCS	30
WECO6FL	*trpC2* Δ*wprA* Δ*epr cwlO*::pCA6FLCO	pCA6FLCO -> WEC
OH013	*trpC2* Δ*wprA* Δ*epr* / pDG-O6FL	pDGO6FL -> WEC
OH014	*trpC2* Δ*wprA* Δ*epr lytF*::pCA6FLCWB$_E$	pCA6FLCWB$_E$ -> WEC
OH015	*trpC2* Δ*wprA* Δ*epr lytE*::pCA6FLCWB$_F$	pCA6FLCWB$_F$ -> WEC
OH016	*trpC2* Δ*wprA* Δ*epr cwlS*::pCA6FLCWB$_S$	pCA6FLCWB$_S$ -> WEC
OH017	*trpC2* Δ*wprA* Δ*epr cwlO*::pCA6FLNTD$_O$	pCA6FLNTD$_O$ -> WEC
OH018	*trpC2* Δ*wprA* Δ*epr* / pDGNO6FL	pDGNO6FL -> WEC
OH019	*trpC2 lytE*::pCA-FbEcII ($N_{LytE}C_{LytF}$) *cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*)	pCA-FbEcII -> OH002
OH020	*trpC2 lytE*::pCA-FbSc ($N_{LytE}C_{CwlS}$) *cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*)	pCA-FbSc -> OH002
OH021	*trpC2 lytE*::pBlue-FtEbkan (*5'-lytF kan*) *cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*)	pBlue-FtEbkan -> OH002
OH022	*trpC2 lytE*::$N_{LytF}C_{LytE}$ *cwlO*::pXyl-cwlO (P_{xyl}-*cwlO*)	Supplementary material
OH023	*trpC2 cwlO*::$N_{CwlO}C_{LytF}$ *lytE*::pM4LYTE (P_{spac}-*lytE*)	Supplementary material
OH024	*trpC2 cwlO*::$N_{CwlO}C_{CwlS}$ *lytE*::pM4LYTE (P_{spac}-*lytE*)	Supplementary material

[a]Sources shown before and after the arrows indicate donor DNA and recipient cells of transformation, respectively.

[b]The previous strain names, WECE6FL and WECF6FL (30), are changed to

WECLytF6FL and WECLytE6FL, respectively, to avoid the confusion of gene names.

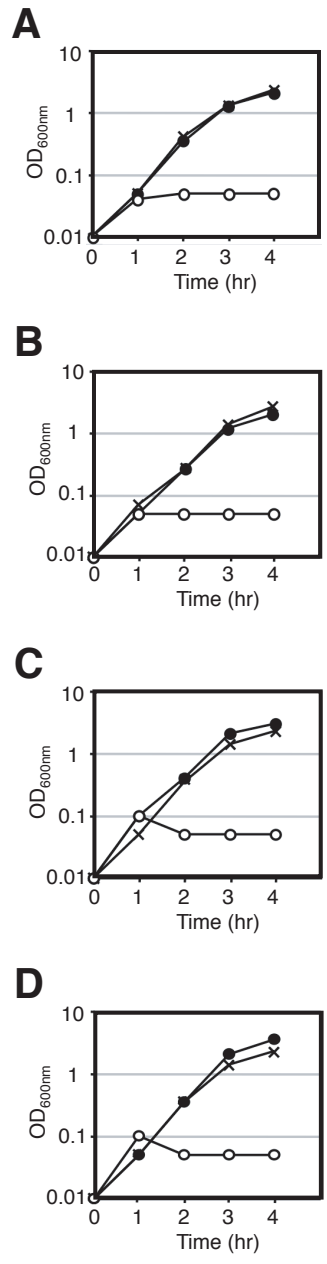


FIG. 1 Hashimoto et al

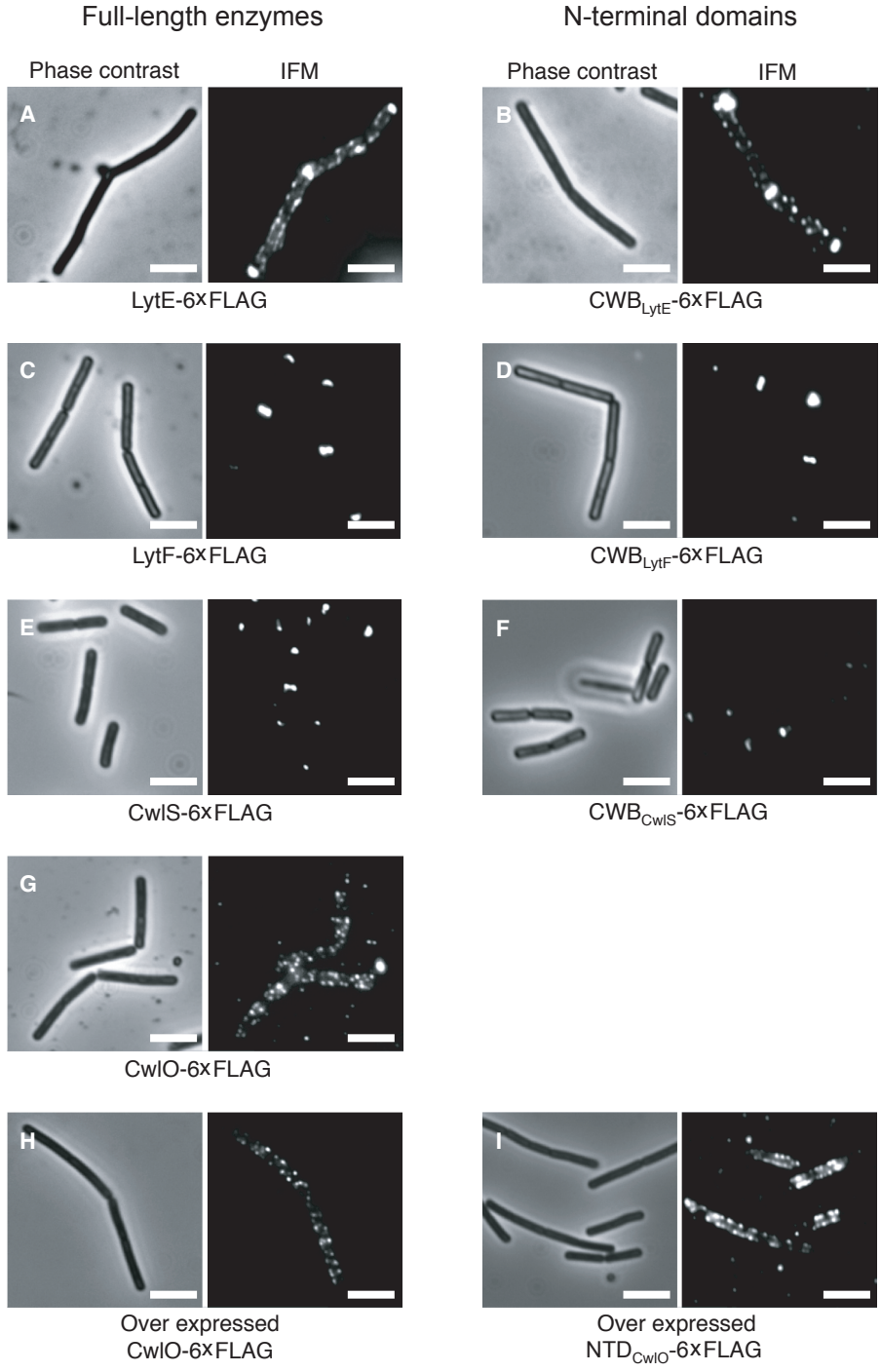


FIG. 2 Hashimoto et al

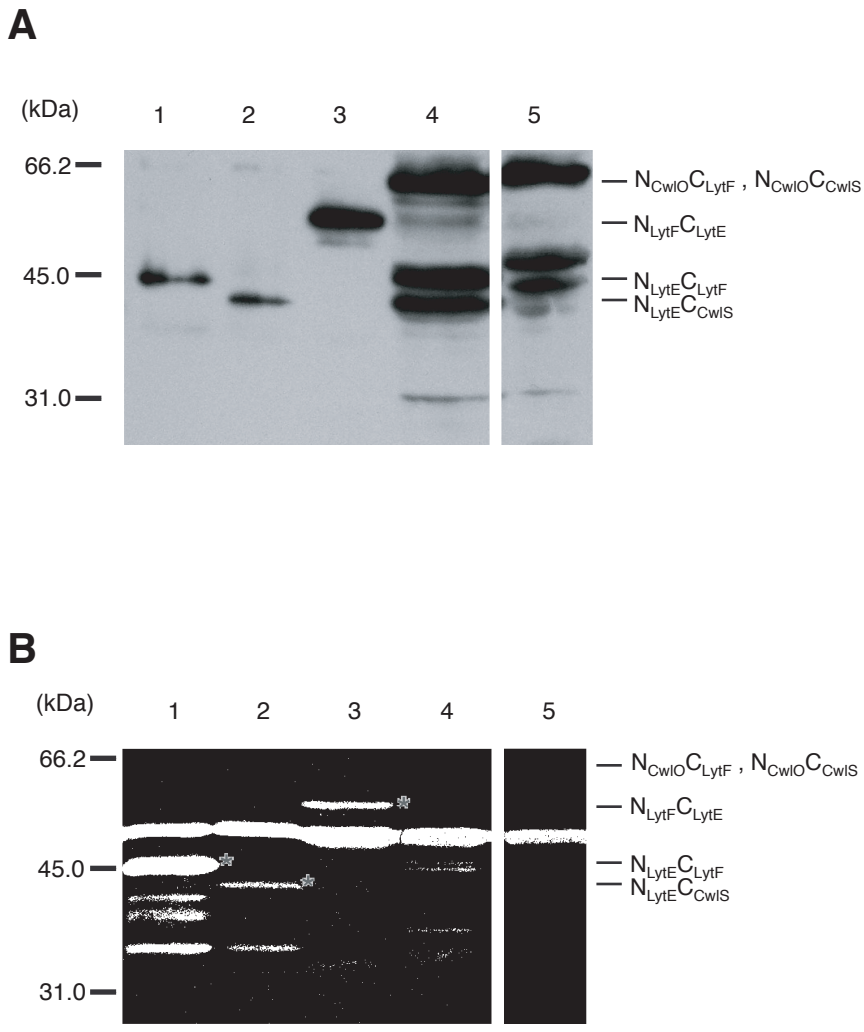


FIG. 3 Hashimoto et al

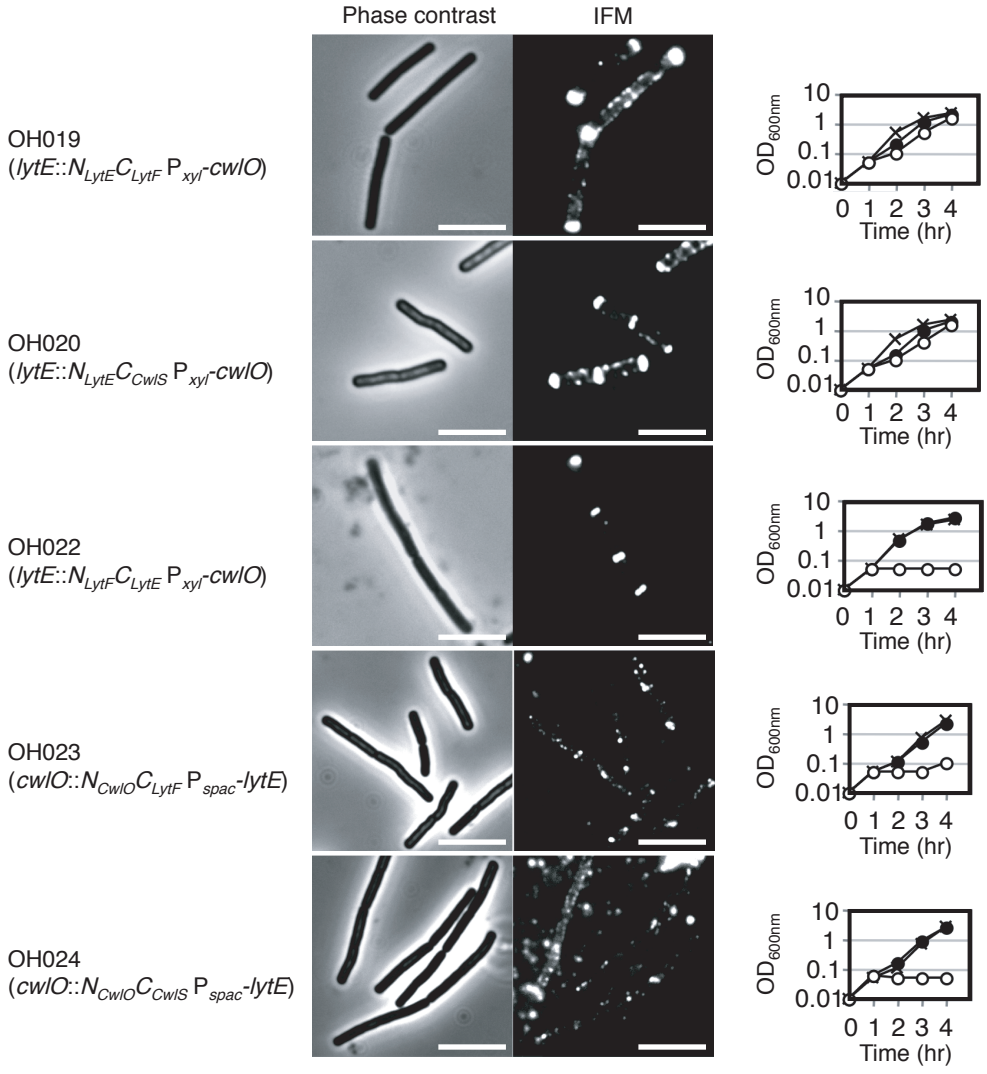


FIG. 4 Hashimoto et al

Supplementary material

SUPPLEMENTARY MATERIALS AND METHODS

Plasmid constructions. The plasmids and primers used in this study are shown in Tables S1 and S2, respectively.

To construct the CwlO inducible strains by xylose, a fragment containing the spectinomycin resistance gene and the P_{xyl} region from pSG1154 was obtained by *Sal*I and *Kpn*I digestion, and cloned into same restriction sites of pUC118 to obtain pUC-Xylspc. The upstream region of *cwlO* and a part of the *cwlO* gene were amplified with two set of primers, CvcD-Fw and CvcD-Rv, and XylvcE-Fw and PQECWB-Rv, respectively, and *B. subtilis* 168 chromosomal DNA as a template. The amplified DNA fragments were digested with *Hin*dIII and *Sal*I, and *Kpn*I and *Mun*I, respectively, and then cloned into the same restriction sites of pUC-Xylspc, step by step, to prepare pXyl-CwlO.

For construction of the FLAG fusion strains, two *cwlO* gene fragments were amplified by PCR using FvcE-Fw and FvcE-Rv, and PQEvcE-Fw and BF-YVCE as primers, respectively, and 168 chromosomal DNA as a template. The amplified fragments were digested with *Hin*dIII and *Bam*HI, and then cloned into the same restriction sites of pCA6×FLAG to obtain pCA6FLCO and pCA6FLNTD$_O$, respectively. A part of the N-terminal domain of the LytE coding region was amplified with CWBF-Ef and CFGFP-RX as primers, and 168 chromosomal DNA as a template, and the amplified fragment was digested with *Eco*RI and *Xba*I, and then cloned into the same restriction sites of pCA6×FLAG to obtain pCA6FLCWB$_F$. Likewise, a part of the N-terminal domain of the CwlS coding region was amplified with CWBS-Ef and CWBS-Xr as primers, and 168 chromosomal DNA as a template, and then digested with *Eco*RI and *Xba*I, and cloned into

the same restriction sites of pCA6×FLAG to obtain pCA6FLCWB$_S$.

To obtain CwlO-6×FLAG (pDGO6FL) and NTD$_{CwlO}$-6×FLAG (pDGNO6FL) overexpression plasmids, DNA fragments were amplified with vcEflag-Fw and vcEflag-Rv as primers, and WECO6FL or OH017 chromosomal DNA as a template, respectively. The amplified DNA fragments were digested with *Sal*I and *Sph*I, and then cloned into the same restriction sites of pDG148 to obtain pDGO6FL and pDGNO6FL, respectively.

For construction of domain-swapped N$_{LytE}$C$_{LytF}$, two DNA fragments were amplified with two sets of primers, FbEc-Fw2 and Ec-Rv, and Fb-Fw and Fb-Rv, respectively, and 168 chromosomal DNA as a template. Next, the amplified fragments were used as templates for 2nd PCR with Ec-Rv and Fb-Fw as primers, and then the 2nd PCR amplified fragment was digested with *Kpn*I and *Bam*HI, and cloned into the same restriction sites of pCA6×FLAG to obtain pCA-FbEcII. To construct pCA-FbSc for N$_{LytE}$C$_{CwlS}$, two DNA fragments were amplified with two sets of primers, FbSc-Fw and Sc-Rv, and Fb-Fw and Fb-Rv, respectively, and 168 chromosomal DNA was used as a template. Next, the amplified fragments were used as templates for 2nd PCR with Sc-Rv and Fb-Fw as primers, and then the 2nd PCR amplified fragment was digested with *Kpn*I and *Bam*HI, and cloned into the same restriction sites of pCA6×FLAG.

For construction of N$_{LytF}$C$_{LytE}$, three plasmids, pBlue-EbKn, pBlue-FtEbKn, and pCA-EbFc, were prepared. For pBlue-FtEbKn, two DNA fragments were amplified with Eb-Fw and Eb-Rv as primers, and 168 chromosomal DNA as a template, and with EbKn-Fw and Kn-Rv as primers, and pDG780 as a template. The amplified DNA fragments were used as templates for 2nd PCR with Eb-Fw and Kn-Rv as primers, and the 2nd PCR amplified fragment was digested with *Eco*RI and *Bam*HI, and then cloned into the same restriction sites of pBluescriptII SK+ to generate pBlue-EbKn. Next, two DNA fragments were amplified

with two sets of primers, CF5-Fw and CF5-Rv, and CF3-Fw and CF3-Rv, and 168 chromosomal DNA as a template. The amplified DNA fragments were digested with *Kpn*I and *Eco*RI, and *Sac*I and *Bam*HI, respectively, and then cloned into the same restriction sites of pBlue-EbKn, step by step, to construct pBlue-FtEbKn. For construction of pCA-EbFc, two DNA fragments were amplified with two sets of primers, EbFc-Fw and Fc-Rv, and Eb-Fw and Eb-Rv, and 168 chromosomal DNA as a template. The amplified fragments were used for 2nd PCR as templates with Eb-Fw and Fc-Rv as primers, and the 2nd PCR amplified fragment was digested with *Kpn*I and *Bam*HI, and then cloned into the same restriction sites of pCA6×FLAG.

To construct pCA-ObEcII for $N_{CwlO}C_{LytF}$, two DNA fragments were amplified with two sets of primers, Ob-Fw and Ob-Rv, and ObEc-Fw and Ec-Rv, and 168 chromosomal as a DNA template. The amplified fragments were used as templates for 2nd PCR with Ob-Fw and Ec-Rv as primers, and the 2nd PCR amplified fragment was digested with *Kpn*I and *Bam*HI, and then cloned into the same restriction sites of pCA6×FLAG. For construction of pCA-ObScII, two DNA fragments were amplified with two sets of primers, Ob-Fw and Ob-Rv, and ObSc-Fw2 and Sc-Rv, using 168 chromosomal DNA as a template. The amplified fragments were used as templates for 2nd PCR with Ob-Fw and Sc-Rv as primers, and the 2nd PCR amplified fragment was digested with *Kpn*I and *Bam*HI, and then cloned into the same restriction sites of pCA6×FLAG.

To construct pQE-$LytE_{C247S}$ for expression of a point-mutated catalytic domain of $LytE_{C247S}$ in *E. coli*, two DNA fragments were amplified with two sets of primers, SR-CwlF and LytE-CSF, and BF-CwlF and LytE-CSR, using pHisktCwlF as a template. The amplified fragments were used for 2nd PCR as a template with SR-CwlF and BF-CwlF, and the 2nd PCR amplified fragment was digested with *Bam*HI and *Sma*I, and then cloned into the same

restriction sites of pQE30. For construction of pQE-CTD-CwlO$_{C377S}$ expressing a point-mutated catalytic domain of CwlO$_{C377S}$ in *E. coli*, a DNA fragment was amplified with BF-YVCE and PQEyFL-Rv as primers, and the described plasmid pCACO$_{C377S}$ as a template. The amplified fragment was digested with *Bam*HI and *Kpn*I, and then cloned into pQE-30 to obtain pQE-CTD-CwlO$_{C377S}$.

To construct point-mutated LytE$_{C247S}$ in *B. subtilis*, two plasmids (pCAlytEfull and pCALE$_{C247S}$) were constructed. For construction of pCAlytEfull, a DNA fragment was amplified with Fb-Fw and Fc-Rv as primers, using 168 chromosomal DNA as a template. The amplified fragment was digested with *Kpn*I and *Bam*HI, and then cloned into the same restriction sites of pCA6×FLAG. Next, a DNA fragment containing point mutations was amplified with LE-CSF and LE-CSR, and pCAlytEfull as a template, and then the resulting fragment was phosphorylated using T4 polynucleotide kinase (Takara) following the manual for the enzyme, and then self ligated to construct pCALE$_{C247S}$. To construct point-mutated CwlO$_{C377S}$ in *B. subtilis*, two plasmids (pCAcwlOfull and pCACO$_{C377S}$) were constructed. A DNA fragment was amplified with PQEvcE-Fw and FvcE-Rv as primers, using 168 chromosomal DNA as a template. The amplified fragment was digested with *Eco*RI and *Bam*HI, and then cloned into the same restriction sites of pCA6×FLAG to construct pCAcwlOfull. Next, a DNA fragment containing a point mutation was amplified with vcE-CSF and vcE-CSR, and pCAcwlOfull as a template, and the resulting fragment was phosphorylated using T4 polynucleotide kinase, and then self ligated to construct pCACO$_{C377S}$.

The DNA sequences of all cloned regions, which were amplified by PCR, were confirmed by DNA sequencing.

Strain constructions. The bacterial strains used in this study are listed in Table 1. The sources of donor DNA and recipient strains for *B. subtilis* mutant construction are also indicated in Table 1.

To construct OH006, two DNA fragments were amplified with PQEvcE-Fw and CM4-CTDr as primers, and plasmid pCA6FLCO as a template, and with 3vcECm-Fw and 3vcECm-Rv as primers, and 168 chromosomal DNA as a template. These amplified fragments were used for 2nd PCR as templates with PQEvcE-Fw and 3vcECm-Rv, and the 2nd PCR amplified fragment was used for transformation of OH003 to obtain OH006. Construction of OH007 was carried out in a similar manner to OH006 except that pCA6xFLAG, as a template, was replaced by $pCACO_{C377S}$.

To construct OH022, OH001 was transformed with pBlue-FtEbKn to obtain OH021. Next, two DNA fragments were amplified with Eb-Fw and CM4-CTDr as primers, and pCA-EbFc as a template, and CmCF3-Fw and cLE-3R as primers and 168 chromosomal DNA as a template. The amplified fragments were used for the 2nd PCR as templates with Eb-Fw and cLE-3R as primers, and the 2nd PCR amplified fragment was used for transformation of OH021 to generate OH022. To construct OH024, two DNA fragments were amplified with Ob-Fw and CM4-CTDr as primers, and pCA-ObScII as a template, and 3vcECm-Fw and 3vcECm-Rv as primers and 168 chromosomal DNA as a template. The amplified fragments were used for 2nd PCR as templates with Ob-Fw and 3vcECm-Rv as primers, and the 2nd PCR amplified fragment was used for the transformation of OH003 to obtain OH024. Construction of OH023 was carried out in a same manner to OH024 except that template DNA pCA-ObScII for OH024 was replaced by pCA-ObEcII.

SUPPLEMENTARY REFERENCES

1. Fukushima, T., Afkham, A., Kurosawa, S., Tanabe, T., Yamamoto, H., Sekiguchi, J. (2006) A new D,L-endopeptidase gene product, YojL (renamed CwlS), plays a role in cell separation with LytE and LytF in *Bacillus subtilis*. J. Bacteriol. **188**: 5541-5550.

2. Yamamoto, H., Hashimoto, M., Higashitsuji, Y., Harada, H., Hariyama, N., Takahashi, L., Iwashita, T., Ooiwa, S., Sekiguchi, J. (2008) Post-translational control of vegetative cell separation enzymes through a direct interaction with specific inhibitor IseA in *Bacillus subtilis*. Mol. Microbiol. **70**: 168-82.

3. Yamamoto, H., Miyake, Y., Hisaoka, M., Kurosawa, S., Sekiguchi, J. (2008) The major and minor wall teichoic acids prevent the sidewall localization of vegetative D,L-endopeptidase LytF in *Bacillus subtilis*. Mol. Microbiol. **70**: 297-310.

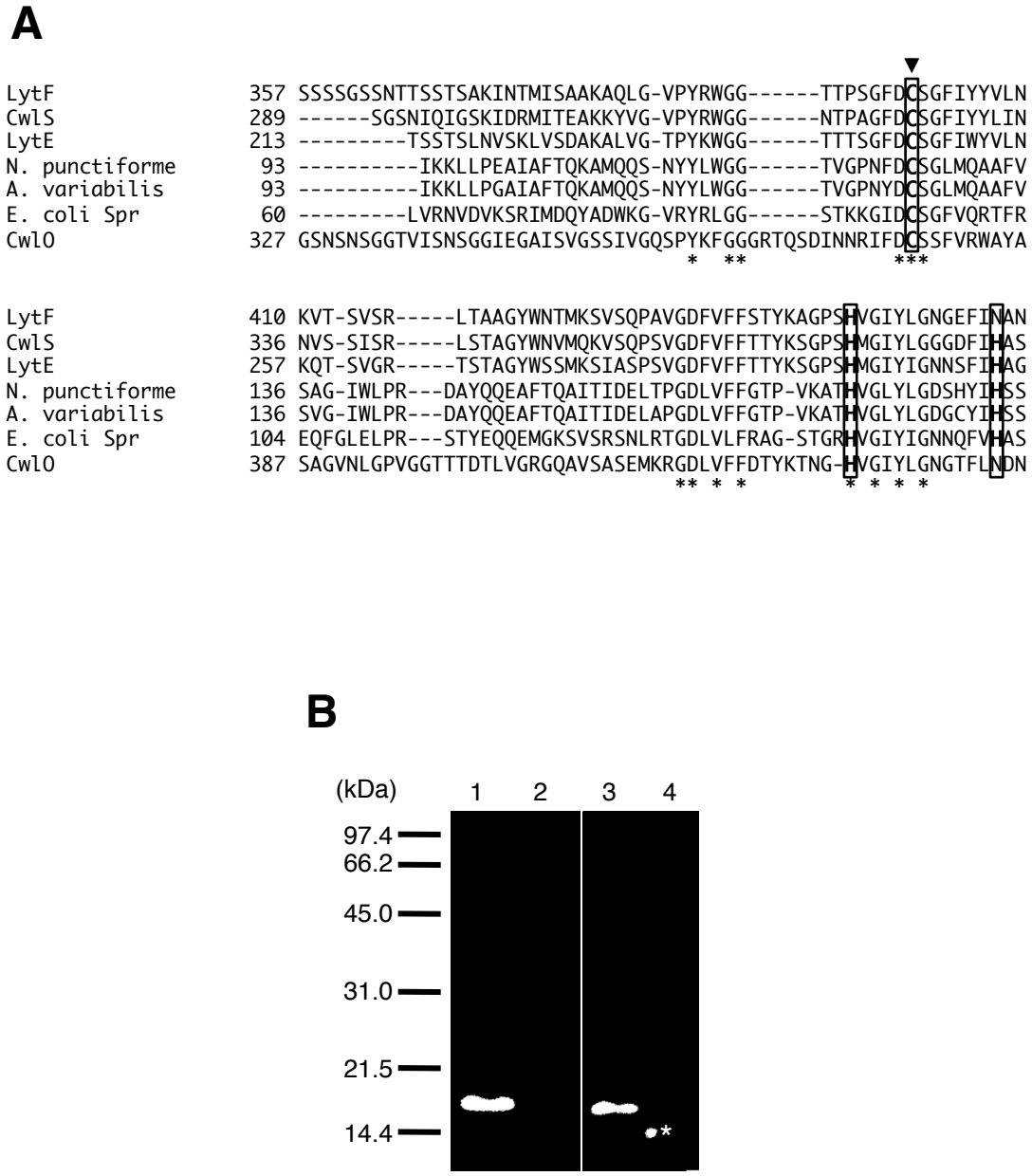
TABLE S1. Plasmids used in this study.

Strains	Relevant genotype	Source or reference [a]
pUC118	*bla*	Takara
pSG1154	*bla spc gfpmut-1 amyE'-'amyE*	BGSC
pUC-Xylspc	*bla spc* P_{xyl}	This study
pXyl-cwlO	*bla spc* P_{xyl}-*cwlO*	This study
pBluescriptII SK+	*bla*	Toyobo
pDG780	*bla kan*	BGSC
pM4LYTE	*bla erm lacI* P_{spac}-*lytE*	2
pCA6FLCE	*bla cat lytF-6xflag*	2
pCA6FLCS	*bla cat cwlS-6xflag*	2
pCA6FLCF	*bla cat lytE-6×flag*	2
pM4LYTF	*bla erm lacI* P_{spac}-*lytF*	2
pM4SDΔojL	*bla erm lacI* P_{spac}-*cwlS*	1
pCA6×FLAG	*bla cat* 6×flag	3
pCA6FLCO	*bla cat cwlO-6×flag*	This study
pDG148	*bla ble kan lacI* P_{spac}	BGSC
pDGO6FL	*bla ble lacI* P_{spac}-*cwlO6FL*	This study
$pCA6FLCWB_E$	*bla cat* cwb_E-*6×flag*	3
$pCA6FLCWB_F$	*bla cat* cwb_F-*6×flag*	This study
$pCA6FLCWB_S$	*bla cat* cwb_S-*6×flag*	This study
$pCA6FLNTD_O$	*bla cat* cwb_O-*6×flag*	This study
pDGNO6FL	*bla ble lacI* P_{spac}-ntd_O*6FL*	This study
pCA-FbEcII	*bla cat* cwb_{lytE}.ctd_{lytF}*II-6×flag*	This study
pCA-FbSc	*bla cat* cwb_{lutE}-ctd_{cwlS}-*6×flag*	This study
pBlue-EbKn	*bla kan* cwb_{lytF} *kan*	This study
pBlue-FtEbkn	*bla kan 3'lytE-*cwb_F*kan-5'lytE*	This study
pCA-EbFc	*bla cat* cwb_{lytF}.ctd_{lytE}-*6×flag*	This study
pCA-ObEcII	*bla cat* ntd_{cwlO}-ctd_{lytF}*II-6×flag*	This study
pCA-ObSc	*bla cat* ntd_{cwlO}-ctd_{cwlS}-*6×flag*	This study
pCA-ObScII	*bla cat* ntd_{cwlO}-ctd_{cwlS}*II-6×flag*	This study
pHistkCwlF	*bla* 6xHis-ctd-*lytE*	3
$pQELytE_{C247S}$	*bla* 6×His-*ctd-*$lytE_{C247S}$	This study
pQE-30	*bla*	Qiagen
$pQE\text{-}CTD\text{-}CwlO_{C377S}$	*bla* 6×His-*ctd-*$cwlO_{C377S}$	This study
pCAlytEfull	*bla cat lytE-6×flag*	This study
$pCALE_{C247S}$	*bla cat* $lytE_{C247S}$-*6×flag*	This study
pCAcwlOfull	*bla cat cwlO-6×flag*	This study
$pCACO_{C377S}$	*bla cat* $cwlO_{C377S}$-*6×flag*	This study

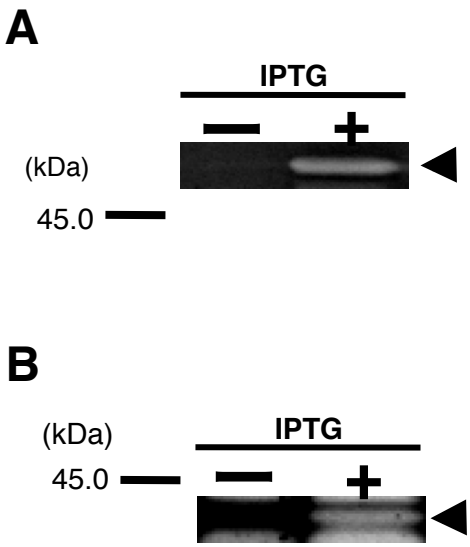
BGSC, *Bacillus* Genetic Stock Center, Ohio State University.

TABLE S2. Oligonucleotide used in this study.

Name	Sequence (5' to 3')
3vcECm-Fw	GAGATAATGCCGACTGTACTGTAAGACGTGTTGTTCAA
3vcECm-Rv	AAGTCAACTTTTTCATTATCA
BF-CwlF	GCGGATCCACATCACTTAATGTGAGCAA
BF-YVCE	GCGCGGATCCGAAGGCGCGATCAGCGTT
CF3-Fw	GCGCGGATCCTTCATTAGACGGAGCACA
CF3-Rv	GCGCGAGCTCCACAATCATCCTGATTACT
CF5-Fw	GCGCGGTACCTGCGGATAACCACAGCT
CF5-Rv	GCGCGAATTCATTTTCCTCCCCAAATGTT
CFGFP-RX	GCGCTCTAGACTTGCTCACATTAAGTGATG
cLE-3R	GCGCGAGCTCCGTATGCGCTCAGGCTT
CM4-CTDr	GTACAGTCGGGCATTATCTC
CmCF3-Fw	TATGAGATAATGCCGACTGTACTTCATTAGACGGAGCACAC
CvcD-Fw	GCGCAAGCTTAGAGAATTACCGCTCCTT
CvcD-Rv	GCGCGTCGACTTATGTTTCAAACAGATGTC
CWBF-Ef	CGCGAATTCTGAAGAAGCTGAATGGC
CWBS-Ef	CGCGAATTCACTTTATCCTAAACAGGTG
CWBS-Xr	GCCGTCTAGAGACATATTTTTTCGCTTCCG
Eb-Fw	GCGCGGTACCGAATTCATGAAAAAGAAATTAGCAGC
Eb-Rv	TGAAGAACCGGATGAAGA
EbFc-Fw	TCTTCATCCGGTTCTTCACTTAATGTGAGCAAGCTG
EbKn-Fw	TCTTCATCCGGTTCTTCAAATGCAAGGAACAGTGAAT
Ec-Rv	GCGCGGATCCGAAATATCGTTTTGCACCG
Fb-Fw	GCGCGGTACCAGCTACGACAGCAGTTG
Fb-Rv	TGATGTAGATGACGTTTTG
FbEc-Fw2	CAAAACGTCATCTACATCACAAAAGCTGGTCATTTCC
FbSc-Fw	CAAAACGTCATCTACATCAGGGTCAAACATTCAAATAGGTTCG
Fc-Rv	GCGCGGATCCGAATCTTTTCGCACCGAG
FvcE-Fw	GCGCAAGCTTACGCACTCAGTCTGATAT
FvcE-Rv	GCGCGGATCCTTGAACAACACGTCTTACA
Kn-Rv	GCGCGGATCCTGTCTAAAAAGCTTGTAGTT
LE-CSF	AGCAGCGGATTCATTTGG
LE-CSR	GTCAAAGCCTGAAGTTGT
LytE-CSF	CAACTTCAGGCTTTGACAGCAGCGGATTCATTTGG
LytE-CSR	CCAAATGAATCCGCTGCTGTCAAAGCCTGAAGTTG
Ob-Fw	GCGCGGTACCGAAACATTAGATGAAAAGAAAC
Ob-Rv	GATCGCGCCTTCAATTC
ObEc-Fw	GAATTGAAGGCGCGATCCAAAAGCTGGTCATTTCC
ObSc-Fw2	GAATTGAAGGCGCGATCCTGACTATTTCGGGAGC
PQECWB-Rv	GCCGCAATTGGGATCCGATCGCGCCTTCAATTCC
PQEvcE-Fw	GCGCGAATTCATTAAAGAGGAGAAATTAACTATGGAAACATTAGATGAAAAGAAAC
PQEyFL-Rv	GCGCGGTACCTTATTGAACAACACGTCTTAC
Sc-Rv	GCGCGGATCCAAAATAACTTCTTGCGCCC
SR-CwlF	GCCCCGGGCGCCTGTGCTCCGTCT
vcE-CSF	AGCTCATCATTCGTACGC
vcE-CSR	GTCAAAAATACGGTTGTTG
vcEflag-Fw	GCGCGAATTCGTCGACTCACAGTAAAAGGGAGGA
vcEflag-Rv	GCGCAAGCTTGCATGCTGTAAAACGACGGCCAG
XylvcE-Fw	GCGCGGTACCTCACAGTAAAAGGGAGGA



Supplementary FIG. S1. The catalytic residues of LytE and CwlO. (A) Amino acid sequence alignment of the NlpC/P60 domain of LytE, CwlO and homologous gene products. Identical amino acid residues are indicated by asterisks, and the predicted active site residues are indicated by boxes. The cysteine residue indicated by an arrowhead in LytE and CwlO was predicted to be a catalytic residue, and was point-mutated to a serine residue in this study. The amino acid sequences of LytE, LytF, CwlS and CwlO are from *B. subtilis* 168. The others are Spr from *Escherichia coli*, ABA23003 from *Anabaena variabilis* and ACC79413 from *Nostoc punctiforme*. (B) Zymography of the intact and mutated D,L-endopeptidase catalytic domains of LytE and CwlO expressed in *E. coli*. Lane 1, CTDCwlO-6×His; lane 2, $CTDCwlO_{C377S}$-6×His; lane 3, CTDLytE-6×His; and lane 4, $CTDLytE_{C247S}$-6×His. The asterisk indicates a nonspecific signal.



Supplementary FIG. S2. Zymography of induced LytF and CwlS. The strains were cultured with 1% xylose for expression of CwlO, and with or without 1 mM IPTG, and harvested 2 hours after inoculation. (A) OH010 (Δ*lytE*Δ*lytC* P_{spac}-*lytF* P_{xyl}-*cwlO*). Since LytF and LytC are similar in size, the *lytC* disruptant was used to investigate LytF expression. (B) OH012 (Δ*lytE* P_{spac}-*cwlS* P_{xyl}-*cwlO*). The arrowheads indicate LytF (A) and CwlS (B).