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Full Paper

Disruption of the cell wall lytic enzyme CwlO affects the amount and molecular size of poly-γ-glutamic acid produced by *Bacillus subtilis* (*natto*)

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Poly-γ-glutamic acid (γPGA), a polymer of glutamic acid, is a component of the viscosity substance of natto, a traditional Japanese food made from soybeans fermented with *Bacillus subtilis* (*natto*). Here we investigate the effects of the cell wall lytic enzymes belonging to the D,L-endopeptidases (LytE, LytF, CwlO and CwlS) on γPGA production by *B. subtilis* (*natto*). γPGA levels in a *cwlO* disruptant were about twofold higher than that of the wild-type strain, whereas disruption of the *lytE*, *lytF* and *cwlS* genes had little effect on γPGA production. The molecular size of γPGA in the *cwlO* disruptant was larger than that of the wild-type strain. A complementary strain was constructed by insertion of the entire *cwlO* gene into the *amyE* locus of the CwlO mutant genome, and γPGA production was restored to wild-type levels in this complementary strain. These results indicated that the peptidoglycan degradation enzyme, CwlO, plays an important role in γPGA production and affects the molecular size of γPGA.

Key Words——*Bacillus subtilis* (*natto*); CwlO; peptidoglycan hydrolase; poly-γ-glutamic acid

Introduction

Poly-γ-glutamic acid (γPGA) is a polymer of glutamic acid in which the residues are linked via γ-bonds. γPGA is a component of the viscosity substance of natto, a traditional Japanese food made from soybeans fermented with *Bacillus subtilis* (*natto*) (Hara et al., 1982). For *B. subtilis* (*natto*), γPGA creates a physical barrier to other organisms and phage (Kimura and Itoh, 2003) and may be required as a nutritional pool under starvation conditions. The viscosity is an important factor contributing to natto quality. Natto containing a larger amount and higher molecular weight of γPGA may have strong viscosity. The viscosity of natto is decreased during the preservation period; therefore, a decrease in γPGA degradation activity may lead to stability of the viscosity. Additively, γPGA is used for various industrial purposes, for example, polluted water treatment (Taniguchi, 2009), calcium absorption (Tanimoto et al., 2007) and the production of cosmetics and medicines (Shih and Van, 2001). Therefore, improvements in γPGA production will be of commercial interest for these industries.

There are several types of γPGAs that are composed of L-glutamic acid only, D-glutamic acid only, or a mixture of both. *Bacillus anthracis* produces γPGA containing only D-glutamic acid. *Natrialba aegyptiaca* and *Bacillus halodurans* synthesize γPGA containing only L-glutamic acid. *B. subtilis* (*natto*), *B. subtilis* (*chung-*

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kookjang), *Bacillus licheniformis* and *Bacillus megaterium* produce γPGA containing D- and L-glutamic acid (Ashiuchi and Misono, 2002).

In *B. subtilis* (*natto*), the enzymes required for γPGA synthesis are encoded by the *pgsBCA* operon, which contains the γPGA synthetase gene *pgsB* (Ashiuchi et al., 1999, 2001; Urushibata et al., 2002). γPGA production is under the control of the ComQXPA quorum-sensing system (Nagai et al., 2000; Tran et al., 2000) and the DegS−DegU two-component system, and DegQ in particular plays an important role in γPGA production (Kimura et al., 2009; Ohsawa et al., 2009; Osera et al., 2009; Stanley and Lazazzera, 2005). Recently, SwrA, a protein associated with swarming motility, was reported to also be required for γPGA production (Osera et al., 2009; Stanley and Lazazzera, 2005).

In addition to producing γPGA, *B. subtilis* (*natto*) also produces γPGA degradation enzymes. YwtD is one such enzyme, located downstream of the γPGA synthetase operon in the *B. subtilis* (*natto*) genome. YwtD has high amino acid sequence similarity to the NLPC/P60 superfamily (Ashiuchi et al., 2006; Suzuki and Tahara, 2003). Cell wall lytic enzymes, LytE, LytF, CwlO and CwlS, also exhibit D,L-endopeptidase activity and belong to the NLPC/P60 superfamily (Fukushima et al., 2006; Ishikawa et al., 1998; Margot et al., 1998, 1999; Ohnishi et al., 1999; Yamaguchi et al., 2004). These enzymes therefore possess the potential for γPGA degradation and may affect γPGA production in *B. subtilis* (*natto*). Thus, the construction of a strain lacking γPGA degradation function would be of commercial interest. In this study, we investigate the effect of these cell wall lytic enzymes on γPGA production in *B. subtilis* (*natto*) and construct a disruptant producing higher amounts of γPGA with greater molecular size.

Materials and Methods

Bacterial strains and plasmids. *B. subtilis* (*natto*) strain NAFM5 (Kimura et al., 2004) and its derivatives, *Escherichia coli* strains, and the plasmids used in this study are listed in Table 1. Strain NAFM5 is a derivative of the major natto starter strain, Miyagino, and has a transformation ability. *B. subtilis* was grown on LB medium (Sambrook et al., 1989) at 37°C overnight, and was then inoculated into γPGA production medium [LB + 1% monosodium L-glutamic acid monohydrate (Glu-Na) and 0.5% sucrose (Suc)] and shaken at 37°C for the required incubation period. If necessary, kanamycin, spectinomycin and tetracycline were added to the medium at final concentrations of 20, 100 and 2 μg/ml, respectively. *E. coli* was grown in LB medium at 37°C. If necessary, spectinomycin and ampicillin were added to the medium at final concentrations of 100 and 50 μg/ml, respectively.

Plasmid construction. To create the strain complementary to *cwlO*, the plasmid pAESP-cwlO was constructed. The upstream region of the *amyE* gene was amplified by PCR using two primers, amyE5XbaI and amyE3KpnI (Table 2), and *B. subtilis* (*natto*) strain NAFM5 genomic DNA as a template. The fragment was digested with *Xba*I and *Kpn*I, ligated to the pUC118 vector (Table 1) at the corresponding sites, then transformed into *E. coli* strain JM109 competent cells. The generated plasmid, pAE, was used for transformation of *E. coli* strain ER2925 (*dam*$^{-}$) and plasmid was extracted from the transformant. The spectinomycin-resistance cassette was amplified using two primers, Sp-Fw and Sp-Rv, and plasmid pUC-Sp (Table 1) as a template. pUC-Sp was constructed by insertion of the spectinomycin-resistance cassette, amplified by PCR from plasmid pDG1727, into the *Eco*RI site of pUC119. The amplified spectinomycin-resistance cassette was digested with *Cal*I and ligated to pAE at the corresponding site to generate plasmid pAESP. The *cwlO* gene was amplified with primers cwlO5BglII and cwlO3SalI and NAFM5 genomic DNA. The PCR product was then digested with *Bgl*II and *Sal*I, and inserted into pAESP at the *Bgl*II and *Xho*I sites, creating plasmid pAESP-cwlO.

Construction of disruptants. To construct the *lytE*-deficient mutant, MIA0603, the 5′ and 3′ regions of the *lytE* gene were amplified using two pairs of primers (lytEUP1 and lytEUP2 for the 5′ region, and lytEDW1 and lytEDW2 for the 3′ region) and NAFM5 genomic DNA. The kanamycin-resistance cassette was amplified using the primers PB-M13-20 and PB-M13Rev, and pUC-Km (Table 1) as a template. Plasmid pUC-Km was constructed by insertion of the kanamycin-resistance cassette amplified by PCR from plasmid pDG783 into the *Eco*RI site of pUC119. The sequences at the 5′end of the primers lytEUP2 and lytEDW1 were complementary to the primers PB-M13-20 and PB-M13Rev, respectively. The fragments comprising the 5′ region of the *lytE* gene, the kanamycin-resistance cassette and the 3′ region of the *lytE* gene, were linked by overlap-

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype	Source or reference
Bacillus subtilis (*natto*)		
NAFM5	Rif^r *bio*	K. Kimura, National Food Research Institute, Japan
MIA0603	Rif^r *bio* Δ*lytE*::*kan*	This study
MIA0604	Rif^r *bio* Δ*lytF*::*kan*	This study
MIA0605	Rif^r *bio* Δ*cwlO*::*kan*	This study
MIA0606	Rif^r *bio* Δ*cwlS*::*spc*	This study
MIA0607	Rif^r *bio* Δ*ywtD*::*tet*	This study
MIA0608	Rif^r *bio* Δ*ggt*::*spc*	This study
MIA0614	Rif^r *bio* Δ*cwlO*::*kan amyE*::*spc*	This study
MIA0615	Rif^r *bio* Δ*cwlO*::*kan amyE*::*spc-cwlO*	This study
Escherichia coli		
JM109	*recA1* Δ (*lac-proAB*) *endA1 gyrA96 thi-1 hsdR17 relA1 supE44* [F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15]	TaKaRa, Kyoto, Japan
ER2925	*ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210*::*Tn10)TetS endA1 rpsL136 dam13*::*Tn9 xylA-5 mtl-1 thi-1 mcrB1 hsdR2*	New England Biolabs, Tokyo, Japan
Plasmid		
pUC118/ pUC119	*bla lacZ*	TaKaRa, Kyoto, Japan
pDG783	*bla kan*	BGSC[#]
pDG1515	*bla tet*	BGSC[#]
pDG1727	*bla spc*	BGSC[#]
pUC-Km	*bla kan*	Laboratory stock
pUC-Sp	*bla spc*	Laboratory stock
pAE	*bla amyE* (upstream)	This study
pAESP	*bla amyE* (upstream)::*spc*	This study
pAESP-cwlO	*bla amyE* (upstream)::*spc-cwlO*	This study

[#]*Bacillus* Genetic Stock Center, Ohio State University, Ohio, USA.

PCR using lytEUP1 and lytEDW2 primers (Kodama et al., 2007). The generated fragment was inserted into the NAFM5 chromosome by double crossover recombination to create mutant strain MIA0603 (Δ*lytE*::*kan*). Disruption of the *lytE* gene was confirmed by PCR using primers lytEUP1 and lytEDW2. Similarly, the following pairs of primers were used for disruption: lytFUP1 and lytFUP2, lytFDW1 and lytFDW2, and PB-M13-20 and PB-M13Rev, for mutant strain MIA0604 (Δ*lytF*::*kan*) along with pUC-Km; cwlOUP1 and cwlOUP2, cwlODW1 and cwlODW2, and PB-M13-20 and PB-M13Rev, for mutant strain MIA0605 (Δ*cwlO*::*kan*) along with pUC-Km; cwlSUP1 and cwlSUP2, cwlSDW1 and cwlSDW2, and PB-M13-20 and PB-M13Rev, for mutant strain MIA0606 (Δ*cwlS*::*spc*) along with pUC-Sp; ywtDUP1 and ywtDUP2, ywtDDW1 and ywtDDW2, and PB-M13-20 and PB-M13Rev, for mutant strain MIA0607 (Δ*ywtD*::*tet*) along with pDG1515; ggtUP1 and ggtUP2, ggtDW1 and ggtDW2, and PB-M13-20 and PB-M13Rev, for mutant strain MIA0608 (Δ*ggt*::*spc*) along with pUC-Sp. For complementation studies, strains MIA0614 and MIA0615 were constructed by transformation of strain MIA0605 with plasmids pAESP and pAESP-cwlO, respectively.

Transformation of E. coli and B. subtilis (natto) strains. *E. coli* transformation was performed as described by Sambrook et al. (1989). *B. subtilis* (*natto*) transformation was performed by the procedure of Anagnostopoulos and Spizizen (1961).

Measurement of poly-γ-glutamic acid amount. Levels of γPGA in the culture media were measured by a modified CET method (Kanno and Takamatsu, 1995). *B. subtilis* (*natto*) strains were shake-cultured in LB medium overnight at 37°C with antibiotics. The precul-

Table 2. Primers used in this study.

Primer name	Sequence (5′→3′)	Restriction site
lytEUP1	cccttcgaccatcaagaaaa	n/a
lytEUP2	cactggccgtcgttttacttcatattttcctccccaaatg	n/a
lytEDW1	catggtcatagctgtttccgttacctcggtgcgaaaaga	n/a
lytEDW2	gtccatgttgtttggcagtg	n/a
lytFUP1	gggtatctgggcaggaaaat	n/a
lytFUP2	cactggccgtcgttttaccagccctgctgctaatttct	n/a
lytFDW1	catggtcatagctgtttcctctcggtgcaaaacgatattt	n/a
lytFDW2	ataaatggcacgccaagaaa	n/a
cwlOUP1	aaaagtggcggaattgagtg	n/a
cwlOUP2	cactggccgtcgttttaccggaagccaaaccaagtgta	n/a
cwlODW1	catggtcatagctgtttccggtgttgtaagacgtgttgttca	n/a
cwlODW2	agcgaaaatcccttcaacct	n/a
cwlSUP1	tcacttgaagattcgcctca	n/a
cwlSUP2	cactggccgtcgttttaccaagccggctacaatcttct	n/a
cwlSDW1	catggtcatagctgtttccttgggcgcaagaagttattt	n/a
cwlSDW2	ataaccgaccaatgcctcag	n/a
ywtDUP1	aacaaccacgcaatggatta	n/a
ywtDUP2	cactggccgtcgttttactcttccagtttgccagtgtg	n/a
ywtDDW1	catggtcatagctgtttccgataaatacgcaggaagtatacgg	n/a
ywtDDW2	ttgatgtaaaagcgctgctg	n/a
ggtUP1	acgttttccggagtgtatgc	n/a
ggtUP2	cactggccgtcgttttaccagacgttccacgttcttttc	n/a
ggtDW1	catggtcatagctgtttcccgatcggcattaatttaaaacg	n/a
ggtDW2	ttgttgatggaacgtcctga	n/a
PB-M13-20	gtaaaacgacggccagtg	n/a
PB-M13Rev	ggaaacagctatgaccatg	n/a
amyE5XbaI	gctctagacatcggtttgaaaggaggaa	*Xba*I
amyE3KpnI	ggggtaccagagttcctcaggctgtcca	*Kpn*I
Sp-Fw	ggccatcgatatcgattttcgttcgtgaataca	*Cla*I
Sp-Rv	gcgcatcgatctcgagccggagatctcatatgcaagggtttattgtttt	*Cla*I, *Xho*I and *Bgl*II
cwlO5BglII	gggaagatctagtcatgagctgctgggttt	*Bgl*II
cwlO3SalI	acgcgtcgaccttccgtattggccatttgt	*Sal*I

The restriction sites are underlined; the *Xho*I site is double underlined. n/a = not applicable.

ture was inoculated into 50 ml of γPGA production medium in 500-ml Sakaguchi flasks and shake-cultured at 37°C without antibiotics. Then, 2 ml of the culture medium was centrifuged and 0.5 ml of the supernatant was transferred to a new tube, to which 25 μl of 50% (w/v) trichloroacetic acid was added. This was followed by incubation for 20 min at 50°C. After centrifugation for 20 min at 20,000 × *g*, the supernatants were transferred to new tubes and neutralized with 1 M NaOH. Then, 1 ml of ethanol was added to the solution, and the solution was centrifuged for 10 min at 20,000 × *g*. The pellets were washed with 70% (v/v) ethanol, air dried, then dissolved in 0.5 ml of distilled water. The γPGA solutions were diluted with distilled water, and 0.2 ml of CET solution (0.1 M cetyltrimethylammonium bromide, 1 M NaCl) was added to 1 ml of the dilute solutions. After 20 min, the absorbance was measured at 400 nm and the γPGA contents (μg/ml) were calculated (absorbance×21.94).

The molecular weight of γPGA was determined by HPLC using a column [GF-7M HQ (7.5×300 mm); Showa Denko, Kanagawa, Japan] and a guard column (GF-1G 7B; Showa Denko) with a refractive index detector (RI) and a UV wavelength of 210 nm (Nagai et al., 1997). The γPGA solutions were eluted with elution buffer [50 mM phosphate buffer, 0.1 M Na_2SO_4 (pH 6.8)] at a flow rate of 0.6 ml/min at 50°C. Pullulans (P-10, P-50, P-200 and P-800; Showa Denko) were used as molecular standards to determine the molecular weight of γPGA. The amounts of L-glutamic acid, su-

crose and D-glucose in the media were determined with an F-kit for L-glutamic acid and an F-kit for sucrose/D-glucose (J. K. International, Tokyo, Japan), respectively, according to the manufacturer's instructions.

Results

Time course of γPGA production

The time course of γPGA production by strain NAFM5 in the γPGA production media was measured, and consumption of the carbon source was determined (Fig. 1). The effect of some additives on γPGA production was also examined. γPGA was found to be poorly produced in LB alone (Fig. 1a) and in LB+1% Glu-Na (Fig. 1b). Addition of sucrose (0.5% final) to LB stimulated the production of γPGA (Fig. 1c), and the addition of sucrose and Glu-Na together led to a further increase in the amount of γPGA (Fig. 1d). γPGA production started at 4 h, which corresponds to the early stationary phase, and increased up to 48 h (Fig. 1d). However, over 80% of γPGA was produced within 24 h, so for the following experiments LB+1% Glu-Na+0.5% Suc was used as the γPGA production medium along with a culture time of 24 h.

Effect of cell wall lysis enzymes on γPGA production

The *ywtD* gene is located near the γPGA synthetic operon (*pgsBCA-ywtC*). YwtD is a γPGA degradation enzyme with high amino acid sequence similarity to the peptidoglycan-hydrolyzing D,L-endopeptidases, *lytE* and *lytF* (37% and 40% identities with those of the catalytic domains, respectively) (Suzuki and Tahara, 2003). Therefore, LytE and LytF could potentially have γPGA degradative characteristics. LytE and LytF are cell wall lytic enzymes. CwlO and CwlS are also cell wall lytic enzymes that have D,L-endopeptidase activi-

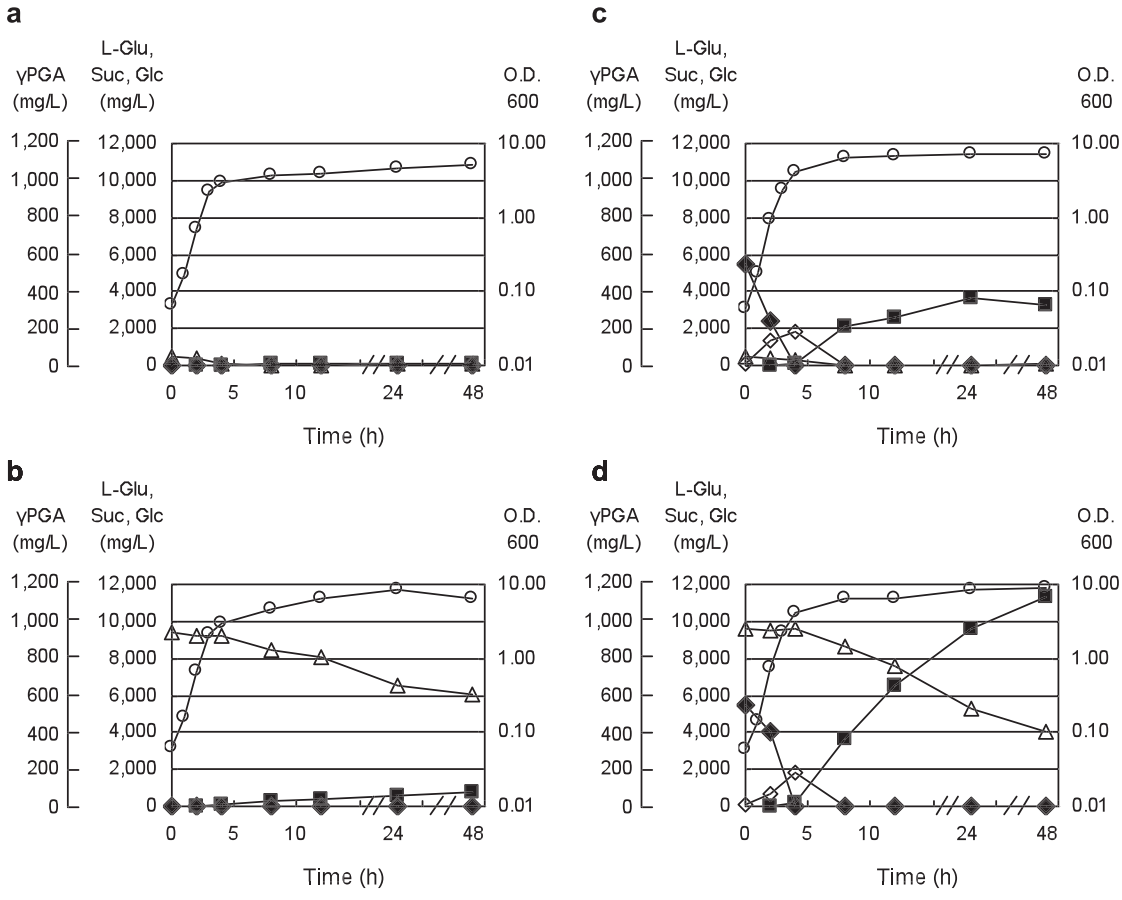

Fig. 1. The time courses of γPGA production and the consumption of carbon sources.

Strain NAFM5 was cultured in: a) LB, b) LB+1% monosodium L-glutamic acid monohydrate (Glu-Na), c) LB+0.5% sucrose, d) LB+1% Glu-Na+0.5% sucrose. γPGA (closed squares), L-glutamic acid (L-Glu: open triangles), sucrose (Suc: closed diamonds) and D-glucose (Glc: open diamonds) levels in the media and the O.D. 600 (open circles) were measured.

ty. Thus, CwlO and CwlS may have the potential to degrade γPGA. To evaluate the effect of cell wall lytic enzymes exhibiting D,L-endopeptidase activity on γPGA production, *lytE*, *lytF*, *cwlO* and *cwlS* disruptants were constructed. Figure 2 shows the amount of γPGA produced by each disruptant. The *lytE*, *lytF* and *cwlS* disruptants showed slightly increased γPGA production compared with the wild-type strain, and the *cwlO* disruptant produced about twofold higher amounts of γPGA (1.8 g/L).

Kimura et al. (2004) have reported that *B. subtilis* (*natto*) possesses another γPGA degradation enzyme, γ-glutamyltransferase (Ggt), that hydrolyzes γPGA from the amino-terminal end, and a *ggt* disruptant accumulated more γPGA than the wild-type strain after a long cultivation period. Disruptants of *ywtD* and *ggt* were constructed and γPGA production was compared with that of the *cwlO* disruptant and the wild-type strain. Increased γPGA accumulation was observed in the *cwlO* disruptant compared with the wild-type strain, whereas γPGA production in the *ywtD* and *ggt* disruptants was almost the same as in the wild-type strain at 24 h (Fig. 3). Since inhibition of γPGA degradation by the *ggt* disruption had been reported after long-term cultivation (Kimura et al., 2004), we also measured the amount of γPGA in all of the strains at 6 days. The γPGA levels at 6 days were slightly increased in all strains compared with the levels at 24 h, but their ratios to wild-type levels remained unchanged.

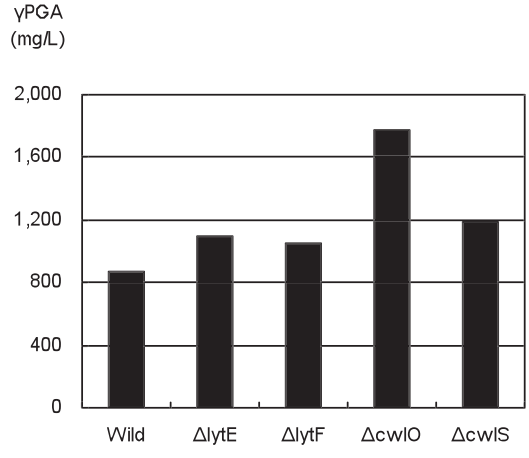

Fig. 2. γPGA production in the wild-type strain and the mutant strains in which cell wall lytic enzymes were disrupted.

The wild-type (Wild) and *lytE* (Δ*lytE*), *lytF* (Δ*lytF*), *cwlO* (Δ*cwlO*) and *cwlS* (Δ*cwlS*) disruptants were cultured in LB+1% Glu-Na+0.5% sucrose medium for 24 h, and the amounts of γPGA were measured. The figure is based on a single experiment.

To confirm the effect of *cwlO* disruption, a *cwlO* complementary strain was constructed by insertion of the *cwlO* gene along with the spectinomycin resistance gene at the *amyE* locus, and the amount of γPGA in this strain was measured (Fig. 4). Insertion of the spectinomycin-resistance cassette did not affect

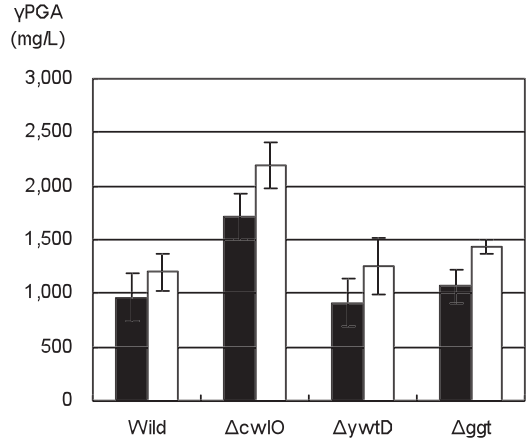

Fig. 3. γPGA production in the wild-type strain and the mutant strains in which γPGA degradation enzymes were disrupted.

The wild-type (Wild) and *cwlO* (Δ*cwlO*), *ywtD* (Δ*ywtD*) and *ggt* (Δ*ggt*) disruptants were cultured in LB+1% Glu-Na+0.5% sucrose medium for 24 h (closed boxes) and 6 days (open boxes), and the amounts of γPGA were measured.

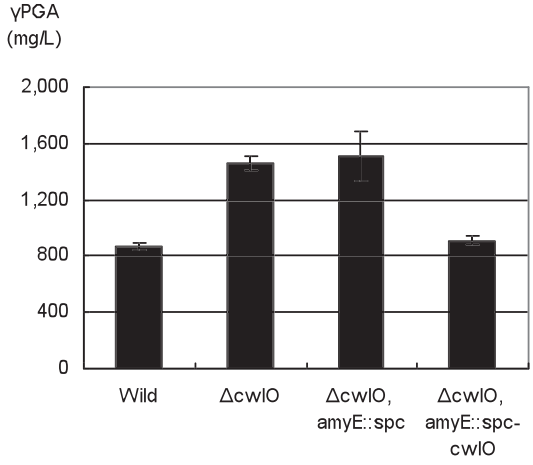

Fig. 4. γPGA production in the wild-type strain and the cwlO disruptant and complementary strains.

The wild-type (Wild), *cwlO* disruptant (Δ*cwlO*), control (Δ*cwlO*, *amyE*::*spc*) and complementary (Δ*cwlO*, *amyE*::*spc-cwlO*) strains were cultured in LB+1% Glu-Na+0.5% sucrose medium for 24 h and the amounts of γPGA were measured. The complementary strain and its corresponding control strain were constructed by insertion of the spectinomycin-resistant gene (*spc*) with or without the *cwlO* gene, respectively, into the *amyE* locus of the *cwlO* mutant genome.

the γPGA level but the *cwlO* gene insertion decreased γPGA production to wild-type levels. Therefore, increased production of γPGA was completely dependent on the deficiency of CwlO. Time courses of γPGA production and cell growth of the *cwlO* disruptant are shown in Fig. 5. The growth of the *cwlO* disruptant was similar to that of the wild-type. γPGA levels in the wild-type and the disruptant began to increase at 4 h and the γPGA levels were consistently higher in the disruptant compared with the wild-type strain. The period from 12 h to 24 h showed high production of γPGA (Fig. 5).

The molecular weight of the γPGA produced by the wild-type strain and the *cwlO* disruptant were investigated. The peak size of γPGA from the wild-type and *cwlO* disruptant were 2.4×10^6 and 4.9×10^6 Da, respectively, indicating that γPGA of the *cwlO* disruptant was larger than that of the wild-type (Fig. 6).

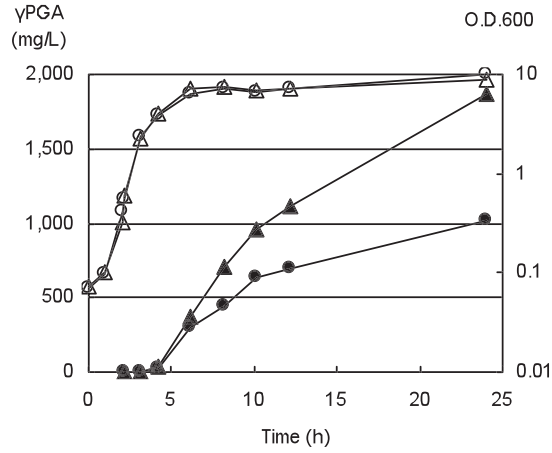

Fig. 5. The time course of γPGA production in the wild-type strain and *cwlO* disruptant strain.

The wild-type and *cwlO* disruptant were cultured in LB+1% Glu-Na+0.5% sucrose medium, and the cell density (O.D. at 600 nm) and γPGA levels were measured until 24 h. The open circles and triangles indicate the O.D. of the wild-type and the *cwlO* disruptant, respectively. The closed circles and triangles indicate the γPGA levels of the wild-type and the *cwlO* disruptant, respectively.

Discussion

B. subtilis (*natto*) is a γPGA producer, but it does not produce γPGA in LB media (Fig. 1). The effects of 1% Glu-Na and/or 0.5% sucrose on γPGA production were investigated (Fig. 1). The addition of 1% Glu-Na alone did not affect γPGA production, whereas the addition of 0.5% sucrose increased γPGA production and the addition of both Glu-Na and sucrose led to the best production of γPGA in this study. Shi et al. (2006) reported that sucrose was the most appropriate carbon source and that addition of L-glutamic acid increased the production of γPGA in this organism; our results were in good agreement with this report. In our study, γPGA production started early in the stationary phase, and sucrose and glucose were consumed at the start of γPGA production (Fig. 1). PgsB is the γPGA synthase dependent on ATP (Ashiuchi et al., 1999, 2001; Urushibata et al., 2002) and the *pgsB* gene is expressed in the early stationary phase (Kimura et al., 2009). These results suggested that the accumulation of ATP and/or an intermediate of γPGA are required for γPGA synthesis.

Among the cell wall lytic enzymes exhibiting D,L-endopeptidase activity, CwlO was found to be the most effective for γPGA production (Fig. 2). The γPGA synthetic enzyme complex was located on the cell membrane (Ashiuchi et al., 2001; Candela and Fouet, 2006), and γPGA accumulated extracellularly. Therefore, γPGA degradation may occur extracellularly. The cell

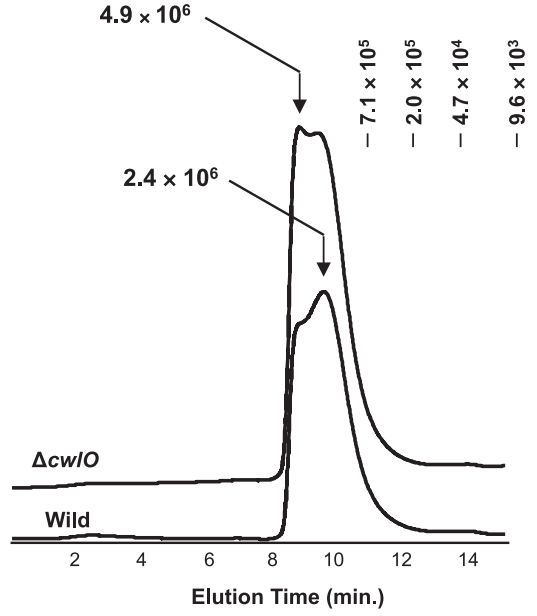

Fig. 6. The molecular size of the γPGA produced by the wild-type strain and *cwlO* disruptant strain.

The wild-type (Wild) and *cwlO* disruptant (Δ*cwlO*) strains were cultured in LB+1% Glu-Na+0.5% sucrose medium for 24 h. The molecular size of γPGA was measured as described in Materials and Methods. The arrows indicate the molecular weights corresponding to the peaks, and the elution times of the pullulan standards are shown to the upper right of the figure along with their molecular weights.

wall hydrolase, CwlO, has been detected in the culture supernatant (Yamaguchi et al., 2004), and it may affect degradation of γPGA. YwtD and Ggt are well recognized as γPGA degradation enzymes (Ashiuchi et al., 2006; Kimura et al., 2004; Suzuki and Tahara, 2003). In this study, we measured the levels of γPGA production in *ywtD* and *ggt* disruptants. Mutation of these genes did not affect γPGA production. In a previous report, Ggt activity was detected after a few days of culture and affected the amount of γPGA (Kimura et al., 2004). In our study, we measured γPGA levels after 6 days of culture, but no decrease in γPGA levels was detected (Fig. 3). Possibly, the differences in results between studies are due to different culture conditions. In our experimental conditions, L-glutamic acid remained at a level of more than 4 g/L after 48 h (Fig. 1), and the high concentration of L-glutamic acid may inhibit the hydrolytic activity of GGT (Kimura et al., 2004). The effect of *ywtD* on γPGA production is not well understood. The *ywtD* expression levels by the quantitative PCR were lower than the *cwlO* expression levels in our conditions (data not shown). Therefore, disruption of *ywtD* may have weakly affected γPGA production in our study.

The production level of γPGA by the *cwlO* disruptant was evaluated as follows. If glutamic acid is only converted to poly-γ-glutamic acid and the efficiency is 100%, 6.90 g of γPGA per liter is produced from 10.0 g of Glu-Na per liter. The wild-type and the *cwlO* disruptants produced 1.0 g and 1.8 g γPGA per liter, respectively, after 24 h (Fig. 5). Thus 14% and 26% efficiencies were achieved for the γPGA production of the wild-type and the *cwlO* disruptant, respectively. If we used the result (2.0 g/L) of γPGA production after 6 days (Fig. 3), the efficiency of production by the *cwlO* disruptant was 29%. Although the contributions of the other substrates, LB and sucrose, are not counted in this calculation, the increased amount by the *cwlO* disruption is very valuable for γPGA production.

We suggest that CwlO is the major enzyme responsible for γPGA degradation and that disruption of the *cwlO* gene results in the production of greater amounts of γPGA of greater molecular size. This is the first report to investigate the role of cell wall lytic enzymes in γPGA production.

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