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Role of Maltogenic Amylase and Pullulanase in Maltodextrin and Glycogen Metabolism of *Bacillus subtilis* 168▽†

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The physiological functions of two amylolytic enzymes, a maltogenic amylase (MAase) encoded by *yvdF* and a debranching enzyme (pullulanase) encoded by *amyX*, in the carbohydrate metabolism of *Bacillus subtilis* 168 were investigated using *yvdF*, *amyX*, and *yvdF amyX* mutant strains. An immunolocalization study revealed that YvdF was distributed on both sides of the cytoplasmic membrane and in the periplasm during vegetative growth but in the cytoplasm of prespores. Small carbohydrates such as maltoheptaose and β-cyclodextrin (β-CD) taken up by wild-type *B. subtilis* cells via two distinct transporters, the Mdx and Cyc ABC transporters, respectively, were hydrolyzed immediately to form smaller or linear maltodextrins. On the other hand, the *yvdF* mutant exhibited limited degradation of the substrates, indicating that, in the wild type, maltodextrins and β-CD were hydrolyzed by MAase while being taken up by the bacterium. With glycogen and branched β-CDs as substrates, pullulanase showed high-level specificity for the hydrolysis of the outer side chains of glycogen with three to five glucosyl residues. To investigate the roles of MAase and pullulanase in glycogen utilization, the following glycogen-overproducing strains were constructed: a *glg* mutant with a wild-type background, *yvdF glg* and *amyX glg* mutants, and a *glg* mutant with a double mutant (DM) background. The *amyX glg* and *glg* DM strains accumulated significantly larger amounts of glycogen than the *glg* mutant, while the *yvdF glg* strain accumulated an intermediate amount. Glycogen samples from the *amyX glg* and *glg* DM strains exhibited average molecular masses two and three times larger, respectively, than that of glycogen from the *glg* mutant. The results suggested that glycogen breakdown may be a sequential process that involves pullulanase and MAase, whereby pullulanase hydrolyzes the α-1,6-glycosidic linkage at the branch point to release a linear maltooligosaccharide that is then hydrolyzed into maltose and maltotriose by MAase.

Bacillus subtilis can utilize polysaccharides such as starch, glycogen, and amylose as carbon sources by hydrolyzing them into smaller maltodextrins via the action of extracellular α-amylase (AmyE) (14). In *B. subtilis*, α-glucosidase encoded by *malL* has been known to contribute to maltodextrin metabolism in the cell (40, 41). Schönert et al. (42) reported that maltose is transported by the phosphoenolpyruvate-dependent phosphotransferase system (PTS) in *B. subtilis*. They also reported that maltodextrins with degrees of polymerization (DP) of 3 to 7 (G3 to G7) are taken up via a maltodextrin-specific (Mdx) ATP-binding cassette (ABC) transport system (42). This system is made up of a maltodextrin-binding protein (MdxE) and two membrane proteins (MdxF and MdxG), as well as an ATPase (MsmX). The basic model proposed for the transport and metabolism of maltooligosaccharides includes a series of carbohydrate-hydrolyzing and -transferring enzymes. However, the enzymatic hydrolysis of maltodextrins and glycogen, providing a major energy reservoir in prokaryotes, was not reflected in the model, due probably to a lack of experimental analysis. Unlike those in *Bacillus* spp., the transport and metabolic systems for maltodextrins in *Escherichia coli* have been investigated extensively (7, 9, 10). A model for maltose metabolism involving an α-glucanotransferase (MalQ), a maltodextrin glucosidase (MalZ), and a maltodextrin phosphorylase (MalP) was proposed previously based on analyses of the breakdown of ^{14}C-labeled maltodextrins in various knockout mutants (10).

Ninety bacterial genomes were analyzed to identify the enzymes involved in sugar metabolism, and the results suggested that bacterial enzymes for the synthesis and degradation of glycogen belong to the glucosyltransferase and glycosidase/transglycosidase families, respectively. Free-living bacteria

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TABLE 1. Bacterial strains and plasmids used or constructed in this study

Bacterial strain or plasmid	Genotype and/or description[a]	Source or reference
E. coli strains		
MC1061	F$^-$ *araD139* Δ(*ara-leu*)*7696 galE15 galK16* Δ(*lac*)*X74 rpsL* (Str^r) *hsdR2*(r_K^- m_K^+) *mcrA mcrB1*; cloning or expression host	39
K-12	Wild type	
JT01	K-12 carrying *malZ*::*yvdF*	This study
B. subtilis strains		
168	Wild-type Marburg strain; *trpC2*	20
yvdF mutant	168 *yvdF* knockout mutant; Cm^r	20
amyX mutant	168 *amyX* knockout mutant; Kan^r	This study
yvdF amyX mutant	168 *yvdF amyX* double mutant; Cm^r Kan^r	This study
glg mutant	168 harboring pWHglg for glycogen production; Tet^r	This study
yvdF glg mutant	*yvdF* mutant strain harboring pWHglg for glycogen production; Cm^r Tet^r	This study
amyX glg mutant	*amyX* mutant strain harboring pWHglg for glycogen production; Kan^r Tet^r	This study
glg DM strain	*yvdF amyX* double mutant harboring pWHglg for glycogen production; Cm^r Kan^r Tet^r	This study
mdxE mutant	*mdxE* knockout mutant	42
Plasmids		
p6×His119	Carries BLMA promoter; Amp^r	23
p6×HisPul	Carries BLMA promoter; Amp^r; used for AmyX expression	This study
pWH1520	Shuttle vector with xylose-inducible expression; Amp^r Tet^r	MoBiTec
pWHglg	Vector for expression of the glycogen synthesis operon; Amp^r Tet^r	This study
pPyvdF-GFP	Vector for expression of the YvdF-GFP fusion protein; Amp^r Tet^r	This study

[a] BLMA, MAase from *B. licheniformis*.

such as *B. subtilis* carry a minimal set of enzymes for glycogen metabolism, encoded by the *glg* operon of five genes. The four genes most proximal to the promoter encode enzymes for the synthesis of glycogen, including a branching enzyme (*glgB*), an ADP-glucose phyrophosphorylase (*glgC* and *glcD*), and a glycogen synthase (*glgA*). On the other hand, the most distal gene, *glgP*, encodes a glycogen phosphorylase (a member of glycosyltransferase family 35) (13, 18), which degrades glycogen branches by forming glucose-1-phosphate (glucose-1-P). *B. subtilis* carries two additional enzymes encoded at separate loci, a maltogenic amylase (MAase [YvdF, encoded at 304°]) and a pullulanase (AmyX, encoded at 262°), which have been known to degrade glycogen in vitro (15, 31). These two enzymes are ubiquitous among *Bacillus* spp. and may play an important role in glycogen and maltodextrin metabolism in the bacteria (see Table S1 in the supplemental material).

The MAase YvdF in *B. subtilis* 168 and its homologue in *B. subtilis* SUH4-2 share 99% identity at both the nucleotide and amino acid sequence levels (4). MAase (EC 3.2.1.133) is a multisubstrate enzyme that acts on substrates such as cyclodextrin (CD), maltooligosaccharides, pullulan, starch, and glycogen (4). MAase belongs to a subfamily of glycoside hydrolase family 13, along with cyclodextrinase (EC 3.2.1.54), neopullulanase (EC 3.2.1.135), and *Thermoactinomyces vulgaris* R-47 α-amylase II (46). Although the catalytic properties and tertiary structure of MAase have been studied extensively (33), its physiological role in the bacterial cell is yet to be elucidated. The expression pattern of MAase in *B. subtilis* 168 has been investigated by monitoring the β-galactosidase activity expressed from the *yvdF* promoter in defined media containing various carbon sources (20). The *yvdF* promoter is induced in medium containing maltose, starch, or β-CD but is repressed in the presence of glucose, fructose, sucrose, or glycerol as the sole carbon source. In a previous study, Spo0A, a master regulator determining the life cycle of *B. subtilis*, was shown to be related to the expression of MAase in a positive manner (20). Kiel et al. (18) reported that the glycogen operon in *B. subtilis* is turned on during sporulation by RNA polymerase containing σ^E. This finding indicated that MAase, along with glycogen phosphorylase and pullulanase, might be involved in the metabolism of maltodextrin and glycogen in vivo.

Pullulanases are capable of hydrolyzing the α-1,6-glycosidic linkages of pullulan to form maltotriose (2, 11, 15, 28, 31, 38). In particular, type I pullulanases have been reported to hydrolyze the α-1,6-glycosidic linkages in branched oligosaccharides such as starch, amylopectin, and glycogen, forming maltodextrins linked by α-1,4-glycosidic linkages (11). Pullulanase is also known as a debranching enzyme. The enzymatic properties and three-dimensional structure of AmyX from *B. subtilis* 168 were investigated by Malle et al. (28). However, to date, the physiological function of pullulanase encoded by *amyX* has not been investigated.

The aim of this study was to elucidate the physiological functions of MAase and pullulanase, specifically concentrating on their roles in the degradation of maltodextrin and glycogen in *B. subtilis*. For this purpose, studies of the localization of the enzymes, the accumulation of glycogen, and the distribution of glycogen side chains were performed using the wild type and knockouts of MAase- and pullulanase-related genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and recombinant DNA constructs that were used or generated in this study are listed in Table 1. *E. coli* and *B. subtilis* strains were cultured in Luria-Bertani (LB) broth (1% Bacto tryptone, 0.5% yeast extract, and 0.5% NaCl) at 37°C with

shaking (250 rpm). The medium was supplemented with ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), or tetracycline (10 μg/ml) where necessary.

TLC. A K5F silica gel plate (Whatman, Maidstone, United Kingdom) was activated at 110°C for 1 h. Prepared samples were spotted onto the plate by using a pipette; the plate was placed in a thin-layer chromatography (TLC) chamber containing a solvent mixture of *n*-butanol–ethanol–water (5:5:3, vol/vol/vol), and results were developed twice at room temperature. The reducing sugar was detected using the naphtol-H_2SO_4 method (37). The plate was dried and the contents were visualized by being dipped into a solution containing 0.3% *N*-(1-naphthyl)-ethylenediamine and 5% H_2SO_4 in methanol and then being heated for 10 min at 110°C.

Degradation of maltodextrin and β-CD in *B. subtilis* strains. The wild-type strain 168, the *yvdF*, *amyX*, and *yvdF amyX* mutants of 168, and the *mdxE* mutant strain were cultured in 50 ml of LB medium to the stationary growth phase at 37°C with shaking (250 rpm). The cells were harvested and washed three times with M9 minimal medium salt (0.6% Na_2HPO_4, 0.3% K_2HPO_4, 0.05% NaCl, 0.1% NH_4Cl) and resuspended in 5 ml of the salt. The wild-type and mutant cells were incubated for 5 min at 37°C in M9 minimal medium containing 500 μM maltoheptaose (G7) or β-CD as the carbon source. Maltooligosaccharides in the cells were analyzed using high-performance anion-exchange chromatography (HPAEC) after the cells were washed twice with ice-cold M9 salt and sonicated three times for 5 min each with a VC-600 instrument (Sonics & Materials, Inc.) at 4°C. The cell lysates were centrifuged, and the supernatants were subjected to further analyses.

HPAEC. The reaction mixtures (or cell lysates) were boiled for 10 min before centrifugation at 12,000 × *g* for 10 min, and the supernatants were filtered using a membrane filter kit (0.2-μm pore diameter; Gelman Sciences). HPAEC was performed using a DX-500 system (Dionex, Sunnyvale, CA) equipped with a pulsed amperometric detector (ED40; Dionex) and a CarboPac PA-1 column (4 by 250 mm; Dionex). Samples were eluted with a linear gradient of 0.6 M sodium acetate in 0.15 M NaOH at a flow rate of 1.0 ml/min, as follows: 10 to 30% from min 0 to 10, 30 to 40% from min 10 to 16, 40 to 50% from min 16 to 27, 50 to 60% from min 27 to 44, and 60 to 64% from min 44 to 60.

Construction of glycogen-overproducing mutants. A 6.0-kb DNA fragment harboring the entire *glg* operon of *B. subtilis* was amplified from the genomic DNA by PCR using the primers Glg/SpeI (5′-ATG GCC GCT ACT AGT CCG ACA GCA CAT G-3′) and Glg/SacI (5′-GCC GCT TAG AGC TCG GCT TTT TCA CAT C-3′). The PCR product was cloned into pWH1520 (MoBiTec, Göttingen, Germany), a shuttle vector. The operon was placed under the control of the *yvdF* promoter (P_{yvdF}), which had been amplified by PCR using the primers PyvdF/SalI (5′-CCT GTT GTT GTC GAC GGG GAA GCC-3′) and PyvdF/SpeI (5′-GCT GCA TAA CTA GTC ATG TTT CCC CC-3′) and replaced the promoter on pWH1520, and the resulting recombinant DNA construct was designated pWHglg. The wild-type and *yvdF*, *amyX*, and *yvdF amyX* mutant strains were transformed with pWHglg, yielding a *glg* mutant strain with the wild-type background, *yvdF glg* and *amyX glg* mutant strains, and a *glg* mutant strain with a double mutant background (the *glg* DM strain), respectively.

Construction of the *E. coli malZ* mutant. To replace the *malZ* gene of *E. coli* K-12 with the *yvdF* gene from *B. subtilis* 168, a PCR template plasmid was constructed as follows. The *cat* gene was introduced into pET29b (Novagen Inc., Madison, WI) after amplification by PCR using pACYCDuet-1 (Novagen Inc., Madison, WI) with the primers Cat/NdeI (5′-CTT TGC GCC CAT ATG ATA CCT GTG AC–3′) and Cat/EcoRV (5′-CCG CTT ATT GA T ATC TAT TCA GGC G–3′). The *yvdF* gene was amplified by PCR from the *B. subtilis* 168 genomic DNA by using the primers YvdF/F-NdeI (5′-CAA TTG TAT CAT ATG ATA GAA AGC GG–3′) and YvdF/R-NdeI (5′-CAA AGG GGG ACA TAT GAT GGA ATA TG-3′) and then inserted close to the *cat* gene on pET29b. The *yvdF-cat* region of the vector was amplified by PCR using forward and reverse primers that each had a 30-nucleotide-long sequence homologous to *malZ* at the 5′ end. The resulting PCR product was used for mutagenesis as described by Datsenko and Wanner (6), and the resulting mutant was designated *E. coli* JT01 (*malZ*::*yvdF*).

Overexpression and purification of pullulanase. The *amyX* gene from the genomic DNA of *B. subtilis* 168 was amplified by PCR using the primers AmyX/NdeI (5′-GGA GAG AGC TTC ATA TGG TCA GCA TCC GCC GC-3′) and AmyX/SalI (5′-GCG CTA TAG CAC AGT CGA CGT CAA GCA AAA CTC-3′) such that the product carried appropriate restriction sites for cloning. The PCR product was digested with NdeI and SalI and ligated into p6×His119 at the corresponding restriction sites. *E. coli* MC1061 was transformed with the resulting construct (p6×HisPul) and cultured on LB agar containing ampicillin (100 μg/ml). Pullulanase produced in *E. coli* was purified by nickel-nitrilotriacetic acid chromatography, and the activity toward pullulan was measured using the 3,5-dinitrosalicylic acid method (30). One unit of enzyme activity was defined as the amount of enzyme that cleaved the equivalent of 1 μmol of glycosidic bonds in the substrate in 1 min under the reaction conditions.

Isolation of glycogen from *B. subtilis*. Four *B. subtilis* strains that overproduced glycogen, i.e., the *glg*, *yvdF glg*, *amyX glg*, and *glg* DM strains, were cultured in LB broth (500 ml) at 37°C with shaking (250 rpm). Maltodextrin (0.5%; dextrose equivalent, 12 [Daesang Co., Seoul, Republic of Korea]) was added as a carbon source for glycogen accumulation when the cultures reached the stationary growth phase. The strains were cultured further, and 100-ml aliquots were taken at 5, 10, 15, and 25 h after the addition of maltodextrin. The cells were harvested by centrifugation at 6,000 × *g* for 20 min, washed with ice-cold water, and resuspended in 5 ml of 50 mM sodium acetate (pH 4.5). The suspensions were boiled for 10 min and sonicated three times for 5 min each with a VC-600 instrument (Sonics & Materials, Inc.) at room temperature. The crude cell extracts were centrifuged at 10,000 × *g* for 20 min at 25°C, and glycogen in the supernatants was precipitated by adding 2 volumes of ethanol. Precipitated glycogen was collected by centrifugation at 10,000 × *g* for 20 min and air dried. The dried glycogen was resuspended in 1 ml of sterile distilled water and stored at −18°C until further analysis.

SEC-MALLS/RI analysis. The distribution of glycogen molecular weights was analyzed based on size by multiangle laser light scattering (MALLS) coupled with size exclusion chromatography (SEC) using a SEC-MALLS/refractive index (RI) system (Dawn DSP; Wyatt Technology) and an RI detector (model 410; Waters). SUGAR KS-804 and KS-806 columns (8 by 300 mm; Shodex, Kawasaki, Japan) were connected in tandem and equilibrated at room temperature. The flow rate of the mobile phase (0.15 M $NaNO_3$ containing 0.02% NaN_3) was 0.4 ml/min. Purified glycogen from each *B. subtilis* strain was filtered through a 0.5-μm-pore-size disposable syringe filter and injected into the SEC-MALLS/RI system. The weight-average molecular weight (M_w) of the sample was calculated using ASTRA V4.90.07 software (Wyatt Technology) with the Berry extrapolation method for curve fitting and a refractive index increment, dn/dc, of 0.146 ml/g.

Localization of MAase. For the immunolocalization study of MAase, *B. subtilis* 168 was cultured overnight in 5 ml of LB broth and then transferred into the same volume of fresh LB broth supplemented with 0.5% maltose and cultured for 3 h to induce the expression of MAase. Specimens were processed and gold labeled as described previously (22). Before energy-filtering transmission electron microscopy (TEM) with a LIBRA 120 microscope (Carl Zeiss, Germany), Western blotting was performed to detect the expression of MAase. Cells treated with the serum of an unimmunized rabbit were used as a control. For the localization of MAase in the endospore, *B. subtilis* was cultured in 5 ml of LB broth supplemented with 0.5% maltose and harvested when the optical density at 600 nm reached 1.5. The pellet was washed twice with ice-cold M9 minimal medium and transferred into modified Shaeffer's sporulation medium [27 mM KCl, 2 mM $MgSO_4$, 1 mM $Ca(NO_3)_2$, 0.1 mM $MnCl_2$, and 0.001 mM $FeSO_4$]. The cells were cultured overnight and prepared for energy-filtering TEM.

Green fluorescent protein (GFP) was fused with MAase as follows. The pEGFP plasmid (BD Biosciences Inc., CA) was digested with KpnI and XhoI (New England Biolabs Inc., MA), and the fragment containing the GFP gene was ligated to pWH1520 (MoBiTec, Germany). The resulting recombinant DNA construct was designated pWHGFP. The whole *yvdF* gene, including the promoter, was amplified by PCR and subcloned into pWHGFP at the SalI and KpnI sites by removing the original promoter on pWH1520. The final recombinant DNA construct (pPyvdF-GFP) expressed the fusion protein YvdF-GFP under the control of the *yvdF* promoter. *B. subtilis* carrying the recombinant plasmid was cultured under the same conditions used for the immunolocalization study. Vegetative cells and endospores of the *B. subtilis* transformant were observed by using an Axio Imager fluorescence microscope (Zeiss, Germany).

RESULTS

Localization of MAase during vegetative growth and sporulation. The vegetative cells of *B. subtilis* 168 were embedded in LR white resin and immunolabeled with an anti-MAase antibody and a secondary antibody conjugated to colloidal gold (Fig. 1A and C). MAase was also fused to GFP in an effort to detect MAase expression in the cell (Fig. 1B and D). Immunogold electron microscopy showed that the gold particles were present predominantly on both sides of the cytoplasmic membrane and in the periplasmic space (Fig. 1A) but were rarely observed in the cytoplasm or outside of the cells during

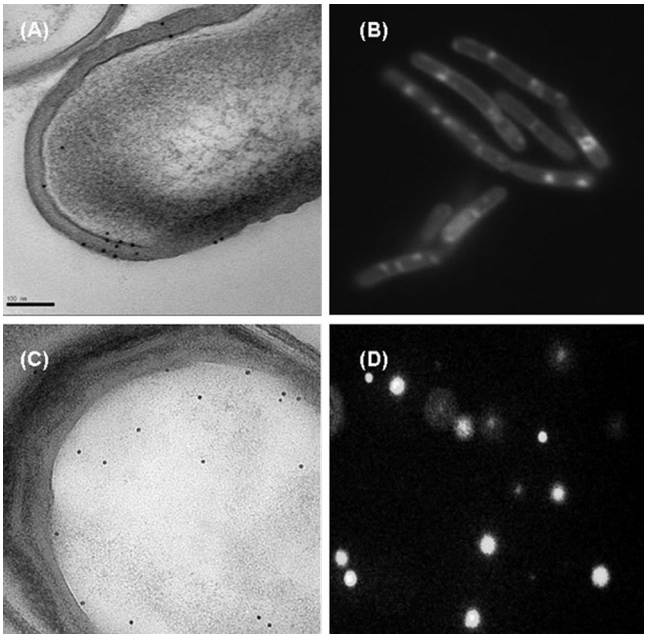

FIG. 1. TEM and fluorescence microscopy images of MAase in *B. subtilis* during vegetative growth (A and B) and sporulation (C and D). The black bar in the TEM image in panel A represents 100 nm and corresponds to the image in panel C as well. Fluorescence microscopy images (B and D) were obtained at an original magnification of ×1,000.

vegetative growth (Fig. 1B). Results from a previous fractionation study of MAase using spheroplasted cells suggested that the enzyme was localized mainly in the periplasmic space (data not shown), as Anderson and Salyers (1) reported for neopullulanase of *Bacteroides thetaiotaomicron*. Using immunogold labeling and a MAase-GFP fusion protein, we demonstrated that MAase is present in the prespores during the sporulative phase (Fig. 1C and D). Therefore, the localization of MAase in the cell varied according to the life cycle of *B. subtilis*; these differential localization patterns are probably related to differences in carbohydrate metabolism during the growth phases.

Use of G7 by MAase in vivo. To investigate the role of MAase in the maltodextrin metabolism of the cell, wild-type *B. subtilis* and the *yvdF* knockout mutant were incubated in M9 minimal medium containing G7. Maltooligosaccharides in the cells were analyzed by HPAEC after 5 min of incubation (Fig. 2). The *mdxE* mutant, which does not transport maltooligosaccharides into the cytoplasm (42), was used as a control. Significant amounts of G7 and its degradation products were detected in the wild-type cells compared with the amounts in the *mdxE* cells. In the *yvdF* mutant, G7 was detected in an amount similar to that in the wild type, but its degradation products were detected only in trace amounts (Fig. 2A). Thus, G7 taken up into the cells via MdxE was likely to be hydrolyzed mainly to maltose by MAase. The significantly larger amounts of G2 and G3 in the *mdxE* mutant than in the *yvdF* mutant may

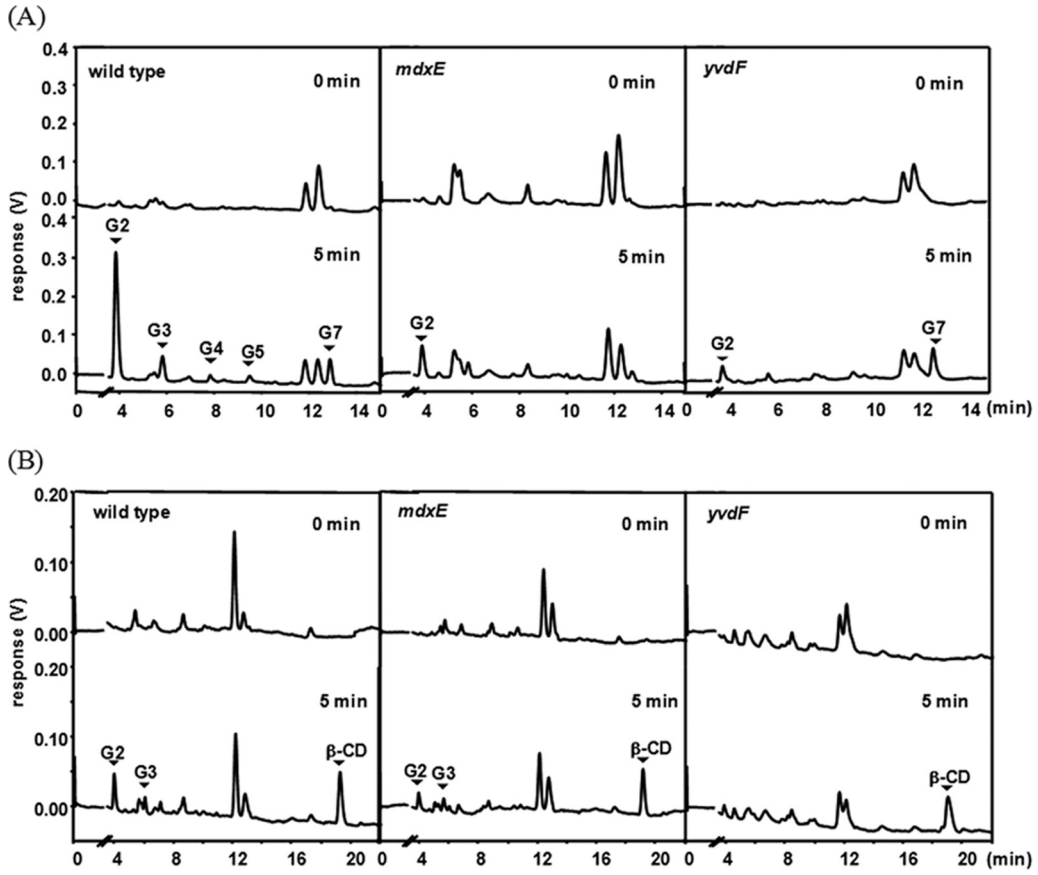

FIG. 2. HPAEC analyses of G7 (A) and β-CD (B) taken up and degraded by wild-type and *mdxE* and *yvdF* mutant strains. The cells were incubated with each carbon source for 5 min and harvested. Total cell extracts were subjected to HPAEC after the removal of proteins.

indicate that MAase degraded maltooligosaccharides in the periplasmic space before transportation into the cytoplasm. In *B. subtilis*, α-glucosidase (MalL) can hydrolyze maltodextrins with DP of <6 but exhibits significantly reduced activity toward those with DP of >7 (40, 41). In contrast, MAase prefers G7, as well as maltopentaose (G5) and maltohexaose (G6), allowing the bacterium to use longer maltooligosaccharides efficiently (21, 34).

To characterize the role of MAase in maltodextrin metabolism further, an *E. coli* K-12 mutant carrying the *malZ*::*yvdF* fusion was constructed by replacing the *malZ* gene that encodes maltodextrin glucosidase with the *yvdF* gene and was designated JT1. MalZ has catalytic characteristics similar to those of MAase, hydrolyzing γ-CD and attacking maltooligosaccharides from the reducing end, but differs in substrate preference, producing glucose from relatively small maltooligosaccharides (G3 and G4) (48). *E. coli* K-12 and JT1 were cultured in 5 ml of LB broth at 37°C overnight. The cells were harvested and washed twice with ice-cold M63 medium and then transferred into 100 ml of M63 medium containing G7 (0.5%) as the carbon source (10). The growth and expression of MAase in JT1 were monitored by measuring the optical density at 600 nm and the β-CD-hydrolyzing activity, respectively, at appropriate time intervals. The expression of MAase in *E. coli* was confirmed by immunolocalization analysis (data not shown). The two strains in cultures containing G7 as the carbon source had logarithmic growth phases of similar lengths (16 to 18 h), longer than those of strains in cultures containing glucose or maltose as the carbon source (data not shown). In the medium containing either glucose or maltose, the mutant grew at a rate that was the same or slightly lower than that of wild-type growth (data not shown). However, the mutant grew 1.7 times faster than the wild type in the medium containing G7, implying a significant effect of MAase on G7 utilization (see Table S2 in the supplemental material). Thus, MAase was confirmed to be involved in the use of maltooligosaccharides in vivo.

Use of β-CD by MAase in vivo. The uptake and subsequent degradation of β-CD by MAase in *B. subtilis* were investigated by adding β-CD to cultures of the wild-type and *yvdF* strains and then analyzing β-CD and the resulting accumulation of maltooligosaccharides in the cells (Fig. 2B). Similar amounts of β-CD were detected in the two strains, indicating that β-CD was taken up by each. However, some hydrolysis products ranging from G2 to G7 were detected in the wild type, but only a limited amount of such products was detected in the *yvdF* mutant. Thus, the wild-type strain degraded β-CD to G2 to G7, whereas the mutant strain was attenuated in this activity because of the absence of MAase. In the case of the *mdxE* mutant, β-CD was likely to be transported into the cell efficiently (Fig. 2B) and then hydrolyzed by MAase. *Bacillus* strains have the CD-binding protein CycB (16), and the promoter for the *yvdF* gene has been shown previously to be induced and expressed fully in the early stationary phase in the presence of β-CD, G2, or starch as the carbon source but repressed in the presence of glucose or fructose (20). The *yvdF* mutant grew poorly when β-CD was added to the growth medium as the sole carbon source. This result suggested that MAase may be involved in the metabolism of maltodextrin and β-CD when readily usable sugars such as glucose and fructose are not available. The results confirm that *B. subtilis* uses β-CD as a carbon source via the action of MAase.

Debranching of the outer side chain of glycogen by pullulanase AmyX. To investigate the specificity of pullulanase from *B. subtilis* in the hydrolysis of side chains, branched β-CDs (1.2 μmol/ml) with various DP (G2 to G6) were incubated with a purified pullulanase (0.5 U/mg of the substrate). TLC analysis of the hydrolysates indicated that branched β-CDs comprising side chains with DP of 3 to 5 decreased rapidly but that branched maltosyl- and hexaosyl-β-CDs decreased slowly as the reaction proceeded (Fig. 3). Thus, the enzyme preferred branched side chains with three to five glucosyl residues for hydrolysis. Similarly, the preference of pullulanase (0.5 U/mg of the substrate) for the side chain length was examined by comparing the distribution of side chain lengths before and after the treatment of glycogen with the enzyme. The numbers of side chains with three to five glucosyl units decreased significantly, by 40% in 30 min, whereas the numbers of side chains with more than six glucosyl units decreased by only 10% (see Fig. S1 in the supplemental material). The side chains with DP of 2 and >9 remained almost intact under the same reaction conditions. Thus, the pullulanase has a specific preference for side chains with DP of 3 to 5, which are the lowest DP that can be hydrolyzed by glycogen phosphorylase (36). This observation correlated well with the results of the degradation of branched β-CDs by the enzyme.

Based on these results, a working hypothesis for the process of sequential degradation of glycogen in *B. subtilis* was established. Glycogen phosphorylase attacks the side chains of glycogen from the nonreducing end to release glucose and stops cleaving α-1,4-linkages when it reaches a terminal residue that is 4 residues away from a branch point. This process exposes four glucosyl residues for hydrolysis by the pullulanase, which has a specific preference for G4 side chains. The resulting free G4, a favored substrate of MAase, may be hydrolyzed immediately into G2 by the enzyme (34).

Accumulation and degradation of glycogen in vivo. *B. subtilis* 168 accumulated glycogen in only a limited amount (18). To investigate the accumulation and degradation of glycogen in *B. subtilis* 168, mutant strains that overproduced glycogen were constructed by introducing the whole *glg* operon on a multicopy plasmid into the wild-type and *yvdF*, *amyX*, and *yvdF amyX* mutant strains and were designated the *glg*, *yvdF glg*, *amyX glg*, and *glg* DM strains, respectively. The glycogen-rich mutants were then cultured to monitor the accumulation and degradation of glycogen. Time course assays of glycogen formation and breakdown indicated that all of the mutants produced more glycogen than the wild type over 5 to 10 h of growth; the accumulation of glycogen in the *amyX glg* and *glg* DM strains was more significant than that in the wild type. Accumulation in the *glg* mutant with the wild-type background and in the *yvdF glg* mutant reached maximal levels after 10 h, and that in the *amyX glg* and *glg* DM strains reached maximal levels at 15 h (Fig. 4). Glycogen breakdown began thereafter. Glycogen was degraded significantly in the wild type when the strain was cultured for longer than 10 h, whereas it decreased only slightly in the *amyX glg* mutant and the *glg* DM strain during the same period (Fig. 4). The *yvdF glg* strain exhibited an intermediate decrease. Therefore, both MAase and pullulanase are likely to be involved in the degradation of glycogen,

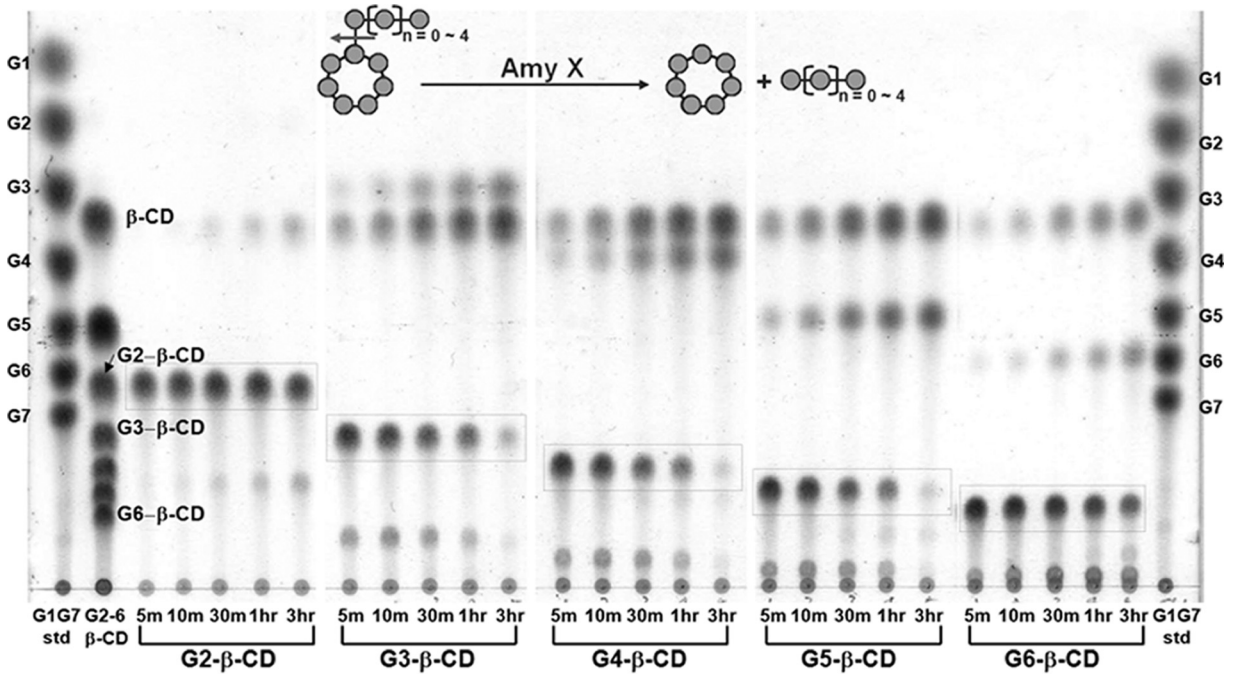

FIG. 3. TLC analysis of the specificity of AmyX in hydrolyzing branched β-CDs comprising branches with DP of 2 to 6. The concentration of each substrate was 1.2 μmol/ml. m, minute; std, standard.

but the latter seems to contribute to the process more directly. Analyses of glycogen degradation carried out in vitro using cell extracts from the wild type, the *yvdF* and *amyX* mutants, and the *yvdF amyX* double mutant demonstrated tendencies similar to the results obtained in vivo (see Fig. S2 in the supplemental material). Glycogen in the reactions with cell extract from the *amyX* or *yvdF amyX* mutant was hardly attacked compared to that in the reaction with cell extract from the wild type. The strains ranked in the following order based on residual amounts of glycogen, from largest to smallest, after cell extract treatment: *yvdF amyX* double mutant > *amyX* mutant > *yvdF* mutant > wild type.

In the *amyX glg* and *glg* DM strains, the numbers of side chains with DP of 4 increased enormously after 5 to 10 h of cultivation, a period during which glycogen accumulated (Fig. 4C and D). This result indicates that pullulanase has a high degree of specificity for side chains consisting of four glucosyl residues. The number of side chains with four glucosyl residues in the *amyX glg* strain increased over 15 to 25 h of culture (Fig. 4C). This finding correlated well with the results obtained in the in vitro study and shows more specifically that pullulanase has a strong preference for maltotetraosyl side chains left at the branch point by the action of glycogen phosphorylase. The variation in side chain distribution among the strains suggested that pullulanase controlled the amount of glycogen in the cell. Thus, pullulanase in combination with MAase is involved in the utilization of glycogen; specifically, pullulanase releases side chains by cleaving the α-1,6-glycosyl linkage at the branch point, and thereafter, MAase hydrolyzes the resulting glucan into maltose.

Molecular masses of glycogens accumulated by *B. subtilis* 168 strains. To investigate the effects of MAase and pullulanase on the sizes and structures of the glycogen molecules, the molecular masses of glycogens in the wild type and the *yvdF*, *amyX*, and *yvdF amyX* mutants over 5 and 10 h of growth were monitored using the SEC-MALLS/RI system. The average molecular masses of glycogens that accumulated in the *amyX* mutant and the *yvdF amyX* double mutant were significantly increased to 4.22×10^7 and 7.90×10^7 g/mol, respectively, nearly two to three times that of glycogen in the wild type. In contrast, the molecular mass of glycogen from *yvdF* was similar to that of glycogen from the wild type (Table 2). Thus, pullulanase remains active during glycogen synthesis and is involved in designing the structure of glycogen by debranching the side chains. Therefore, the role of pullulanase is clearly to facilitate the degradation of glycogen, resulting in the production of smaller glycogen molecules by reducing the frequency of external side chains.

DISCUSSION

The gene encoding MAase (*yvdF*) is a member of the gene cluster for maltodextrin utilization, which includes *malL* for α-glucosidase. During the early stationary phase of vegetative growth, MAase was located adjacent to both sides of the cell membrane, where it would immediately hydrolyze any incoming maltodextrin (G7) into maltose. α-Amylase was not likely to be responsible for the transport of small maltodextrins such as G7, since no difference in the profiles of maltodextrins taken up by the *mdxE* and *mdxE amyE* mutants was observed (data not shown). MAase on both sides of the membrane may function in coordination with sugar uptake systems such as the PTS and Mdx during vegetative growth. However, the enzyme may

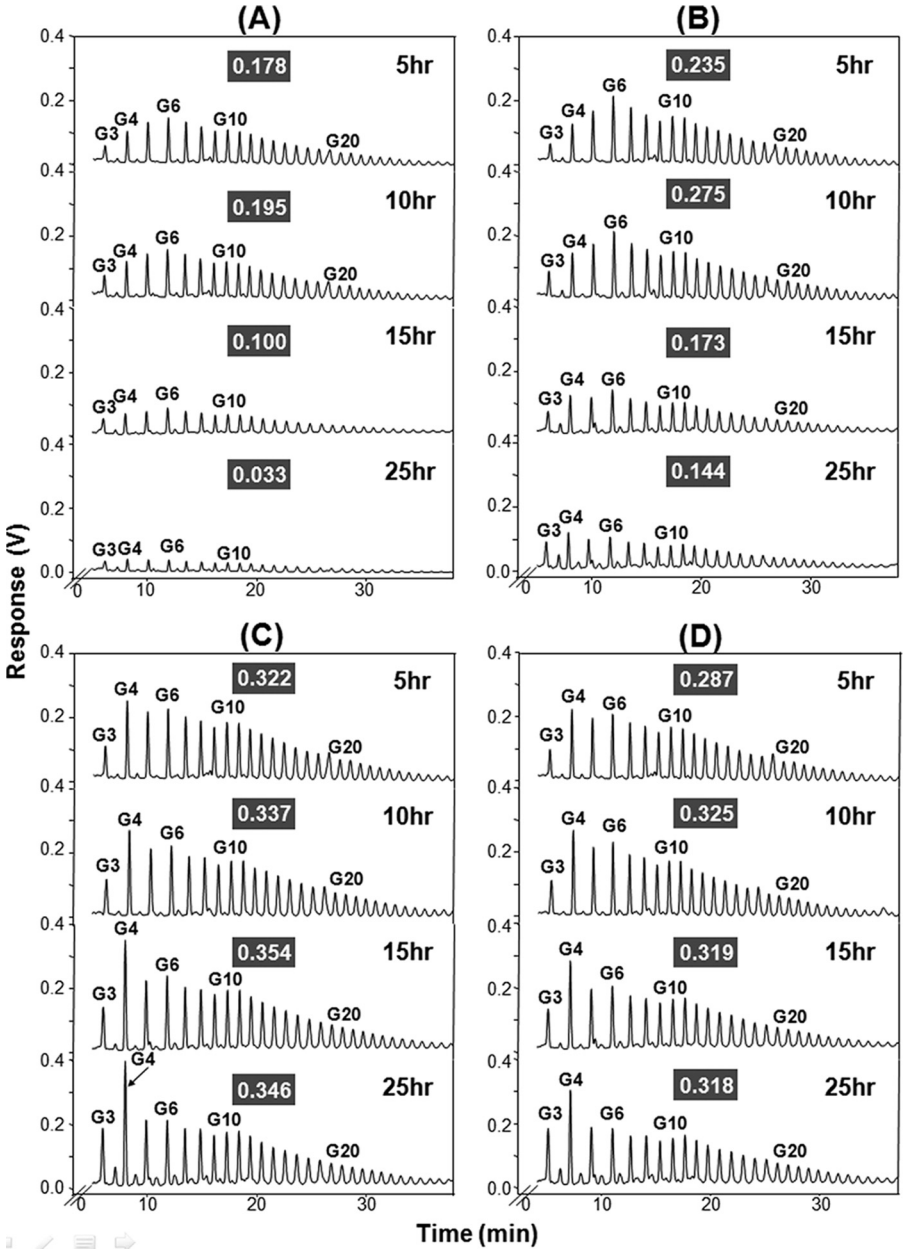

FIG. 4. HPAEC analyses of the distribution of glycogen side chain lengths in the cells of *glg* (A), *yvdF glg* (B), *amyX glg* (C), and *glg* DM (D) strains. The glycogen amount (in milligrams per milliliter of culture medium, indicated by the numbers in the dark gray boxes) was calculated from the sum of peak areas. Total areas in the HPAEC chromatogram for bovine glycogen at various concentrations were used as standards.

TABLE 2. Average molecular masses of glycogens from *B. subtilis* 168 strains

Incubation time[b] (h)	Avg molecular mass[a] (10^7 g/mol) of glycogen from:			
	Wild type	*yvdF* mutant	*amyX* mutant	*yvdF amyX* double mutant
5	1.01 ± 0.05	1.26 ± 0.12	1.57 ± 0.34	1.37 ± 0.10
10	2.46 ± 0.38	2.57 ± 0.40	4.22 ± 0.46	7.90 ± 1.8

[a] Molecular masses were determined using SEC-MALLS/RI analysis, and values are expressed as averages ± standard deviations.
[b] Incubation time after the addition of the carbon source.

be present in the cytoplasm of a prespore for another role in carbohydrate metabolism during the process of sporulation.

An operon consisting of a regulatory gene (*susR*) and seven structural genes (*susABCDEFG*) that are involved in the degradation of starch in *Bacteroides thetaiotaomicron* has been identified previously (5, 8, 43, 45). Two of the genes, one encoding neopullulanase (*susA*) and the other encoding α-glucosidase (*susB*), are involved in starch utilization, along with the pullulanase gene (*pulI*) at another locus. Most of the neopullulanase and α-glucosidase activities appear to be associated with the membrane, whereas the pullulanase activity is in the soluble fraction (43). When the *susA* gene, whose prod-

uct is highly homologous to MAase, is disrupted, *Bacteroides thetaiotaomicron* can still grow on starch but only at a rate reduced by 70% (8). Recently, an unusual starch metabolism pathway in a hyperthermophilic sulfate-reducing archaeon, *Archaeoglobus fulgidus*, was described (25). The pathway comprises the combined action of an extracellular CD glucanotransferase (CGTase) converting starch into CD and an intracellular cyclodextrinase, a homologue of MAase, hydrolyzing the intermediate into maltose.

The expression of many degradative enzymes that are produced in the late exponential phase of bacterial growth was reported to be under the regulation of *spo0A*, which serves as a master regulator in controlling the life cycle of *B. subtilis* (12). Previously, the *yvdF* promoter has been shown to be regulated by the *spo0A* gene in a positive manner (20). In an experiment with heat treatment (80°C for 20 min), the *yvdF* mutant produced roughly twice as many endospores as the wild type, which led to a phenotype of darker pigmentation (data not shown). Therefore, MAase seemed to be involved in the metabolism of carbohydrates in the stationary phase or during the process of sporulation.

In bacteria, the lack of nitrogen-based nutrients in the presence of excess carbon causes the intracellular accumulation of glycogen. However, glycogen is barely detected in *B. subtilis* 168, probably because it accumulates in insignificant amounts. In contrast, many other *Bacillus* spp. accumulate glycogen in fairly large amounts (17, 19, 35, 44). The mechanism of glycogen degradation in *Bacillus* spp. compared to that in mammalian and other bacterial systems is not clearly understood. Many *Bacillus* spp. possess MAase and pullulanase as a pair, which can degrade glycogen efficiently (34). MAase that was present in the vicinity of the cellular membrane during vegetative growth was redistributed into the cytoplasm of a prespore during sporulation (Fig. 1C and D), indicating that the enzyme may make contact with intracellular materials such as glycogen in the prespore. Therefore, we postulated that MAase and pullulanase are involved in the degradation of glycogen in *B. subtilis*, as in *B. cereus* and *Clostridia* sp. (27), mainly during the process of sporulation.

Pullulanase in *B. subtilis* exhibited substrate preference for maltodextrin with a DP of 4 and was likely involved in structuring glycogen during its synthesis, as well as during degradation. In the absence of the enzyme, bacteria accumulated structurally modified glycogen forms comprising a high number of branches with DP of 4. This finding implies that pullulanase is expressed under maltodextrin-rich conditions, that is, in a period of glycogen accumulation as well as in a period of glycogen breakdown. Isoamylase, a debranching enzyme that is found mainly in plants, is also expressed constitutively and involved in the synthesis of starch (3). A mutant that was deficient in isoamylase accumulated phytoglycogen (24, 32). In many bacteria and archaea, the activities of debranching enzymes are detected throughout the whole cell cycle and are even upregulated by maltose or starch (7, 26, 29, 47).

Based on previous results and those obtained in this study, we propose a model for the roles of MAase and pullulanase in *Bacillus* spp. (Fig. 5). Maltodextrins that are derived from starch by the action of α-amylase are transported into the cell by binding with MdxE and are hydrolyzed to maltose by MAase inside the cell. Maltodextrins with DP of ≥7 may also

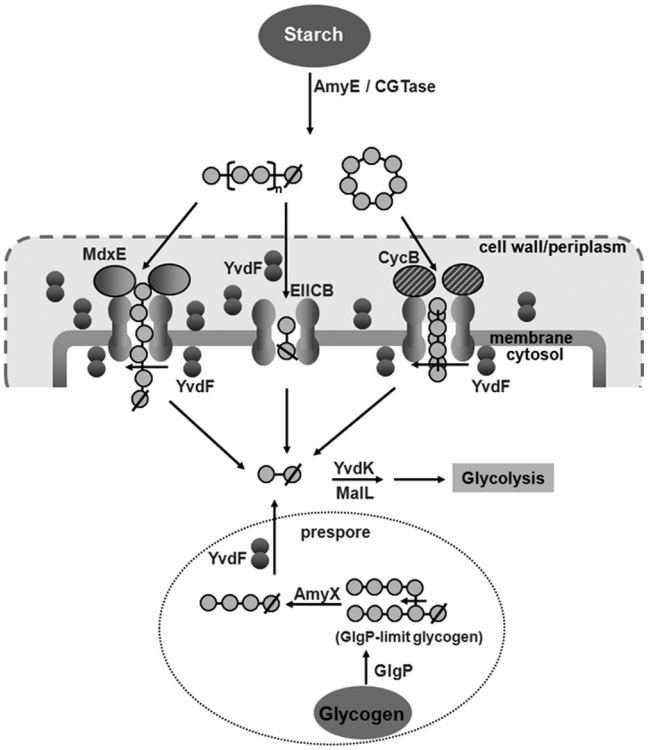

FIG. 5. Proposed model of starch and glycogen use in *Bacillus* spp. AmyE, α-amylase; MalL, α-glucosidase; GlgP, glycogen phosphorylase; YvdK, maltose phosphorylase; CycB, CD-binding protein; MdxE, maltodextrin-binding protein; EIICB, phosphoenolpyruvate-dependent PTS. The events occurring in the cytoplasm during sporulation are indicated in the circle.

be degraded by MAase in the periplasm for efficient transportation. β-CD can be hydrolyzed to maltose by MAase after cellular uptake via the Cyc ABC transporter. The resulting maltose would be hydrolyzed by YvdK, the maltose phosphorylase, to glucose and glucose-1-P, which would then be metabolized via glycolysis. When glycogen accumulates in *B. subtilis*, it may be used by the cell as follows. Glycogen may first be attacked by glycogen phosphorylase, releasing glucose at the nonreducing end in the form of glucose-1-P and leaving glycogen phosphorylase-limited glycogen. Four glucosyl residues remaining in the side chain of glycogen would then be preferably cleaved at the branch point by pullulanase. The resulting G4 would then be hydrolyzed by MAase to maltose as a major reaction product. Thus, the absence of MAase would slow down glycogen degradation, not only due to the malfunction of MAase, but also due to the inhibition of pullulanase by G4 accumulation. YvdK readily catalyzes the phosphorylysis of maltose to yield glucose-1-P and glucose. The accumulation of glucose-1-P via YvdK has not been observed in cell extracts because of the immediate use of glucose-1-P in subsequent metabolism.

The starch and glycogen utilization systems of *B. subtilis* appear to differ from those of *E. coli.* The enzymes for starch and glycogen utilization in *B. subtilis* include α-amylase, MAase, pullulanase, YvdK, and α-glucosidase, whereas the corresponding enzymes in *E. coli* are GlgX (glycogen debranching enzyme), MalQ (α-glucanotransferase), MalP (mal-

todextrin phosphorylase), and MalZ (maltodextrin glucosidase) (see Table S1 in the supplemental material). Notably, *Bacillus* spp. require enzymes that are capable of hydrolyzing large carbohydrates, such as starch, pullulan, and glycogen, that are obtained from the environment to produce maltose as a final hydrolysis product. In contrast, *E. coli* that grows in a maltose-rich environment needs only maltose-processing enzymes to use the major carbon source. Our results allow a better understanding of carbohydrate metabolism in bacteria; further investigation will help to elucidate carbon metabolism in *B. subtilis* at the onset of and during sporulation.

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