Expression of the Promoter for the Maltogenic Amylase Gene in *Bacillus subtilis* 168

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An additional amylase, besides the typical α-amylase, was detected for the first time in the cytoplasm of *B. subtilis* SUH4-2, an isolate from Korean soil. The corresponding gene (*bbmA*) encoded a malto-genic amylase (MAase) and its sequence was almost identical to the *yvdF* gene of *B. subtilis* 168, whose function was unknown. Southern blot analysis using *bbmA* as the probe indicated that this gene was ubiquitous among various *B. subtilis* strains. In an effort to understand the physiological function of the *bbmA* gene in *B. subtilis*, the expression pattern of the gene was monitored by measuring the β-galactosidase activity produced from the *bbmA* promoter fused to the amino terminus of the *lacZ* structural gene, which was then integrated into the *amyE* locus on the *B. subtilis* 168 chromosome. The promoter was induced during the mid-log phase and fully expressed at the early stationary phase in defined media containing β-cyclodextrin (β-CD), maltose, or starch. On the other hand, it was kept repressed in the presence of glucose, fructose, sucrose, or glycerol, suggesting that catabolite repression might be involved in the expression of the gene. Production of the β-CD hydrolyzing activity was impaired by the *spo0A* mutation in *B. subtilis* 168, indicating the involvement of an additional regu-latory system exerting control on the promoter. Inactivation of *yvdF* resulted in a significant decrease of the β-CD hydrolyzing activity, if not all. This result implied the presence of an additional enzyme(s) that is capable of hydrolyzing β-CD in *B. subtilis* 168. Based on the results, MAase encoded by *bbmA* is likely to be involved in maltose and β-CD utilization when other sugars, which are readily usable as an energy source, are not available during the stationary phase.

Key words: Maltogenic amylase, *B. subtilis*, *yvdF*, cyclodextrin utilization, catabolite repression, *spo0A*

Alpha-amylase (EC 3.2.1.1.) is one of the enzymes that have been investigated most thoroughly and used most widely in the starch industry. In particular, α-amylase encoded by the *amyE* gene in *B. subtilis* has been char-acterized in depth for its catalytic properties, enzymatic and genetic structures, secretion mechanism, and regula-tion of the gene expression (Vihinen and Mantsala, 1989). Until recently, the extracellular α-amylase has been con-sidered as the only amylolytic enzyme produced by *B. subtilis*, although Haddaoui *et al.* (1995) reported a cell-bound enzyme that is immunologically related to α-amy-lase in the *B. subtilis* 168 Marburg strain. On the other hand, various amylolytic enzymes such as pullulanase (EC 3.2.1.41; Kuriki *et al.*, 1988), cyclodextrin glucan-otransferase (EC 2.4.1.19; Sakai *et al.*, 1987), cyclodext-rinase (EC 3.2.1.54; Kitahata *et al.*, 1983), and maltogenic amylase (EC 3.2.1.133; MAase; Kim *et al.*, 1992) have been reported in other *Bacillus* species. These enzymes

exhibited novel enzymatic properties that were clearly discernible from those of α-amylase, being capable of hydrolyzing poly- and/or maltooligosaccharides such as pullulan and cyclodextrins (CDs).

MAases are very unique in that they have multi-sub-strate specificity toward starch, pullulan, and CDs, which is modulated by dimerization of the enzymes (Park *et al.*, 2000). Dimeric MAase catalyzes β-CD most efficiently, while the hydrolytic activity toward starch increases as the monomeric form becomes dominant. They not only hydrolyze these carbohydrates but also transfer the hydro-lyzed sugar moiety simultaneously to an acceptor mole-cule by forming α-1,3-, α-1,4-, and α-1,6-glycosidic linkages. Moreover, the enzymes hydrolyze acarbose, a potent α-amylase inhibitor (Park *et al.*, 1998). In their study, the enzyme activity was found cell-bound and it was also evidenced by the predicted amino acid sequence with no evident signal sequence necessary for secretion outside the cell. MAases have been isolated in *B. licheni-formis* (Kim *et al.*, 1992), *B. stearothermophilus* (Cha *et al.*, 1998), and a *Thermus* species (Kim *et al.*, 1999). Recently, an isolate producing MAase was obtained from

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Korean soil and identified as a strain of *B. subtilis* (Cho *et al.*, 2000). The gene responsible for the enzyme activity was cloned in *E. coli* and its sequence was determined. A Blast search of a similar sequence in the *B. subtilis* 168 genome revealed that it had a homologue of a MAase encoded by the *yvdF* gene at 304 degrees on the chromosome. The *yvdF* gene was listed as a gene with unknown function and located in a cluster of genes that were likely to be involved in maltose transport and utilization in *B. subtilis* 168 (SubtiList, 1997).

MAases with unique catalytic properties have been utilized in the production of branched oligosaccharides and various modified sugars (Kim *et al.*, 1994; Lee *et al.*, 1995). However, their physiological roles in bacteria have not been elucidated yet. Since the *yvdF* gene was highly homologous to *bbmA*, the catalytic properties of the enzyme encoded by *yvdF* might resemble those of BbmA. Therefore, genetic analysis of the gene for MAase became possible by using the well-established genetic system of *B. subtilis* 168 to investigate the physiological roles of the enzyme in the cell. In this study, the expression pattern of the *bbmA* gene under various growth conditions was monitored by following the β-galactosidase activity that was produced from the *bbmA* promoter fused to the *lacZ* gene in *B. subtilis* 168 wild-type and a sporulation mutant (*spo0A*). The effects of the *yvdF* mutation on the β-CD hydrolyzing activity and bacterial growth were also investigated in defined media containing various carbohydrates.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

The *bbm*A gene of *Bacillus* SUH4-2 was cloned on pUC119 in *E. coli* (pBMA119) and used for the construction of the *yvdF* mutant and an expression vector in *B. subtilis* 168 (Marburg strain, *trpC2*) and a *spo0A* mutant (JH13528). *E. coli* harboring pBMA119 was cultured in Luria-Bertani (LB; Bacto-tryptone 1%, yeast extract 0.5%, NaCl 0.5%) medium containing ampicillin (100 µg/ml) at 37°C with moderate shaking (250 rpm). *B. subtilis* was cultured in defined media [0.3 mM $(NH_4)_2SO_4$, 3 nM $FeCl_2 \cdot H_2O$, 0.028 nM $MnSO_4$, 16.2 nM $MgSO_4$, 1.25 mM Trizma base (pH 7.0), 4 mM K_2HPO_4, 0.01 mM $ZnCl_2$] containing various carbon sources (1 or 2%) at 37°C with vigorous shaking (300 rpm). LB agar plates containing starch (1%), chloramphenicol (5 µg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 100 µg/ml) were used for selecting and screening mutants or transformants.

Genetic Transformation

Transformation into *E. coli* was carried out using the $CaCl_2$ method (Sambrook *et al.*, 1986). For *B. subtilis*, transformation was carried out using natural competency (Dubnau and Davidoff-Avelson, 1971) or electroporation.

For electroporation, the *Bacillus* cells were harvested by centrifugation when the OD_{540} reached 1.5. The cells were resuspended in 1/500 volume of 10% glycerol and subjected to electric shock (350V, 200Ω; Jetgene A-3010, Bioneer, Korea) after a wash with PEB buffer [272 mM sucrose, 1 mM $MgCl_2$, 7 mM K_2HPO_4 (pH 7.4)] three times. Electroporated cells were recovered by shaking (180 rpm) at 37°C for 30 min in SOC medium (10 mM $MgCl_2$, 10 mM $MgSO_4$, 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM glucose) and selected on LB agar medium containing appropriate antibiotics.

Primer Extension

RNAs of *B. subtilis* or *E. coli* cultured in LB broth containing 2% soluble starch for 12 hr were isolated using Trizol reagent (Life Technologies, USA) according to the manufacturer's instructions. A primer (5'-AGTATCAAA-CTTTCATAGTACTAC-3') complementary to nucleotides 231 to 254 was end labeled with $[\gamma^{-32}P]ATP$ (Pharmacia Biotech, USA) and T4 PNK (Gibco BRL, USA). The end-labeled primer (12 ng) was annealed to RNA (30 µg) in a hybridization buffer [125 mM NaCl, 1.0 mM Tris-HCl (pH 8.0), 0.1 mM EDTA] by incubating at 60°C for 3 min and slowly cooling down to room temperature over an hour. The RNA-primer hybrid was recovered by ethanol precipitation and 50 µl of a reaction mixture [10 mM $MgCl_2$, 5 mM DTT, 770 µM dNTPs, 20 mM Tris (pH 8.0), 30 U RNAsin, 150 U Superscript II] was added before subjected to incubation at 40°C for 70 min. Extended products were separated by electrophoresis on 6% polyacrylamide gel containing 8 M urea along with a sequencing ladder that was generated by annealing the same primers to pBMA119 and by the dideoxy-chain termination method of Sanger *et al.* (1977).

Construction of an expression system in B. subtilis 168

A DNA fragment of 263 bp, 5' upstream to the *bbmA* gene and likely to contain the promoter for the gene, was amplified by PCR using two primers (BBf, 5'-TGATCG-CATCTCCT-3'; BBr, 5'-CCCTTTGATCTCCGTT-3') and pBMA119 as the template. The PCR product was restricted with *Sau*3AI sites (underlined) and ligated to an integration vector, pDH32 (Shimotsu and Henner, 1986), at the *Bam*HI site. The ligation mixture was transformed into *E. coli* MC1061 [*hsd*R, *mer*B, *ara*D139Ä(*ara*ABC-*leu*)7679, Ä*lac*X74, *gal*U, *gal*K, *rps*L, *thi*] and the resulting transformants were screened based on their capability to form blue colonies on LB plates supplemented with chloramphenicol and X-gal. The resulting recombinant DNA, pIM321, was linearized at the *Pst*I site and transformed into *B. subtilis* strains for gene replacement at the *amyE* locus.

Mutagenesis of the yvdF gene

In order to inactivate the *yvdF* gene in *B. subtilis* 168, a

1.7 kb *Hin*dIII fragment that was internal to the *bbmA* structural gene was subcloned in pJM103 (Brosius, 1984) at the corresponding restriction sites. The resulting recombinant DNA was electroporated into *B. subtilis* 168 as described above and the putative transformants were selected on LB agar plates containing chloramphenicol. The transformants were screened for their decreased growth in the presence of β-CD or maltose as putative *yvdF* mutants, and the introduced construction was confirmed by Southern blot analysis (Southern, 1975) using an ECL kit (Amersham, USA) and pJM103 as a probe.

Enzyme assay

The β-CD hydrolyzing activity of *B. subtilis* was determined using cell extracts and following the method described by Nicholson and Chambliss (1985). An aliquot of cell culture (1 ml) taken every hour was centrifuged (10,000 rpm, 1 min) and the cell pellet was washed once with a defined medium without a carbon source. The washed cells were resuspended in 1 ml of Tris-HCl (pH 6.8) and lysed by adding toluene (20 μl). Cell debris was removed by centrifugation and the supernatant (250 μl) was mixed with 1 ml of 0.1% β-CD in 50 mM Tris-HCl (pH 6.8). The mixture was incubated at 37°C for 30 min and the reaction was stopped by adding 0.5 ml of an iodine solution (0.01% I_2 and 0.1% KI in 1 N HCl). The blank was prepared by incubating the mixture of substrate and iodine solution under the same conditions and by subsequently adding the cell extract to the mixture. The optical density of the reaction was measured at 620 nm against the control. Each reaction was carried out in duplicate and one unit of the enzyme activity was defined as a decrease in the OD_{620} of 0.1 and the linear range of the assay was found to extend to a decrease of 0.6 absorbance units. The specific activity was defined as units of enzymatic activity per milligram of cellular protein, as determined by the Bradford protein assay (Bradford, 1976) with bovine serum albumin as a standard. The specific activity of β-galactosidase was determined as described by Ferrari *et al.* (1986) using the cell extract and *o*-nitrophenyl-β-D-galactoside (ONPG) as a substrate.

Results and Discussion

Ubiquity of the MAase gene among B. subtilis spp.

MAases have been detected in *B. licheniformis* and *B. stearothermophilus* (Kim *et al.*, 1992, Cha *et al.*, 1998), but not in *B. subtilis* until recently. Since the blast search of the genome sequence of *B. subtilis* 168 indicated that a homologue of *bbmA* was on the chromosome, the presence of the MAase gene among *B. subtilis* spp. was investigated. Southern blot analysis was carried out to investigate the chromosomal DNA of various *B. subtilis* spp. using the 1.7 kb *Hin*dIII fragment of *bbmA* at high stringency. Chromosomal DNAs of five *B. subtilis* spp.
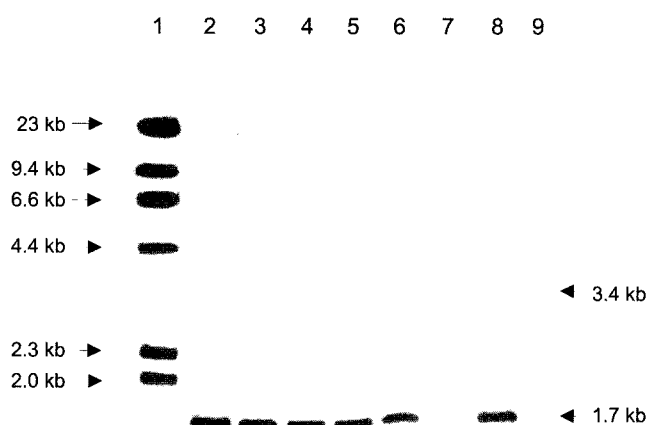

Fig. 1. Southern blot analysis to examine the ubiquity of the maltogenic amylase gene among various *Bacillus* strains. All DNAs were restricted with *Hin*dIII and probed with the 1.7kb *Hin*dIII fragment carrying the *bbmA* gene except λ DNA. Lane 1 was loaded with λ DNA; lane 2, pBMA119; lane 3, genomic DNA of *B. subtilis* SUH4-2; lane 4, *B. subtilis* LKS87; lane 5, *B. subtilis* 168; lane 6, *B. subtilis* DB104; lane 7, *B. licheniformis* MC14; lane 8, *B. subtilis* ATCC 12053; lane 9, *B. stearothermophilus*.

including *B. subtilis* SUH4-2 showed a 1.7 kb *Hin*dIII fragment that hybridized to the probe and that of *B. licheniformis* showed a 3.4 kb *Hin*dIII fragment hybridized to the probe, while *B. stearothermophilus* DNA showed none (Fig. 1). The results correlated well with the sequence comparison data, which showed that *yvdF* of *B. subtilis* 168 was 99% identical to *bbmA* at the nucleotide sequence level, the *B. licheniformis* MAase gene 70%, and the *B. stearothermophilus* MAase gene only 53%. These results suggested that MAase was ubiquitous among *Bacillus* spp., possibly having some undetermined physiological role in the cell. The intracellular enzyme was distinguished from the extracellular α-amylase encoded by *amyE* in many aspects of physicochemical and catalytic properties (Park *et al.*, 2000). A sequence comparison of the α-amylases and MAases showed that their $(\beta/\alpha)_8$ barrel catalytic core structures were highly conserved among these strains, suggesting that they originated from a common ancestor gene (Jespersen *et al.*, 1993). However, the polyclonal antibody raised against α-amylase of *B. licheniformis* did not cross-react with MAase of the same species (Jang *et al.*, 1994), indicating structural differences in these enzymes. The 82 kD cell-bound α-amylase of *B. subtilis* 168 Marburg cross-reacted with the polyclonal antibody raised against the extracellular α-amylase (Haddaoui *et al.*, 1995).

Production of maltogenic amylase in B. subtilis SUH4-2

The patterns of MAase production in *B. subtilis* SUH4-2 were monitored by a time-course assay of the β-CD
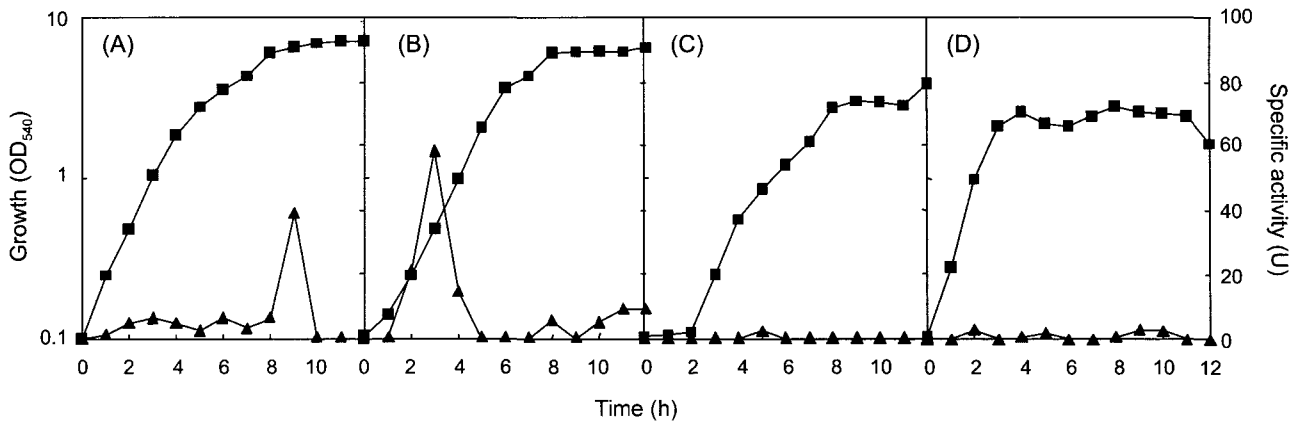
Fig. 2. Time-course assay of β-CD hydrolyzing activity in *B. subtilis* SUH4-2. The strain was cultured in a defined medium containing β-CD (A), starch (B), glucose (C), or fructose (D) as the carbon source. The graphs marked with squares represent growth and triangles, β-CD hydrolyzing activity.

hydrolyzing activity during growth in a defined medium containing various carbon sources. Production of the β-CD hydrolyzing activity was regulated by carbon sources in the growth medium. This was induced by β-CD and starch, while repressed tightly by glucose and fructose (Fig. 2). Maltose also induced the enzyme activity as much as β-CD did but only a low level of the enzyme activity (5-15 units) was observed with sucrose and glycerol (data not shown). These results suggested that MAase production might be under catabolite repression in *B. sub-*

tilis. Temporal regulation exerting control on the production of MAase or the β-CD hydrolyzing activity was also observed depending on the carbon source in the growth medium. In the presence of β-CD, the activity was induced by almost eight fold at the early stationary phase (Fig. 2A). On the other hand, the highest enzyme activity was detected at the early exponential phase and it dropped to the basal level (less than 10 units) during the rest of the growth phase when starch was added to the medium (Fig. 2B). A similar pattern was observed with maltose as the
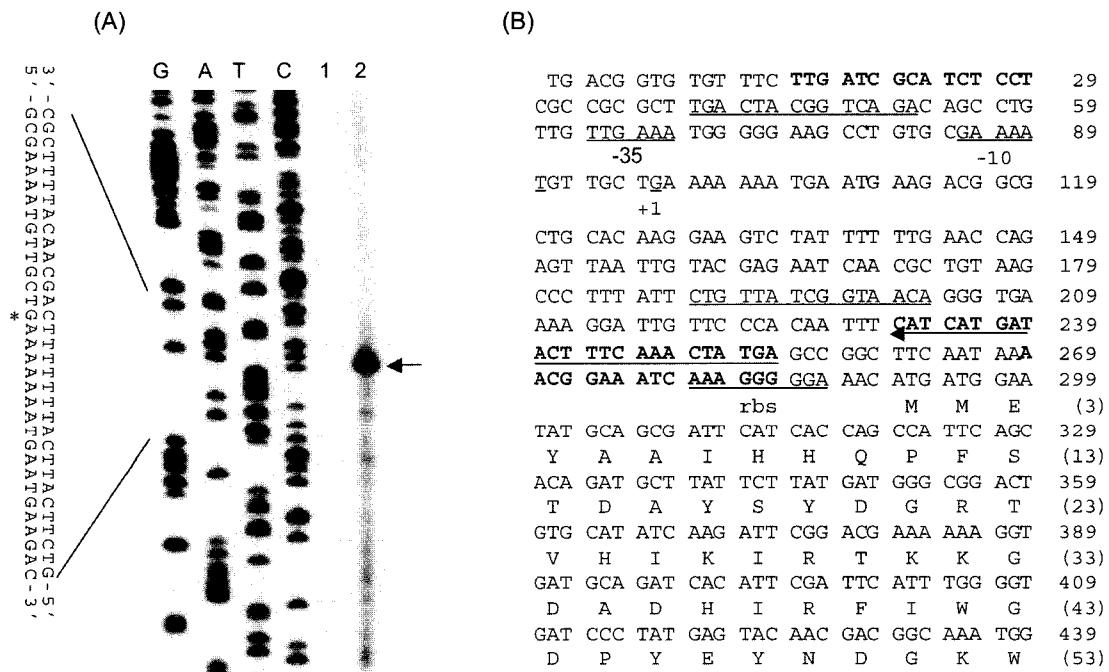
Fig. 3. Mapping of the transcription start site of the *bbmA* gene by primer extension. Panel A. Total RNAs were extracted from *B. subtilis* 168 (lane 1) and *E. coli* MC1061 harboring pBMA119 (lane 2) grown in LB medium containing 2% starch. The primer used for reverse transcription is indicated in panel B. Sequencing ladders were obtained using the same oligonucleotide as a primer and pBMA119 as the template. Panel B. The nucleotide sequence of the *bbmA* promoter region. The transcription initiation site, putative promoter sequences (-10 and -35), and the ribosome binding site (rbs) are marked. The primers for PCR amplification of the promoter region are shown in bold face and the sequence of the primer for primer extension is shown in bold face and marked with an arrow. The putative cre sites are underlined.

carbon source (data not shown). The difference in the temporal control on the production of the β-CD hydrolyzing activity according to the carbon source might suggest an existence of multiple genes responsible for the activity or multiple regulatory systems exerting control on the expression of a gene. Based on the results, the expression pattern of the *bbmA* gene that originated from *B. subtilis* SUH4-2 was analyzed in *B. subtilis* 168 by monitoring the β-galactosidase activity produced from the transcriptional fusion between the *bbmA* promoter (P_{bbmA}) and the *lacZ* gene of *E. coli*.

Effect of carbon sources on the expression of the *bbmA* promoter in *B. subtilis* 168 using the P_{bbmA}-*lacZ* fusion
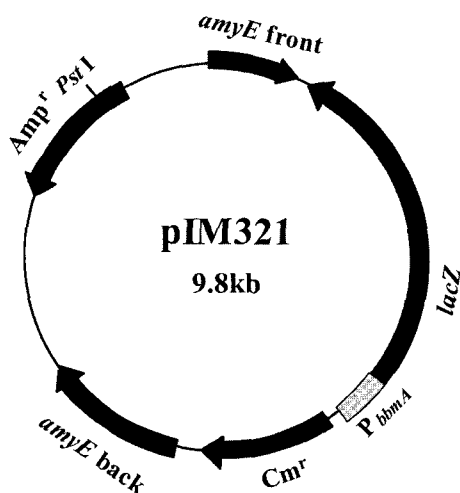
In order to investigate the expression and regulation of the promoter for *bbmA*, the P_{bbmA}-*lacZ* transcriptional fusion was constructed in *B. subtilis* 168, since the strain had a well-established genetic system and its whole genome sequence was available. First, the promoter region for *bbmA* was defined by mapping the transcription initiation site by primer extension. Transcription of the gene was likely to be initiated from the guanine residue at nucleotide 97 in *E. coli* (Fig. 3A, lane 2). Based on it, the putative conserved sequences for -10 and -35 regions, GAAAAT and TTGAAA, respectively, were localized upstream from the site with a spacing of 16 nucleotides (Fig. 3B). Unfortunately, no signal was detected from *B. subtilis* 168, probably due to a low level of the corresponding mRNAs under the growth conditions (Fig 3A, lane 1). A 263 bp DNA fragment containing the promoter region was amplified by PCR and ligated to the integrative

Fig. 4. Structure of the integrational expression vector, pIM32. A transcriptional fusion between the *bbmA* promoter and the promoter-less *lacZ* gene was constructed using the integrational expression vector, pDH32. The construct linearized at the *PstI* site was integrated at the *amyE* locus when transformed into *B. subtilis* 168, thereby making it possible to monitor the expression of the *bbmA* promoter in a single copy by measuring the β-galactosidase activity.

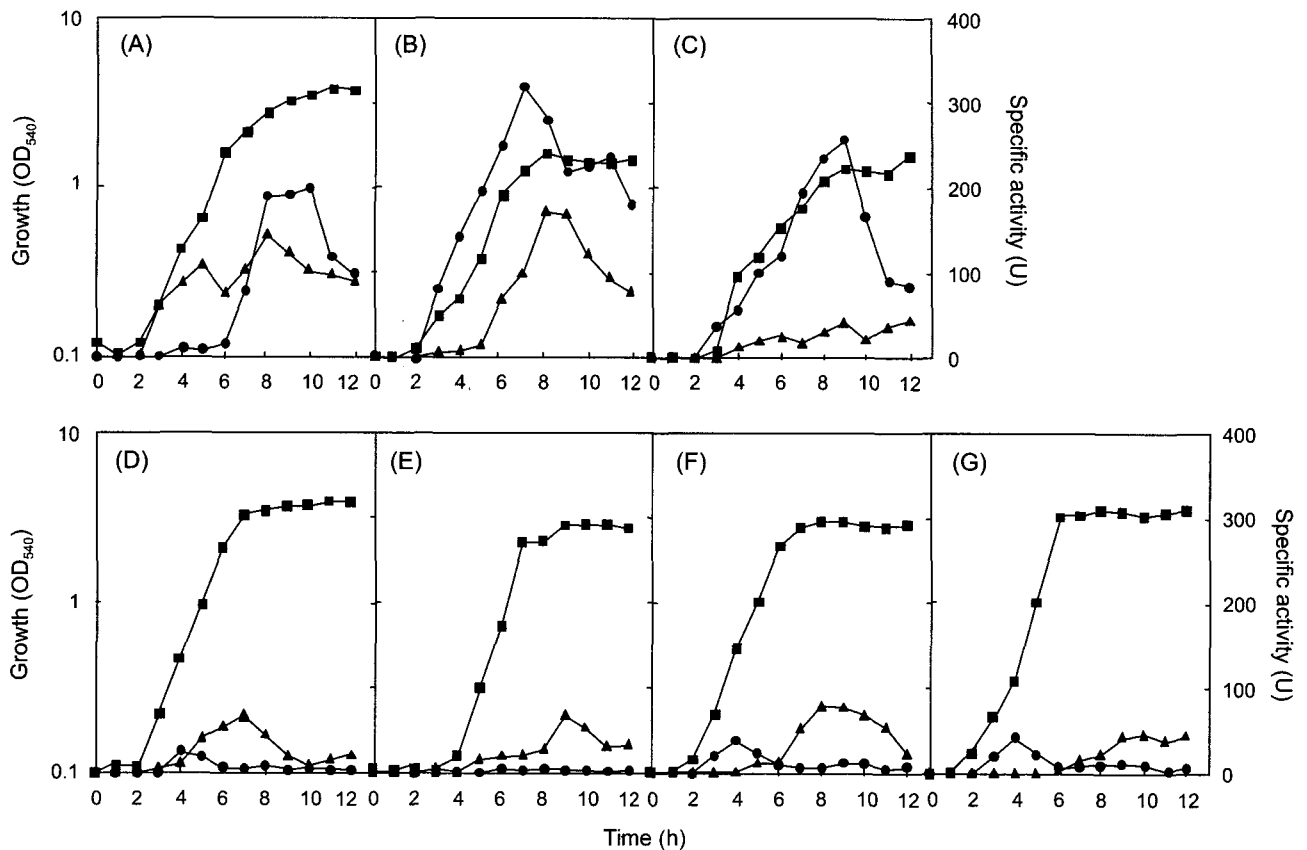expression vector, pDH32, at the *BamHI* site after it was digested with *Sau3AI*.

The resulting recombinant DNA, pIM321, carried the transcriptional fusion between the promoter for *bbmA* and the promoter-less *lacZ* structural gene proceeded by the ribosome-binding site for *spoVG* (Fig. 4). It was then integrated into the chromosome at the *amyE* locus via gene replacement when linearized with *PstI* and transformed into *B. subtilis* 168. The resulting transformants were selected and screened based on their capability to grow and develop blue colonies on a LB agar plate containing chloramphenicol and X-Gal, and based on a loss of the starch hydrolyzing phenotype on a starch agar plate. The DNA structure of the transformant with a blue colony phenotype was confirmed by Southern blot analysis using the 4.5 kb *EcoRI* fragment of pBMA119, which carried the *bbmA* gene as the probe (data not shown).

The expression of the *bbmA* promoter according to the carbon source was monitored by measuring the β-galactosidase activity from the *lacZ* gene fused to the promoter during the growth in a defined medium containing various carbon sources (Fig. 5). No β-galactosidase activity was detected from the transformant that carried the promoter-less pDH32 at the *amyE* locus (data not shown). A time-course assay was also carried out for the β-CD hydrolyzing activity of the transformant, which could be produced by the gene(s) on the chromosome of *B. subtilis* 168 including *yvdF*. The promoter began to be induced at the end of the exponential growth phase when maltose was the carbon source (Fig. 5A), while it began to be induced at the early exponential growth phase when β-CD or starch was the carbon source (Fig. 5B, C). It was β-CD that induced the highest β-galactosidase activity from the *bbmA* promoter, followed by starch and then by maltose. The β-CD hydrolyzing activity, which was detected during the early exponential phase in *B. subtilis* SUH4-2 when starch was the carbon source (Fig. 2B), was not observed in *B. subtilis* 168. Production of the β-CD hydrolyzing activity was mostly in good correlation with that of the β-galactosidase activity. However, in the presence of starch, the β-CD hydrolyzing activity was not induced as much as the β-galactosidase activity was. This could be due to the *amyE* negative background introduced by the fusion construction at the locus. Under these conditions, the cells might not grow enough to fully induce the β-CD hydrolyzing activity. Cell growth was retarded since α-amylase, which is needed to degrade starch was not produced and subsequently no signal was introduced to trigger additional β-CD hydrolyzing activity that was not in accordance with the expression of the *bbmA* promoter. Additional β-CD hydrolyzing activity was detected in the early exponential phase during cell growth in the maltose medium (Fig. 5A).

On the other hand, the promoter was not induced in the presence of glucose, fructose, sucrose, or glycerol (Fig.

Fig. 5. Time-course assay of β-galactosidase expressed from the *bbmA* gene promoter in *B. subtilis* 168. *B. subtilis* 168 carrying the P_{bbmA} *lacZ* fusion was cultured in defined media containing maltose (A), β-CD (B), starch (C), glucose (D), fructose (E), sucrose (F), or glycerol (G). The graphs marked with squares represent growth; circles, β-galactosidase activity; triangles, β-CD hydrolyzing activity.

5D-G). Induction of β-CD hydrolyzing activity decreased significantly as well. This indicated that the promoter and the production of β-CD hydrolyzing activity were also under catabolite repression in *B. subtilis* 168. The gene, *yvdL*, which encodes a maltose inducible oligo-1,4-1,6-glucosidase, was present in the vicinity of *yvdF*, the homologue of *bbmA*, and was subjected to catabolite repression by glucose and fructose (Schönert *et al.*, 1998). CcpA and HPr mediated catabolite repression in *B. subtilis* has been known to be involved in the regulation of various genes including *amyE* (Voskuil and Chambliss, 1995). Catabolite control protein A (CcpA) has been shown to interact specifically with a catabolite responsive element (cre) (Kim *et al.*, 1995). In the upstream sequence of the *bbmA* gene, two sequences with high homology to the cre consensus sequence were observed; 11 out of 14 consensus nucleotides were matched in the sequence 5' to the putative promoter, while 13 were matched in the sequence found 3' to the promoter (Fig. 3B). This suggested that the gene might be under the control of CcpA. The cluster of nine genes including *yvdF* and *yvdL* has high homology to maltose utilization systems. However, repression by glucose in *B. subtilis* 168 was not as tight as in *B. subtilis* SUH4-2, suggesting the presence of an addi-

tional repression mechanism in the latter strain. On the other hand, the β-CD hydrolyzing activity was not repressed as much as the promoter was (Fig. 5D-G), indicating the presence of other gene(s) producing such activity in *B. subtilis* 168. So far, no gene for an enzyme that could hydrolyze β-CD has been reported except *yvdF*. Recently, Kamionka and Dahl (2001) reported the presence of a cyclodextrin-binding protein encoded by *yvfK*, supporting the role of *yvdF* to enable *B. subtilis* 168 to utilize CDs as a carbon source.

Cyclodextrin hydrolyzing activity in the *yvdF* mutant

Since the results of the expression studies suggested that other β-CD hydrolyzing activities might be produced independently of MAase in *B. subtilis* 168, the effect of the *yvdF* mutation on the production of the β-CD hydrolyzing activity was investigated by inactivating the gene. The 1.7 kb *Hind*III fragment internal to the *bbmA* structural gene was subcloned onto pJM103, a suicidal vector with genetic markers of ampicillin and chloramphenicol resistance in *E. coli* and *B. subtilis*, respectively. The resulting recombinant DNA, pBJM103 (Fig. 6), was transformed into *B. subtilis* 168 and integrated into the chromosomal DNA by Campbell-type recombination. Interruption of the *yvdF*
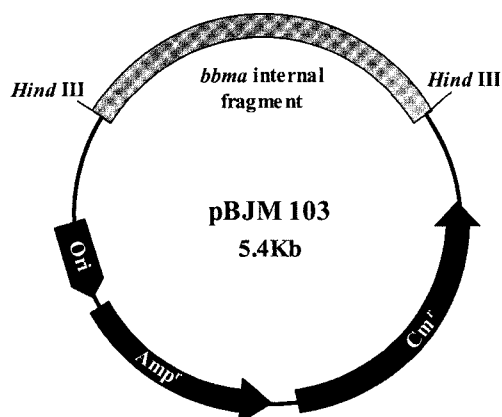
Fig. 6. Structure of the suicidal vector, pBJM103. The 1.7 kb *Hind*III fragment internal to the *bbmA* structural gene was inserted into pJM103, which carried an origin for *E. coli* and antibiotic markers for *E. coli* (Amp^r) and *B. subtilis* (Cm^r). The suicidal vector was integrated into the chromosomal DNA at the homologous *yvdF* locus by Campbell-type recombination when transformed into *B. subtilis* 168. The portion of pJM103 is shown in black lines and arrows.

gene on the chromosome was confirmed by Southern blot analysis using pJM103 as the probe (data not shown).

The time-course assay was carried out to monitor the profile of the β-CD hydrolyzing activity in the *yvdF* mutant with various carbon sources in the culture medium (Fig. 7). The growth pattern of the mutant was almost similar to that of the wild-type except that the former reached the stationary phase earlier than the latter. Inactivation of the *yvdF* gene did not cause significantly delayed or decreased growth of the mutant in the medium containing β-CD as the only carbon source. The β-CD hydrolyzing activity in the mutant decreased significantly if not completely when maltose or β-CD was the only carbon source (Fig. 7A, B), while almost no activity was detected in the medium containing glucose or fructose (Fig. 7C, D). This indicated that the *yvdF* gene was

responsible for most β-CD hydrolyzing activity during cell growth using various carbon sources. However, an additional gene(s) producing the enzyme activity was likely to be present in *B. subtilis* 168 based on the growth of the *yvdF* mutant in the medium containing β-CD and on the residual activity in the culture containing either maltose or β-CD.

Effect of the spo0A mutation on the expression of the bbmA promoter

Spo0A is a member of two component regulatory systems and it plays a role as a master regulator in determining the life cycle of *B. subtilis* 168 (Ferrari *et al.*, 1986). The expression of many degradative enzymes that are produced at the end of the exponential phase has been known to be under the control of *spo0A*. In order to examine if the *bbmA* promoter is under the regulation of *spo0A*, the P$_{bbmA}$-*lacZ* fusion construction was introduced into a *Bacillus spo0A* strain (JH13528) at the *amyE* locus. The construction on the chromosomal DNA of *B. subtilis* 168 was confirmed by Southern blot analysis (data not shown) and the β-galactosidase and β-CD hydrolyzing activities were monitored during cell growth in the culture media containing various carbon sources (Fig. 8). Both enzyme activities in the *spo0A* mutant were detected at significantly lower levels than those in the wild-type strain under all growth conditions tested (Fig. 8). Therefore, the *spo0A* gene was likely to regulate the *bbmA* promoter in a positive manner. This supported the assumption of the presence of multiple regulatory systems exerting control on the expression of the gene. *B. subtilis* SUH4-2 was selected as a MAase producer since it produced more of the enzyme than other *B. subtilis* strains. This may indicate that regulatory systems for the enzyme have been diverged among the bacterial spp. as evidenced by the data obtained in this study. *B. subtilis* SUH4-2 exhibited phenotypes that are distinguishable from other *B. subtilis*
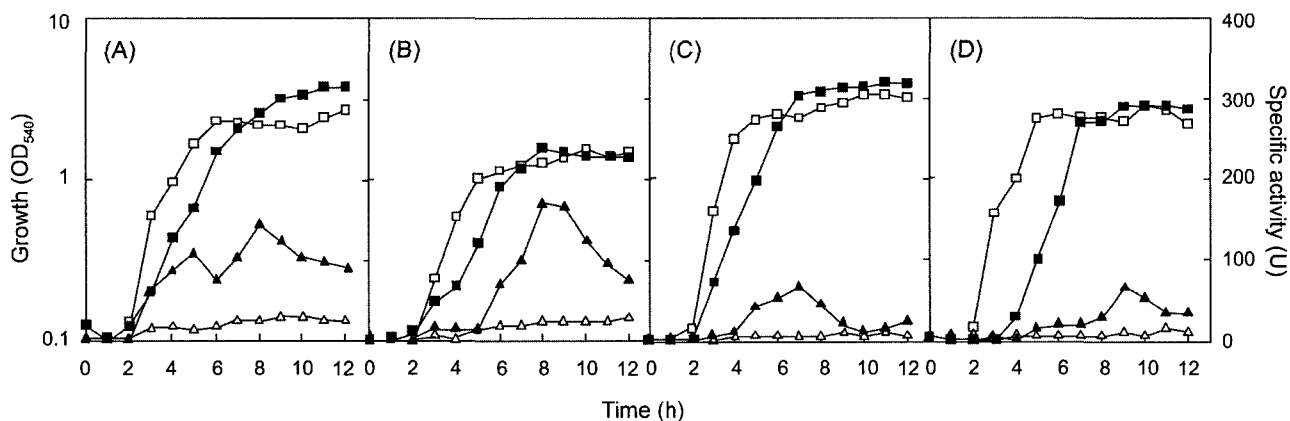
Fig. 7. Time-course assay of β-CD hydrolyzing activity in the *yvdF* mutant strain. The *yvdF* mutant and wild-type *B. subtilis* 168 strains were cultured in defined media containing maltose (A), β-CD (B), glucose (C), or fructose (D). The graphs marked with squares represent growth; triangles, β-CD hydrolyzing activity. The graphs with open symbols are for the *yvdF* strain and closed symbols for the wild-type.
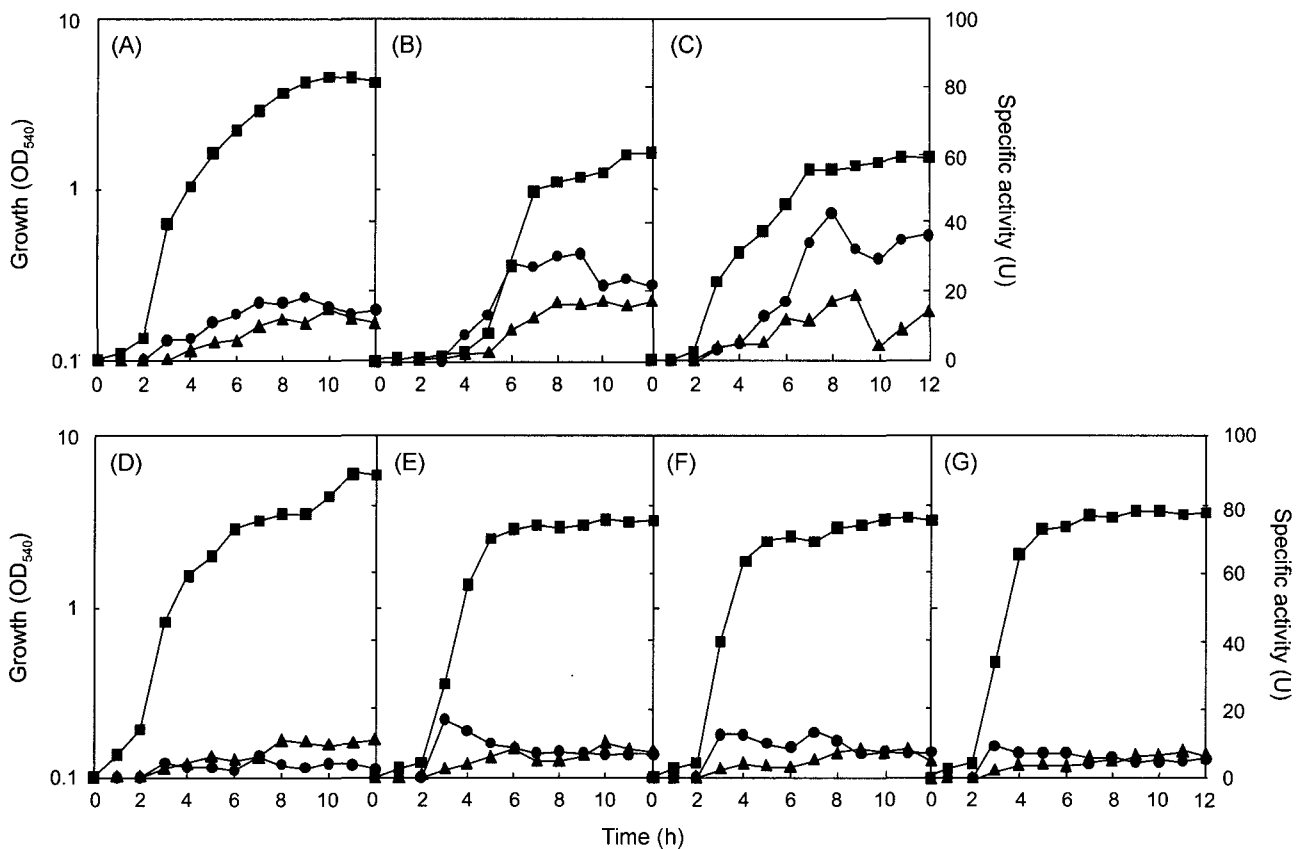
Fig. 8. Time-course assay of β-galactosidase expressed from the *bbmA* gene promoter in the *spo0A* mutant strain. *B. subtilis* 168 carrying the P_{bbmA} *lacZ* fusion in the genetic background of *spo0A* was cultured in a defined medium containing maltose (A), β-CD (B), starch (C), glucose (D), fructose (E), sucrose (F), or glycerol (G). The graphs marked with squares represent growth; circles, β-galactosidase activity; triangles, β-CD hydrolyzing activity.

strains; the cells could grow at 45°C very well, they could aggregate themselves predominantly in a liquid culture, and they could form biofilm more efficiently (unpublished data).

Based on the results obtained in this study, one possible role of MAase in the cytoplasm of *Bacillus* could be hydrolysis of linear maltodextrins, which were produced by extracellular amylolytic enzymes including α-amylase, pullulanase, and/or CGTase, and taken up via a binding protein-dependent ABC transporter(s), to mainly maltose. Since the *yvdF* gene was highly homologous to *bbmA*, the catalytic properties of the enzyme encoded by *yvdF* might resemble those of BBMA. Maltodextrins shorter than maltohexaose, including maltose, could also be hydrolyzed to glucose by the action of oligo-1,4-1,6-glucosidase encoded by *yvdL* in the gene cluster of *yvdE* to *yvdM*, which is likely to be involved in maltose transport and utilization (Schönert *et al.*, 1998). Lee *et al.* (2001) reported that *yvdF* was one of the 230 genes that was poorly or not expressed during the exponential phase but expressed threefold or more in the stationary phase, based on the RNA expression analysis using an antisense *B. subtilis* genome array. A cyclodextrin-binding protein that is encoded by *yvfK* and constitutes a putative ABC-trans-

porter in *B. subtilis* has been documented (Kamionka and Dahl, 2001). At least one of the genes in the cluster to which *yvfK* belonged was specifically induced during sporulation, suggesting a possible link between CD utilization and sporulation. The *yvdE* gene, with an unknown function, is located just upstream of *yvdF* and has some homology to transcriptional regulators. In an effort to elucidate the physiological role of *yvdF* and other genes in the cluster, the expression and function of the *yvdE* gene as the positive regulator for *yvdF* and other gene(s) for the growth of the *yvdF* mutant in the medium containing β-CD hydrolyzing activity have been investigated in the laboratory and the results will be published elsewhere.

Acknowledgement

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