J. Microbiol. Biotechnol. (1998), **8**(1), 21-27

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY
© Korean Society for Applied Microbiology

Catabolite Repression of the *Bacillus stearothermophilus* β-Xylosidase Gene (*xylA*) in *Bacillus subtilis*

CHO, SSANG-GOO AND YONG-JIN CHOI*

Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea

Received: August 7, 1997

Abstract The *xylA* gene of *Bacillus stearothermophilus* encoding the major β-xylosidase was previously cloned and sequenced. In the present study we examined the regulation of the cloned *xylA* gene expression in *Bauillus subtilis* MW15 carrying the *xylA::aprA* fusion plasmids. The induction of the fused *xylA* gene expression remained uninfluenced by any of the carbon sources tested but the gene expression was repressed about 2~3 fold in the presence of glucose. Two CRE-like sequences (CRE-1: nucleotides +124 to +136 and CRE-2: +247 to +259) were recognized within the reading frame region of the *xylA* gene. The deletion experiments showed that the CRE-2 sequence had a role in catabolite repression (CR) as a true CRE of the *xylA* gene, but the CRE-1 had no effect on CR of the *xylA* gene expression. Surprisingly, the deletion of the CRE-1 sequence reduced about 2~3 fold of the expression of the *xylA* fused gene. The repression ratios of the *xylA* gene expression were estimated to be about 0.4 from the assay of subtilisin activity, and about 0.3 at the level of transcription by determining the amounts of *xylA* transcripts in *B. subtilis*. While, the level of CR of the *xylA* gene was assessed to be about 10-fold in previous work when the relative amounts of the *xylA* transcripts were measured in *B. stearothermophilus*.

Key words: Catabolite repression, β-xylosidase, *xylA*, *Bacillus stearothermophilus*, *Bacillus subtilis*

β-Xylosidase is one of the enzymes needed for microbial degradation of xylan, which are β-1,4-linked D-xylopyranoside polymers. The enzyme has been characterized from many organisms including gram-negative and gram-positive bacteria, and fungi.

In our laboratory, three distinct β-xylosidase genes have been cloned [15, 23] from *Bacillus stearothermophilus*, a gram-positive bacterium isolated from soil [21]. The β-xylosidase gene (*xylA*) encoding the major intracellular β-xylosidase of *B. stearothermophilus* has been cloned and sequenced [14, 15]. The β-xylosidase (XylA) produced from the cloned *xylA* gene has also been purified and characterized [13]. In previous study, we also found that the expression of β-xylosidase in *B. stearothermophilus* was fully induced by xylan and repressed about 40-fold by glucose (S. G. Cho and Y. J. Choi. 1998. Regulation of β-xylosidase (XylA) synthesis in *Bacillus stearothermophilus*. *J Microbiol. Biotechnol* **8**: 14-20), although little has been known about the mechanisms controlling β-xylosidase gene expression. The level of glucose repression of the *xylA* gene was, however, assessed as about 10-fold when the relative amounts of *xylA* transcripts were assayed with the same strain.

This glucose-mediated regulation known as catabolite repression (CR) is explained to be a mechanism by which the cell coordinates metabolism of carbon and energy sources to maximize efficiency and regulates other metabolic processes as well. CR in *Escherichia coli* is performed by interfering with a positive regulatory mechanism in which the catabolite repressor protein (CRP or CAP) in complex with the cyclic AMP (cAMP) binds to a specific site in the promoter region of CR-sensitive genes or operons [16, 17]. In contrast, catabolite repression in *B. subtilis* and other gram-positive bacteria examined so far is accomplished by a negative regulatory mechanism in which a repressor protein CcpA (catabolite control protein) in complex with the heat-stable protein HPr (a component of PTS system) binds to catabolite responsive element (CRE) [8, 19]. CRE was found to have a consensus 14-bp palindromic sequence, TGT/AAANC ↓ GNTNA/TCA (where underlined letters represent the most critical bases,

*Corresponding author
Phone: 82-2-3290-3417; Fax: 82-2-923-9923;
E-mail: choiyj@kuccnx.korea.ac.kr

N is any base, and the vertical arrow denotes an axis of symmetry) and showed a striking variability of their positions with respect to the transcriptional start site of the regulated genes [8, 25]. From the sequence data of the *xylA* gene of *B. stearothermophilus* [14], we found two CRE-like sequences (CRE-1: nucleotides +124 to +136 and CRE-2: +247 to +259) within the reading frame of the *xylA* gene.

In this study, we did experiments with *B. subtilis* MW15 strains carrying the *xylA::aprA* fusion plasmids to understand the repression mechanism controlling the β-xylosidase gene expression.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

B. stearothermophilus, which produces the β-xylosidase A and other xylanolytic enzymes, was isolated from soil in our laboratory [21]. As host strains for various plasmids used in this work, *E. coli* JM109 (*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ (*lac-proAB*)/F'[*traD36 proAB*$^+$ *lacI*^{q}*Z* Δ M15]) [28] and *B. subtilis* strain MW15 (*his nprR2 nprE18* Δ *aprA3* Δ *eglS102* Δ *bglT bglSRV* Δ *xynA* Cm^R), which is a mutant deficient in extracellular alkaline and neutral proteases, carboxymethylcellulase, β-1,3 [4]-glucanase, and xylanase [26], were used.

A promoter-probe plasmid, pWP18, which was derived from plasmid pSB [24], is a pUB101 derivative containing a promoterless subtilisin gene (*aprA*) preceded by the pUC18 polylinker. To construct deletion derivatives, plasmid pBluescript and pMG1 carrying the entire *xylA* gene [14], were used.

Culture and Growth Conditions

B. subtilis strain MW15 bearing plasmid was grown in modified Schaeffer's medium (2×SG) [9] containing each of the various carbon sources examined and 10 μg/ml kanamycin at 37°C. *E. coli* containing plasmid was propagated at 37°C in Luria broth supplemented with 50 μg/ml ampicillin.

General Methods

Standard methods of molecular biology [20] were used unless otherwise specified. Enzymes were purchased from New England Biolabs, Promega, United States of Biochemicals, or Boehringer Mannheim and chemicals were from Sigma Co.

DNA was transferred to the competent cells of *B. subtilis* MW15 prepared according to the procedure of Spizizen [22]. In 10 ml of SPI medium (0.2% $(NH_4)_2SO_4$, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.1% Na Citrate·$2H_2O$, 0.02% $MgSO_4$·$7H_2O$, 0.5% glucose, 0.02% casamino acids, and 0.1% yeast extrast), 0.1 ml of the overnight culture was inoculated and grown at 37°C until the cell growth reached the early stationary phase. One milliliter of the early stationary phase culture was mixed with 9 ml of SPII medium (SPI medium plus 0.5 mM $CaCl_2$ and 2.5 mM $MgCl_2$). After incubation for 90 min at 37°C, 0.1 ml of 100 mM EGTA was added and the culture was incubated for an additional 10 min at 37°C. DNA sample (0.1 μg) and 0.2 ml of the competent cells were mixed and incubated for 90 min at 37°C. The transformed cells were plated on Schaeffer's sporulation agar plates [9] containing 10 μg/ml of kanamycin and 1% skim milk.

Transformation into *E. coli* JM109 was performed as described previously [2].

Construction of Deletion Derivatives

Plasmids pMGWPB1, pMGWPS1, pMGWPP1, and pMGWPP2 were constructed as follows: plasmid pMG1 was double digested with *Hin*dIII and one of three enzymes, *Bam*HI, *Sma*I, and *Pst*I to obtain *Hin*dIII-*Bam*HI, *Hin*dIII-*Sma*I, and *Hin*dIII-*Pst*I respective fragments containing different parts of the *xylA* gene (Fig. 1 and Fig. 2). These three fragments obtained were ligated with the *Hin*dIII-*Bam*HI, *Hin*dIII-*Sma*I, or

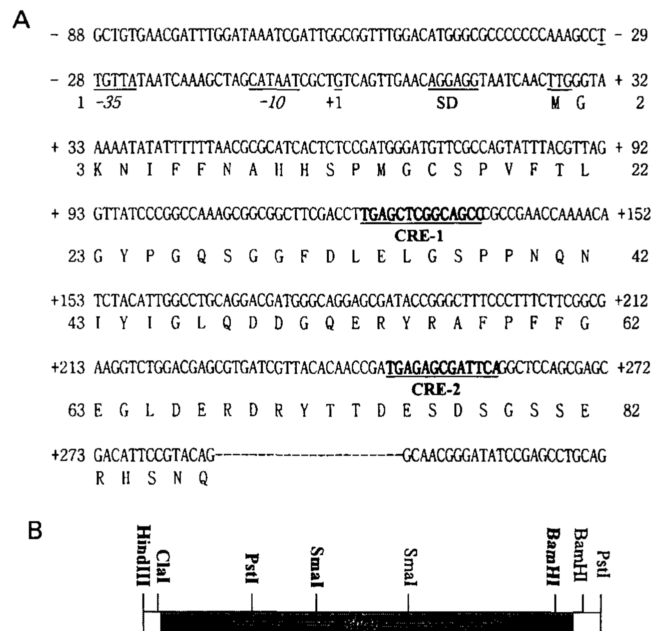

Fig. 1. CRE-like sequences in the *xylA* gene of *B. stearothermophilus* and the restriction map of the insert DNA of pMG1 containing the *xylA* gene (■).

(A) The CRE-like sequences (CRE-1 and CRE-2) in the reading frame region of *xylA* gene. Promoter regions (-35 and -10 region), Shine-Dalgarno (SD) region, the transcription initiation nucleotide, which was mapped by S1 nuclease analysis and is assigned as +1, and the translation initiation codon are underlined. (B) The restriction map of the 2.8-kb insert DNA of pMG1. The restriction sites used in this study for constructing the deletion derivatives of *xylA* gene are indicated in bold letters.

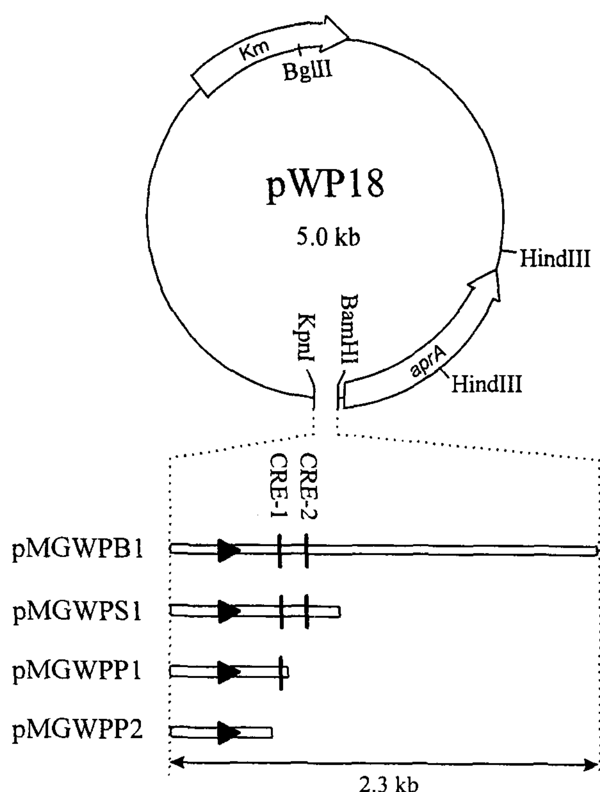

Fig. 2. Structures of plasmid pMGWPB1 and its deletion derivatives.

Plasmid pWP18 is a plasmid pUB110 derivative containing a promoterless subtilisin gene (*aprA*). Km denotes the gene encoded in plasmid pUB110 for kanamycin nucleotidyltransferase. Construction of plasmid pMGWPB1 and its deletion derivatives were described in the text.

*Hin*dIII-*Pst*I digests of the plasmid pBluescript to obtain pMGBB1, pMGBS1, or pMGBP1, respectively. To construct pMGBP2 which contained no CRE-like sequence (Fig. 1 and Fig. 2), the plasmid pMGBP1 was double digested with *Sac*I and *Bam*HI, deleted with exonulease III, treated with Mung bean nuclease, and self-ligated sequentially. After sequencing by the dideoxy chain termination method, we selected the deletion derivative having optimum size, and designated as pMGBP2.

To construct pMGWP series (Fig. 2), the plasmids, pMGBB1, pMGBS1, pMGBP1, and pMGBP2 were digested with *Kpn*I and *Bam*HI, and the respective *Kpn*I-*Bam*HI fragments containing different parts of the *xylA* gene were ligated with the *Kpn*I-*Bam*HI arm of plasmid pWP18.

Measurements of β-Xylosidase and Subtilisin Activity

β-Xylosidase activity was assayed as described previously [13].

For subtilisin assay, *B. subtilis* strain MW15 bearing the recombinant plasmid was grown at 37°C in modified Schaeffer's medium (2×SG) [9] containing 10 μg/ml of kanamycin with or without carbon source as described in the text. A 0.5 ml of the culture was harvested when the OD_{600} reached 0.6 and then centrifuged for 5 min at 16,000×*g* at 4°C. Subtilisin was assayed according to the method of Millet [11] with modifications [5]. A 0.3 ml of the culture fluid was mixed with 0.7 ml of 0.1 M Tris-Cl, pH 8, containing 5 mg of Hide Powder Azure (Sigma) which had been homogenized with a tissue grinder, and followed by incubation for 15 min at 37°C. The mixture was centrifuged for 10 min at 16,000×*g* at 4°C, and then the A_{595} of the supernatant fluid was measured. One unit of subtilisin activity was defined as the amount of enzyme that produced soluble dye giving an A_{595} of 1 in 1 ml.

RNA Preparation

Total cellular RNA was isolated according to the rapid procedure described by Barry *et al.* [1] from *B. subtilis* MW15 bearing the recombinant plasmids, which had been grown to OD_{600}=0.6 in various media. To remove any contaminated DNA , the isolated RNA was treated with RNase-free DNase I, extracted with phenol-chloroform, precipitated with ethanol, and dissolved in DEPC-treated water.

Slot-blot Hybridization and Detection

Quantification of *xylA* mRNA was performed by slot blot hybridization according to the following protocol. Various amounts (1, 5, and 20 μg) of total RNA from each culture were dissolved in 500 μl of ice-cold 10 mM NaOH and 1 mM EDTA solution, and filtered onto a Hybond-N^+ nylon filter (Amersham) using the Bio-Dot SF microfiltration apparatus (Bio-Rad) according to the supplier's recommendation.

Hybridization of the air-dried membrane was performed using a DNA labeling and detection kit (Boehringer Mannheim). The DNA probe, which is a *Cla*I-*Pst*I fragment (nucleotides -66 to +168; Fig. 1A) prepared by digesting the insert DNA of pMGBP1 with *Cla*I (Fig. 1B), was labeled with digoxigenin dUTP, and used for hybridization reaction as recommended by the manufacturer. The intensities of the hybridization signals were compared using the Scanner CS-9000 (Shimadzu, Japan).

RESULTS

Search for the CRE-like Sequences in the *xylA* Gene and Construction of the *xylA::aprA* Fusion Plasmids

CREs, the binding sites of CcpA, show a striking variability in their position with respect to the transcriptional start site of regulated genes [8]. In the

previous study (S. G. Cho and Y. J. Choi. 1998. Regulation of β-xylosidase (XylA) synthesis in *Bacillus stearothermophilus*. *J. Microbiol. Biotechnol.* **8**: 14–20), we found two potential CRE's (CRE-1: nucleotides +124 to +136 and CRE-2: +247 to +259) within the reading frame region of the *xylA* gene (Fig. 1A).

For the molecular study of catabolite repression (CR) of the *xylA* gene, we constructed the *xylA::aprA* transcriptional fusion plasmids, pMGWPB1, pMGWPS1, pMGWPP1, and pMGWPP2 as described in Materials and Methods (Fig. 1B and Fig. 2). As shown in Fig. 2, pMGWPB1 and pMGWPS1 contained both of the CRE-like sequences (CRE-1 and CRE-2). The pMGWPP1 had only CRE-1, and pMGWPP2 carried none of them. We could not developed a satisfactory transformation protocol to introduce plasmids into *B. stearothermophilus*, so we transferred the fusion plasmids to *B. subtilis* MW15, a mutant strain deficient in extracellular alkaline protease (subtilisin: AprA).

CRE sequences are reported to be conserved in the genes from various gram-positive bacteria including *B. subtilis* [8], and we assumed that expression of the *xylA* gene could be also regulated in *B. subtilis* in the same way as in *B. stearothermophilus*.

Analysis of CR in *B. subtilis* MW15 Bearing Deletion Derivatives of pMGWPB1

The *B. subtilis* strains carrying the *xylA::aprA* transcriptional fusion plasmids were grown in 2×SG media containing 10 μg/ml kanamycin and one of the carbon sources indicated in Table 1. The activity of the reporter protein, subtilisin, was determined as described in Materials and Methods, and the results obtained are presented in Table 1. In the pMGWP series, pMGWPB1, pMGWPS1, pMGWPP1, and pMGWPP2, the deleted *xylA* gene fragments which contain complete *xylA* gene promoter were inserted in the upstream region of promoterless subtilisin gene (*aprA*), so each subtilisin activity indicates the transcriptional level of respective *xylA* gene promoter.

Nearly the same subtilisin activities (from 92.4 to 115.1%) were measured from the cells carrying pMGWPB1 grown in the media supplemented with xylan, galactose, xylose, xylitol or none of the carbon sources. But about half (from 52.3 to 61.4%) of the full activity (100%: the relative activity from the cells grown with no additional carbon source) was observed when glycerol or maltose was used as the sole carbon source, and about one third of the activity (from 36.4 to 44.1%) was detected when the cells were grown in the media supplemented with glucose or xylan plus glucose.

This results indicate that expression of the *xylA* gene was also repressed in the *B. subtilis* strains although the level of CR in these strains (about threefold) was somewhat lower than that displayed in *B. stearothermophilus*. The level of glucose repression of the β-xylosidase synthesis had been assessed to be about 40-fold when the relative β-xylosidase activities had been assayed in *B. stearothermophilus*, but the value was about 10-fold when the relative amounts of *xylA* transcripts had been analyzed in the same organism (S. G. Cho and Y. J. Choi. 1998 Regulation of β-xylosidase (XylA) synthesis in *Bacillus stearothermophilus*. *J. Microbiol. Biotechnol.* **8**: 14–20).

The strain MW15 carrying the plasmid pMGWPS1 which contained both the CRE-1 and CRE-2, showed 91.4% subtilisin activity relative to the activity given by the MW15/pMGWPB1 grown in the presence of no additional carbon source. Like the MW15/pMGWPB1, almost the same activity was detected from the MW15/pMGWPS1 cells grown in the medium cantaining arabinose, galactose, xylose, xylitol, or xylan and these strains produced about half activity (from 37.2 to 61.1%) in the medium containing the other carbon sources besides glucose.

The highest subtilisin activity (159.2%) was detected from the MW15 carrying pMGWPP1 in the medium supplemented with no additional carbon source, and almost the same high activity (from 117.2 to 149.6) was appeared from the cells grown with or without either of the carbon sources used in this study. On the other hand, nearly the same activities (from 50.1 to 65.4%) were

Table 1. Catabolite repression of subtilisin synthesis by various sugars in *B. subtilis* strain MW15 bearing pMGWPB1 or its deletion derivative.

Plasmid in strain MW15	Subtilisin activity level (%)													
	None	Glc	Fru	Gal	Xyl	Ara	Rib	Xyt	Gly	Cel	Mal	Suc	Xyn	X+G
pWP18	3.9	4.9	1.8	2.9	2.8	3.8	1.9	2.7	1.9	1.9	2.9	2.9	3.9	3.8
pMGWPB1	100.0	40.1	36.4	99.4	97.3	92.4	44.1	115.1	52.3	38.4	61.4	41.2	103.2	41.1
pMGWPS1	91.4	47.3	39.2	89.4	94.3	88.1	41.2	90.4	48.1	37.2	61.1	46.1	94.3	47.5
pMGWPP1	159.2	137.4	128.4	141.1	141.2	139.2	117.2	142.4	137.5	123.5	146.2	95.4	149.6	139.8
pMGWPP2	65.4	57.2	50.4	65.3	61.1	61.3	50.1	61.6	54.6	56.1	59.6	58.4	65.1	57.9

[a]Subtilisin activities obtained with various sugars are given as percentages of the value from the strain pMGWPB1/MW15 grown in 2×SG medium without exogenous sugar. Each medium contained 1% (w/v) of glucose, fructose, galactose, xylose, arabinose, ribose, xylitol, glycerol, cellobiose, maltose, sucrose, xylan, or 0.5% xylan+0.5% glucose. The values shown are the averages of three independent experiments.

detected from all the cultures regardless of the carbon source used when the MW15 carrying pMGWPP2 carrying none of the CRE-like sequences was used for subtilisin assay. This implies that no specific regulation for the *xylA* gene expression was operated in this strain. However, the activity of MW15/pMGWPP2 was less than half that of MW15/pMGWPP1.

Taken together, these results suggest that the expression of the cloned *xylA* gene in *B. subtilis* did not require any special carbon source for its induction, and was repressed about 2~3 fold in the presence of the carbon source, such as sucrose, glycerol, ribose, fructose, maltose, cellobiose, or glucose. We also noted that CR of the *xylA* gene in the MW15/pMGWPB1 and MW15/pMGWPS1 strains could be removed by deleting the CRE-2 as shown by the experiment with the plasmid pMGWPP1. In addition, deletion of the CRE-1 made MW15/pMGWPP2 produce about half of the subtilisin activity given by MW15/pMGWPP1. The CRE-1 sequence was, therefore, thought to participate in the expression of the *xylA* gene in some other unknown way.

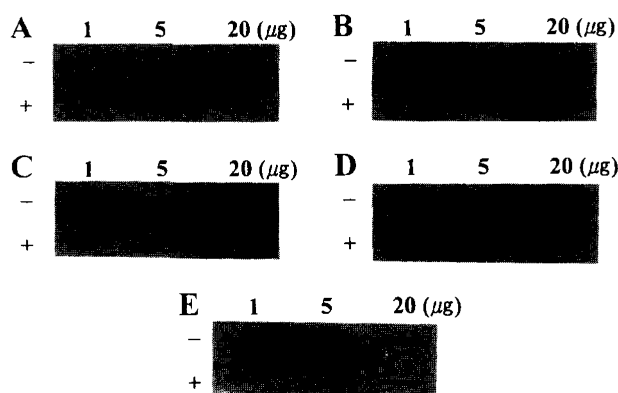

Fig. 3. Slot blotting analysis of *xylA* mRNA synthesized in *B. subtilis* MW15 containing the following plasmid: A, pWP18; B, pMGWPB1; C, pMGWPS1; D, pMGWPP1; E, pMGWPP2. Total RNAs prepared from cells grown with (+) or without (-) glucose were blotted onto nylon membranes and then hybridized as described in the text. The amounts of blotted RNAs were 1, 5, 20 μg per slot, as indicated. Each slot blotted with 5 μg of total RNA was used to obtain the relative amounts of *xylA* mRNA in Table 2.

Determination of the Level of CR

To analyze the extent of CR, we measured subtilisin activities from the fusion plasmid-containing MW15 strains grown with or without additional glucose. As shown in Table 2, repression ratios were estimated to be 0.4 and 0.51 for the strains carrying pMGWPB1 and pMGWPS1, respectively, both of which contained the CRE-1 and CRE-2 sequences. In cases of the pMGWPP1 devoid of the CRE-2, and pMGWPP2 devoid of both the CRE-1 and CRE-2, the ratios were 0.86 and 0.88, respectively. This means these two strains had no CR control.

Next, to determine the repression ratio at the level of transcription, we determined the amounts of the *xylA* transcripts by slot blotting analysis performed as described in Materials and Methods (Fig. 3). From the density patterns on slot blotting and the repression ratios estimated by densitometric measurements of the blottings from multiple independent experiments (Table 2), glucose addition was found not to reduce the amounts of the *xylA* transcripts from the cells carrying pMGWPP1 or pMGWPP2. However, analysis with the plasmids possessing the CRE-2 sequence such as pMGWPB1 and pMGWPS1 showed that glucose reduced considerably the *xylA* transcription rate with the measured repression ratio of 0.33. As shown in Table 2, the subtilisin activity and the amount of *xylA* transcript were highest (164±8 and 108±18, respectively) with pMGWPP1, while the lowest values (67±5 and 30±3, respectively) were displayed by pMGWPP2.

DISCUSSION

B. stearothermophilus produces multiple extracellular and intracellular β-xylosidases [23] and synthesis of the

Table 2. Catabolite repression of subtilisin and *xylA* transcript synthesis in *B. subtilis* strain MW15 bearing pMGWPB1 or its deletion derivative[a].

Plasmid in strain MW15	Subtilisin activity (units × 10^3)[b]		Ratio (Glc/None)	*xylA* transcript level (%)[c]		Ratio (Glc/None)
	None	Glc		None	Glc	
pWP18	4±0.5	5±0.4	1.25	0.9±0.1	0.8±0.1	0.89
pMGWPB1	103±7	41±3	0.40	100±13	33±4	0.33
pMGWPS1	94±5	48±2	0.51	96±10	32±3	0.33
pMGWPP1	164±8	141±9	0.86	108±18	99±15	0.91
pMGWPB2	67±5	59±3	0.88	32±4	30±3	0.94

[a]The values shown are the averages (±standard deviation) of three independent experiments. [b]Cells bearing each plasmid were grown with or without 1% glucose, and then subtilisin in the medium was assayed as described in the Materials and Methods. [c]The integrated values (area) of densities for each blotting from multiple independent experiments (including the blottings shown in Fig. 3) were obtained using a chromatoscanner (CS-9000: Shimadzu, Kyoto). The value from each slot blotted with 5 μg of total RNA (Fig. 3) was given as percentage of that of pMGWPB1-containing MW15 cell which was grown without glucose.

enzymes in this bacterium was found to be induced by xylan and repressed greatly by glucose (S. G. Cho and Y. J. Choi. 1998. Regulation of β-xylosidase (XylA) synthesis in *Bacillus stearothermophilus*. *J. Microbiol. Biotechnol.* **8**: 14−20). From this study, we found that CRE-1 (nucleotides +124 to +136) is located in the reading frame region of *xylA* gene and critical for CR of the gene. The CREs of *acsA* [6], *hutP* [27], and *gntR* [12] genes of *B. subtilis* are also reported to be located in the reading frame region, where binding of the CcpA and Hpr complex probably interferes with transcription elongation [12]. The *xylA* gene of *B. stearothermophilus* was found to contain an additional CRE-like sequence (Fig. 1A; -62 TGGCGGTTTGGACA -49) in the promoter region even though it had barely recognizable similarities to consensus CRE sequence. Therefore, it is also conceivable that a looping mechanism involving cooperative binding of the CcpA and Hpr complex (es) on the CREs could interfere with transcription initiation of the *xylA* gene [27]. The possible regulation associated with the additional CRE-like sequence was not investigated in this study.

In the previous study (S. G. Cho and Y. J. Choi. 1998. Regulation of β-xylosidase (XylA) synthesis in *Bacillus stearothermophilus*. *J. Microbiol. Biotechnol.* **8**: 14−20), the carbon sources described in Table 1 were also used to show their effcets on β-xylosidase synthesis in *B. stearothermophilus* No. 236. In the case, only xylan gave the highest induction of the enzyme synthesis while xylose induced partly (about 30%) and all other carbon sources didn't induce the synthesis. In this study, however, the experiments done with *B. subtilis* MW15 strains carrying the *xylA::aprA* fusion plasmids, pMGWPB1, pMGWPS1, pMGWPP1, and pMGWPP2, revealed that the expression of the cloned *xylA* gene did not require any carbon source for its induction, but was repressed about 2~3 fold in the presence of the carbon sources such as sucrose, glycerol, ribose, fructose, maltose, cellobiose, or glucose.

We also found that CRE-1 has no effect on CR of the *xylA* gene but the deletion of CRE-1 reduced the expression of the *xylA* gene by about 2~3 fold. This suggests that the CRE-1 sequence and/or its flanking sequences deleted from pMGWPP1 may have a role in expression of the *xylA*. Studies are in progress to define the function of the CRE-1 sequence mentioned above.

In the previous study, the level of glucose repression for the β-xylosidase synthesis in *B. stearothermophilus* had been assessed to be about 40-fold when the relative β-xylosidase activity was measured in the presence of glucose, and approximately 10-fold repression was estimated when the relative amounts of *xylA* transcript were assayed in the same organism (S. G. Cho and Y. J. Choi. 1998. Regulation of β-xylosidase (XylA) synthesis in *Bacillus stearothermophilus*. *J. Microbiol. Biotechnol.* **8**: 14−20). Whereas, in the present study with *B. subtilis*, the repression ratios of the *xylA* gene were determined to be about 0.4 and 0.3, from the assay of the subtilisin activity and from the amounts of the *xylA* transcripts produced, respectively.

This level of CR in *B. stearothermophilus* is very low compared with that of the *B. subtilis* β-xylosidase gene (*xynB*; 100-fold) [10], but higher than that for the *xylA* gene assessed to be about 2~3 fold in *B. subtilis* MW15 strains by using the *xylA::aprA* fusion plasmids. Therefore, we suspect that the expression of the *xylA* may be repressed about threefold in *B. stearothermophilus* by the CR executed by the CcpA-HPr repressor complex and additionally about threefold by inducer exclusion and/or expulsion [19]. Nevertheless, this level of CR is even lower than those for many other catabolite genes including *B. subtilis acsA*, *acuA* [6], *amyE* [7], *gntK* [3], *gntB* [3], *hut* [27], and *B. megaterium xylA* (xylose isomerase) gene [8]. Then, we suppose that the efficiency of CR by CRE-2 of the *xylA* gene could be actually over threefold in *B. stearothermophilus*, but the system of CR in *B. subtilis* may be a little different from that in *B. stearothermophilus*, for example, a difference of binding affinity of the CcpA and HPr complex to CRE. This may be one reason why the CR of the *xylA* gene was underestimated in *B. subtilis* MW 15 strains.

The CcpAs from three gram-positive bacteria show over 55% identity to one another [4] and the HPrs from many gram-positive bacteria are also reported to be more than 60% identical [18]. Hence, in order to investigate CR of the *xylA* gene in molecular details, we will clone the *ccpA* and *ptsH* genes from *B. stearothermophilus*, and the binding affinity of the CcpA and HPr complex to CRE-2 of the *xylA* will be compared with that of other gram positive strains. By developing a transformation protocol to introduce plasmids into *B. stearothermophilus*, we will examine directly CR of the *xylA* and other genes encoding the enzymes essential for degradation of xylan.

Acknowledgments

We thank Dr. Rainer Borriss for providing the strain B. subtilis MW15 and Dr. Sungsoo Park for the plasmid pWP18 used in this study. This work was supported by a Genetic Engineering Research grant from the Ministry of Education of Korea.

REFERENCES

1. Barry, T., S. Geary, S. Hannify, C. MacGearailt, M. Shalloo, D. Heery, F. Gannon, and R. Powell. 1992. Rapid

mini-preparations of total RNA from bacteria. *Nucleic Acids Res.* **20**: 4940.

2. Cho, S. G. and Y. J. Choi. 1995. Nucleotide sequence analysis of endo-xylanase gene (*xynA*) from *Bacillus stearothermophilus. J. Microbiol. Bictechnol.* **5**: 117–124.
3. Deutscher, J., J. Reizer, C. Fischer, A. Galinier, M. H. Jr, Saier, and M. Steinmetz. 1994. Loss of protein kinase-catalyzed phosphorylation of HPr, a phospho-carrier protein of the phosphotransferase system, by mutation of the *ptsH* gene confers catabolite repression resistance to several catabolite genes of *Bacillus subtilis. J. Bacteriol.* **176**: 3336–3344.
4. Egeter, O. and R. Brückner. 1996. Catabolite repression mediated by the catabolite control protein CcpA in *Staphylococcus xylosus. Mol. Microbiol.* **21**: 739–749.
5. Fujita, Y. and Y. Miwa. 1989. Identification of an operator sequence for the *Bacillus subtilis gnt* operon. *J. Biol. Chem.* **264**: 4201–4206.
6. Grundy, F. J., A. J. Turinsky, and T. M. Henkin. 1994. Catabolite regulation of *Bacillus subtilis* acetate and acetoin utilization genes by CcpA. *J. Bacteriol.* **176**: 4527–4533.
7. Henkin, T. M., F. J. Grundy, W. L. Nichoson, and G. H. Chambliss. 1991. Catabolite repression of alpha-amylase gene expression in *Bacillus subtilis* involves a trans-acting gene product homologous to the *Escherichia coli lacI* and *galR* repressors. *Mol. Microbiol.* **5**: 575–584.
8. Hueck, C. J. and W. Hillen. 1995. Catabolite repression in *Bacillus subtilis*: a global regulatory mechanism for the gram-positive bacteria? *Mol. Microbiol.* **15**: 395–401.
9. Leighton, T. and R. H. Doi. 1971. The stability of messenger ribonucleic acid during sporulation in *Bacillus subtilis. J. Biol. Chem.* **246**: 3189–3195.
10. Lindner, C., J. Stke, and M. Hecker. 1994. Regulation of xylanolytic enzymes in *Bacillus subtilis. Microbiology* **140**: 753–757.
11. Millet, J. 1970. Characterization of proteinases excreted by *Bacillus subtilis* Marburg strain during sporulation. *J. Appl. Bacteriol.* **33**: 207–219.
12. Miwa, Y. and Y. Fujita. 1993. Promoter-independent catabolite repression of the *Bacillus sbutilis gnt* operon. *J. Biochem.* **113**: 665–671.
13. Mun, A. R. and Y. J. Choi. 1992. Purification and characterization of exo-xylanase from *Escherichia coli* cells harboring the recombinant plasmid pMG1. *Kor. J. Appl. Microbiol. Biotechnol.* **20**: 574–582.
14. Oh, H. J. and Y. J. Choi. 1995. Sequence analysis of β-xylosidase gene from *Bacillus stearothermophilus. Kor. J. Appl. Microbiol. Biotechnol.* **22**: 134–142.
15. Oh, S. W., S. S. Park, Y. I. Park, and Y. J. Choi. 1992. Molecular cloning and expression of *Bacillus stearothermophilus* β-D-xylosidase gene in *Escherichia coli. Kor. J. Appl. Microbiol. Biotechnol.* **20**: 136–142.
16. Postma, P. W., J. W. Lengeler, and G. R. Jacobson. 1993. Phosphoenol pyruvate: carbohydrate phosphotransferase systems of bacteria. *Mocrobiol. Rev.* **57**: 543–594.
17. Saier, M. H., Jr. 1993. Regulatory interactions involving the proteins of the proteins of the phosphotransferase system in enteric bacteria. *J. Cell. Biochem.* **51**: 62–68.
18. Saier, M. H., Jr., M. J. Fagan, C. Hoischen, and J. Reizer. 1993. Transport mechanisms, pp. 133–156. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis and other gram-positive bacteria: biochemisty, physiology, and molecular genetics*. American Society for Microbiology, Washington, DC.
19. Saier, M. H., Jr., S. Chauvaux, G. M. Cook, J. Deutsher, I. T. Paulsen, J. Reizer, and J. J. Ye. 1996. Catabolite repression and inducer control in gram-positive bacteria. *Microbiology* **142**: 217–230.
20. Sambrook, J., T. Maniatis, and E. F. Fritsch. 1989. *Molecular cloning, A laboratory manual.* Cold Spring Harbor Laboratory, New York.
21. Song, H. S. and Y. J. Choi. 1989. Production of xylanase by *Bacillus stearothermophilus. Kor. J. Appl. Microbiol. Biotechnol.* **17**: 289–294.
22. Spizizen, J.. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleotide. *Proc. Natl. Acad. Sci. USA* **44**: 1072–1078.
23. Suh, J. H., S. J. Eom, S. G. Cho, and Y. J. Choi. 1996. Molecular cloning and expression of the β-xylosidase gene (*xylB*) of *Bacillus stearothermophilus in Escherichia coli. J. Microbiol. Biotechnol.* **6**: 331–335.
24. Wang, L. and Doi, R. H. 1987. Promoter switching during development and the termination site of the sigma 43 operon of *Bacillus subtilis. Mol. Gen. Genet.* **207**: 114–119.
25. Weickert, M. J. and G. H. Chambliss. 1990. Site-directed mutagenesis of a catabolite repression operator sequence in *Bacillus subtilis. Proc. Natl. Acad. Sci. USA* **87**: 6238–6242.
26. Wolf, M., A. Geczi, O. Simom, and R. Borriss. 1995. Genes encoding xylan and β-glucan hydrolysing enzymes in *Bacillus subtilis*: characterization, mapping and construction of strains deficient in lichenase, cellulase and xylanase. *Microbiology* **141**: 281–290.
27. Wray, L. W., Jr., J. K. Pettengill, and S. H. Fisher. 1994. Catabolite repression of the *Bacillus subtilis hut* operon requires a cis-acting site located downstream of the transcription initiation site. *J. Bacteriol.* **176**: 1894–1902.
28. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**: 103–199.