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Regulation of β-Xylosidase (XylA) Synthesis in *Bacillus stearothermophilus*

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Abstract Syntheses of the *B. stearothermophilus* xylanolytic enzymes such as xylanases, β-xylosidases, α-arabinofuranosidases, and esterases, were observed to be regulated by the carbon source present in the culture media. Xylan induced synthesis of β-xylosidase at the highest level while xylose gave about 30% of the β-xylosidase activity induced by xylan. The lowest syntheses of the xylanolytic enzymes above mentioned were detected in the basal medium containing glucose as a sole carbon source. When a mixture of xylan and glucose was used as a carbon source, we could observe glucose repression of xylanase (about 70-fold) and β-xylosidase (about 40-fold) syntheses. Whereas, the level of the glucose repression of the expression of the *xylA* gene encoding the major β-xylosidase of *B. stearothermophilus* was assessed to be about 10-fold when the relative amounts of the *xylA* transcript were determined. From the sequence of the *xylA* gene, we could find two CRE-like sequences (CRE-1: nucleotides +124 to +136 and CRE-2: +247 to +259) within the reading frame of the *xylA* gene, either or both of which were suspected to be involved in catabolite repression of the *xylA* gene.

Key words: Regulation, β-xylosidase, *xylA*, *Bacillus stearothermophilus*

Xylan, the primary component of plant pentosan, is the most abundant biomass in nature next to cellulose [2]. This polymer consists of a backbone chain of 1,4-linked β-D-xylopyranosyl residues and side chains of arabinose, acetic acid, glucuronic acid, or methylglucuronic acid. Therefore, the complete enzymatic degradation of xylan requires several xylanolytic enzymes including xylanase, β-xylosidase, α-arabinofuranosidase, esterase, and α-glucuronidase [32].

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Recently, we have isolated a strain of *Bacillus stearothermophilus* capable of producing all the enzymes necessary for xylan biodegradation [27], and cloned a xylanase gene (*xynA*) [4], three β-xylosidase genes [23, 29] including the *xylA* gene [23], an α-arabinofuranosidase gene (*arfI*) [6], and two esterase genes (*estI* and *estII*) [10, 13] from the genomic DNA of this strain. We have also sequenced some of the cloned genes described above [5, 16, 22] and shown the synergism among xylanase, β-xylosidase, α-arabinofuranosidase, and esterase in hydrolytic reactions of xylan substrate [27, 30]. Furthermore, in the previous study on the pentose utilization of *B. stearothermophilus*, it was known that the strain produced hexokinase constitutively but production of D-xylose isomerase, D-xylulokinase, and D-arabinose isomerase was induced by xylose, xylan, and xylitol and repressed by glucose [15].

Many xylanolytic enzymes have been characterized for their potential use in biotechnology, but little is known at the molecular level about the regulatory mechanisms controlling their gene expression.

As the initiatation of such study, we examined in detail the effect of carbon sources on syntheses of xylanase, β-xylosidase, α-arabinofuranosidase, and esterase in this study. We found that synthesis of each of the xylanase, β-xylosidase, and α-arabinofuranosidase was induced at the highest level by xylan while the production of the above enzymes were repressed significantly by glucose. To assess the glucose repression on expression of the *xylA* gene encoding the major β-xylosidase of *B. stearothermophilus* at the transcriptional level, we compared relative amounts of the *xylA* transcripts synthesized from the cells grown with or without glucose. Furthermore, from the sequence of the *xylA* gene [22], two catabolite responsive element (CRE)-like sequences (CRE-1: nucleotides +124 to +136 and CRE-2: +247 to +259) were found within the reading frame region of the *xylA* gene. This suggested that

formation of XylA in *B. stearothermophilus* could also be regulated by CRE-mediated catabolite repression. The results obtained by studying the problems mentioned above are reported and discussed in this paper.

MATERIALS AND METHODS

Bacterial Strains, Plasmid, and Culture Conditions

B. stearothermophilus, which produces β-xylosidases and other xylanolytic enzymes, was isolated from soil in our lab [27]. The recombinant plasmid pMG1 [22] carrying the entire *xylA* gene was used to prepare the DNA probe for slot blotting. *Escherichia coli* JM109 (*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ (*lac-proAB*)/F'[*traD36 proAB*$^+$ *lacI*^{q}*Z* Δ M15]) [34] was used as a host strain for the plasmid.

B. stearothermophilus was grown in the basal medium (BM; 10.6 g K_2HPO_4, 6.1 g $NaH_2PO_4 \cdot 2H_2O$, 2 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4$ and 3.5 g yeast extract per liter) containing carbon sources [15], which were supplied at different concentrations as indicated in the text, under vigorous aeration at 45°C. *E. coli* containing plasmid pMG1 was propagated at 37°C in Luria broth supplemented with 50 μg/ml ampicillin.

General Methods

Standard methods of molecular biology [26] 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.

Transformation into *E. coli* JM109 was performed as described previously [4].

The protein concentration of enzyme samples was determined by Lowry's method [17] using bovine serum albumin as a standard.

Enzyme Preparation

B. stearothermophilus cells cultured in various media were harvested by centrifugating with a Beckman JA10 rotor at 6000 rpm for 20 min at 4°C and washed twice in half the volume of the culture with 50 mM phosphate buffer (pH 7.0). After centrifugation, the collected cells were resuspended in the same buffer and sonicated until they were lysed. Cell debris was spun down with a Beckman JA20 rotor at 10,000 rpm for 15 min. We used the culture fluid and the two washes as extracellular enzyme solution and the supernatant fluid after the last centrifugation as intracellular enzyme solution.

Measurements of Enzyme Activities

Xylanase activity was measured by incubating 0.5 ml of birchwood xylan (2% in 50 mM phosphate buffer (pH 7.0)) with the same volume of the enzyme solution for 30 min at 55°C. Released sugar content was quantified by the DNS method [18]. One unit of enzyme was defined as the amount of enzyme required to release 1 μmole of xylose equivalents per min.

β-Xylosidase and α-arabinofuranosidase activities were assayed by monitoring the liberation of *p*-nitrophenol from 10 mM of *p*-nitrophenyl-β-D-xylopyranoside (pNPX) or *p*-nitrophenyl-α-L-arabinofuranoside (pNPAf) in 50 mM phosphate buffer (pH 7.0), respectively [6, 23]. The point one ml of the enzyme solution was incubated at 45°C for 30 min with the same amount of the substrate and the reaction was terminated by adding 2 ml of 0.4 M Na_2CO_3. The released *p*-nitrophenol was determined by reading the absorbance at 405 nm. One unit of enzyme was defined as the amount of enzyme required to release 1 μmole of *p*-nitrophenol per min.

Esterase activity was assayed by adding 550 μl of the enzyme solution to 3 ml of 10 mM methylacetylsalicylate (MAS) in 50 mM phosphate buffer (pH 7.0) and incubating at 45°C for 30 min, followed by reading the absorbance at 300 nm [13]. One unit of enzyme was defined as the amount of enzyme required to produce 1 μmole of methylsalicylate per min.

RNA Preparation

From *B. stearothermophilus* which had been grown in the medium containing 0.5% of glucose, xylan, or both of the carbon sources, total cellular RNAs were isolated by a rapid procedure described by Barry *et al.* [1]. To remove contaminated DNA, the isolated RNAs were 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 by 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) prepared by digesting the insert DNA of pMG1 with *Cla*I and *Pst*I [22], 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

Regulation of Syntheses of Xylanolytic Enzymes by Carbon Sources

From previous studies, *B. stearothermophilus* was found to produce many kinds of xylanolytic enzymes such as xylanases [3], β-xylosidases [20], α-arabinofuranosidases [7], esterases [11, 12], and α-glucuronidases (unpublished data). Several genes encoding the xylanolytic enzymes have been cloned and characterized [4, 6, 10, 13, 23, 29] but little is known about the mechanisms controlling their expression in *B. stearothermophilus*. To have insight into the mechanisms, we first examined the effects of carbon sources on the syntheses of xylanase, β-xylosidase, α-arabinofuranosidase, and esterase.

For this purpose, *B. stearothermophilus* was grown in BM containing each of the various carbon sources indicated in Table 1 and the extracelluar and intracellular xylanase, β-xylosidase, α-arabinofuranosidase, and esterase activities were assayed on each culture as described in Materials and Methods.

As shown in Table 1, in BM containing each of the carbon sources examined except for glycerol, the cells grew well in the 12-h culture at 45°C, and produced all the xylanolytic enzymes, but when glycerol was used as a sole carbon source, no growth and no enzyme activity were detected (data not shown). Xylanase and α-arabinofuranosidase were found to be mainly localized in extracellular fractions, while most esterase was detected in intracellular fractions. β-Xylosidase activity was assessed almost at the same levels in both fractions. We could also note that syntheses of all the enzymes examined were lowest in BM supplemented with glucose as a sole carbon source, while xylan induced full syntheses of xylanase, β-xylosidase, and α-arabinofuranosidase. When a mixture of xylan and glucose was used as a carbon source, the extracellular xylanase activity was determined as 0.89 unit/mg, indicating that glucose mediated about 70-fold repression of the xylanase synthesis (The activity induced by xylan was 62.19 units/mg). In the case of the extracellular α-arabinofuranosidase, arabinose induced about half (8.19 units/mg) of the full activity (15.53 units/mg), and other sugars such as fructose, galactose, xylose, and maltose resulted in 10~30% induction. On the other hand, synthesis of esterase was increased slightly only by xylan but other carbon sources tested in this experiment were observed to have little effect on the esterase production.

Regulation of β-Xylosidase Synthesis by Carbon Sources

As shown in Table 1, xylan gave the highest induction of the β-xylosidase synthesis while xylose induced about 30% (24.61 units/mg) of the fully induced activity (65.99 units/mg; the intracellular activity from the cells cultured in the medium supplemented with xylan as a sole carbon source). All the other carbon sources tested in this experiments showed less than 9% activities compared to the highest β-xylosidase activity. When glucose or a mixture of xylan and glucose were used as a carbon source, the β-xylosidase was measured to be less than 2.5% (0.71 and 1.68 units/mg, respectively) of the activity (65.99 units/mg) obtained from the cells cultured in BM

Table 1. Inducibility of xylan-hydrolytic enzymes of *B. stearothermophilus*[a].

Carbon source	Specific activity[b] (unit/mg)							
	Xylanase		β-Xylosidase		α-Arabinofuranosidase		Esterase	
	I	E	I	E	I	E	I	E
Glucose	0.01	0.19	0.71	0.84	0.07	0.01	3.73	0.16
Fructose	0.56	3.41	3.71	3.19	0.13	4.65	4.55	1.79
Galactose	0.24	4.15	2.84	3.26	0.39	4.27	3.14	0.12
Xylose	1.02	5.78	24.61	29.14	0.38	3.46	4.92	0.30
Arabinose	0.73	4.92	4.33	2.16	0.51	8.19	8.99	0.88
Ribose	0.47	3.25	3.91	3.13	0.37	1.41	8.53	0.36
Xylitol	0.95	3.46	5.16	9.55	0.31	1.72	9.71	1.01
Cellobiose	0.34	4.27	2.05	1.21	0.15	1.33	6.72	0.24
Maltose	0.56	6.24	2.81	2.01	0.48	5.46	8.94	0.54
Sucrose	0.42	3.01	4.09	2.32	0.39	1.92	8.75	0.71
Xylan	4.85	62.19	65.99	72.28	0.56	15.53	13.08	2.79
Xylan+Glucose	0.57	0.89	1.68	1.97	ND[c]	ND	ND	ND

[a]Cells were grown in BM containing carbon source (0.5% (w/v) glucose, fructose, galactose, xylose, arabinose, ribose, xylitol, cellobiose, maltose, sucrose, xylan, or 0.5% xylan+0.5% glucose) for 12 h at 45°C. [b]Enzyme activities (unit/ml) were determined from the intracellular fraction (I) or extracellular fraction (E) of the cultured *B. stearothermophilus* cells and divided by the concentration of total protein (mg/ml) of each fraction. [c]ND: Not determined.

containing 0.5% xylan as a sole carbon source. This result indicates that glucose mediated about 40-fold repression of the β-xylosidase formation in *B. stearothermophilus*.

Effect of Glucose on Expression of the *xylA* Gene

The results of zymogram performed in previous study showed that *B. stearothermophilus* produced multiple β-xylosidases [29], and the enzyme synthesized by the *xylA* gene was the major β-xylosidase formed in this bacterium. Therefore, to examine the effect of glucose on synthesis of the major β-xylosidase (XylA) in more detail, the *B. stearothermophilus* cells were grown in BM supplemented with 0.5% xylan, 0.5% glucose or a mixture of the two carbon sources. Then, the amounts of the *xylA* transcript synthesized were monitored during the cultures.

As seen in Fig. 1A, nearly the same rates of the cell growth were obtained from the three cultures, but the β-xylosidase activity and the *xylA* transcript produced were determined to be high only in the cultures supplemented with 0.5% xylan as a sole carbon source (Fig. 1B and C). We could also observe that the intracellular β-xylosidase production and the *xylA* mRNA synthesis were increased with almost the same rate during the culture period.

Next, to analyze the effects of glucose concentrations on the expression of the *xylA* gene in *B. stearothermophilus*, the β-xylosidase activities and the amounts of the *xylA* mRNA synthesized were measured from the cells grown in BM containing 0.5% xylan and various concentrations of additional glucose ranging from 0.1% to 2% (w/v). As shown in Fig. 2A and B, production of the *xylA* mRNA and the β-xylosidase activity sharply decreased with the increase in the concentrations of glucose up to 0.5%, but no further decreases were detected with the glucose concentrations higher than that.

From this experiment, about 40-fold repression of the β-xylosidase activity was estimated in the presence of 0.5% glucose while the level of repression was assessed to be about 10-fold when the relative amounts of the *xylA* transcripts were assayed with the cells cultured in the presence of the same concentration of glucose. This

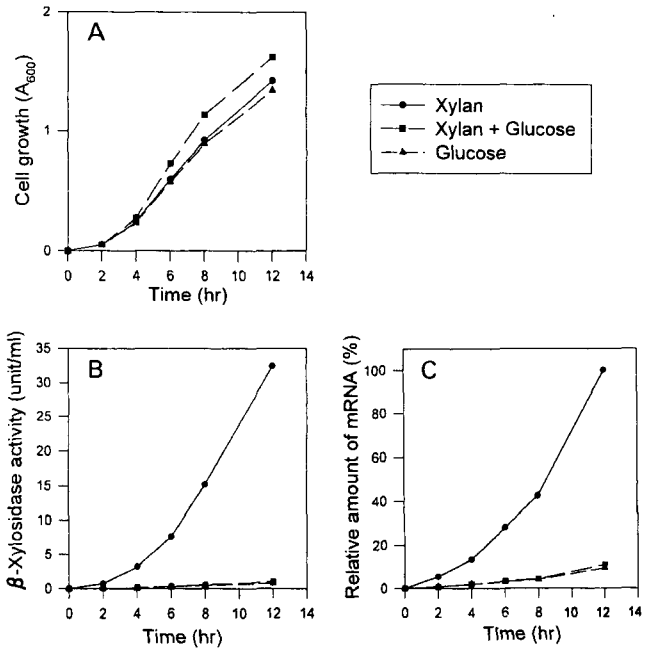

Fig. 1. Effect of glucose on cell growth (A) and synthesis of the β-xylosidase (B) and *xylA* mRNA (C) in *B. stearothermophilus*.
The cells were grown in BM containing either 0.5% xylan or glucose, or both carbon sources at 45°C and the values shown are the averages of three independent experiments.

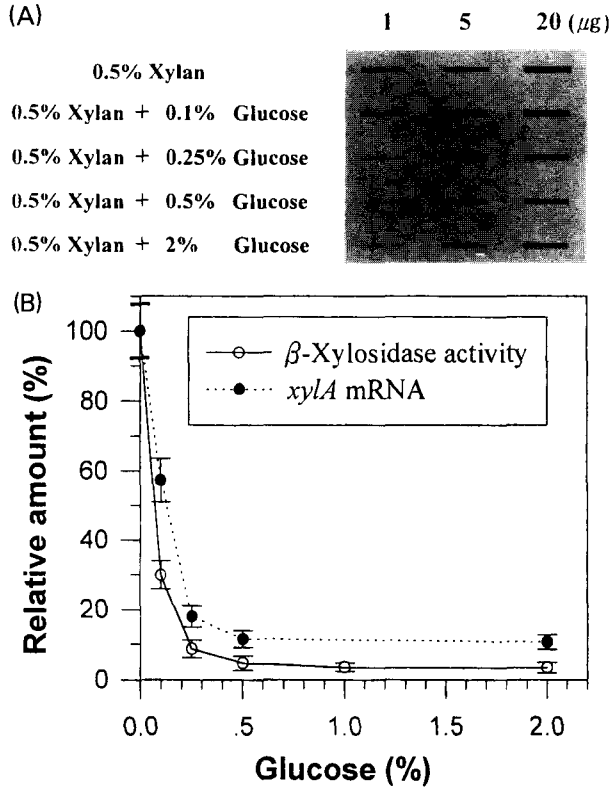

Fig. 2. Dependence of synthesis of the β-xylosidase and *xylA* mRNA in *B. stearothermophilus* on the concentration of glucose.
(A) Slot blotting analysis of *xylA* mRNAs synthesized in the presence of various concentrations of glucose. Total RNAs were prepared from cells grown in BM containing carbon sources indicated above for 12 h at 45°C. The amounts of blotted RNAs were 1, 5, 20 μg per slot, as indicated. The details of the slot blotting analysis are given in the text. (B) Relative amounts of β-xylosidase activity and *xylA* mRNA synthesized in the presence of various concentrations of glucose. The β-xylosidase activities and *xylA* mRNA levels are given as percentages of that obtained with 0.5% xylan. To compare the amount of *xylA* mRNAs, the integrated values (area) of densities for each blotting from multiple independent experiments (including the blottings shown in Fig. 2A) were obtained using a chromatoscanner (CS-9000: Shimadzu, Kyoto). The value from each slot blotted with 5 μg of total RNA was used to obtain the relative amounts of *xylA* mRNA.

level of repression control is much lower than the overall rate of the β-xylosidase repression in *B. stearothermophilus*. Therefore, it is proposed that glucose could repress only about 10-fold the expression of the *xylA* gene in *B. stearothermophilus* at the transcriptional level through the mechanism (s) of catabolite repression.

DISCUSSION

B. stearothermophilus is found to produce nearly all types of xylanolytic enzymes including xylanases, β-xylosidase, α-arabinofuranosidase, α-glucuronidase, and acetyl xylan esterase. In our laboratory, the *B. stearothermophilus* xylanolytic enzymes described above have been characterized, and the genes encoding xylanase [4, 5], β-xylosidase [22, 23, 29], α-arabinofuranosidase [6], and acetyl xylan esterase [10, 13, 16] have been cloned and sequenced.

In other previous studies, we examined the effects of potential inducers and repressors on synthesis of the two major xylanolytic enzymes, xylanase and β-xylosidase (Kwon, Y. M. 1991. M. Agr. thesis. Korea University, Seoul), but regulations of the two corresponding gene expression have never been investigated in molecular details. We confirmed in the present study that the synthesis of all the xylanolytic enzymes were regulated by the carbon source present in the culture media (α-glucuronidase was not included in this work).

Production of the β-xylosidase like the other xylanolytic enzymes was greatly induced by xylan in *B. stearothermophilus*. On the other hand, about 40-fold repression of the β-xylosidase synthesis was observed when the bacterium was grown in BM medium containing glucose as a carbon source. However, from the results obtained by analyzing the levels of the *xylA* mRNA synthesis, we could assess about 10-fold catabolite repression when the bacterium was cultured in the presence of glucose. This lower repression rate is supposed to be caused partly by the multiplicity of β-xylosidase in *B. stearothermophilus* even though the *xylA* gene product is the major β-xylosidase in this organism [23, 29].

The glucose-mediated regulation is known as catabolite repression (CR), a mechanism by which the cell coordinates metabolism of carbon and energy sources to maximize efficiency and regulates other metabolic processes as well [24]. CR in gram-positive bacteria, which is studied primarily with *B. subtilis*, is reported to be 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 a catabolite responsive element (CRE) [9, 25].

The catabolite responsive element (CRE) was reported to have a consensus 14-bp palindromic sequence, TGT/AAANC ↓ GNTNA/TCA, where underlined letters represent the most critical bases, N is any base, and the vertical arrow denotes an axis of symmetry [9, 31]. In addition, the CRE elements show a striking variability of their positions with respect to the transcriptional start sites of regulated genes [9]. Then, we searched the CRE-like sequence from the sequence not only of the promoter region but also of the reading frame of the *xylA* gene [22] whose transcription initiation nucleotide was mapped by S1 nuclease analysis and assigned as +1 (data not shown).

B. amyloliquefaciens	*bglA*	-6	TGTATGATC#AATA	+7
B. subtilis	*bglS*	-6	TGTAAGATC#AACA	+7
B. subtilis	*amyE*	-3	TGTAAGCGTTAACA	+11
B. subtilis	*xylA*	+134	TGAAAGCGTTACCA	+147
B. subtilis	*gntR*	+141	TGAAAGCGGTACCA	+154
B. subtilis	*hutP*	+203	TGAAACCGCTTCCA	+216
B. stearothermophilus	*xylA*	+124	TGAGCTCGGCAGCC	+137
		+247	TGAGAGCGAT#TCA	+259
Consensus sequence			TGWNANCGNTNWCA	
Optimal sequence			TGTAAGCGCTTACA	

Fig. 3. Comparison of the CRE sequences involved in catabolite repression of gram-positive bacteria.
The sequences and their positions from the transcription initiation base (+1) have been reported, as follows; *B. subtilis amyE* [31], *bglS* [21], *gntR* [19], *hutP* [33], *xylA* [14], and *B. amyloliquefaciens bglA* [8]. (W= A or T, N=any base, #=gap). The underlined numbers are estimated positions from the possible transcription initiation site of *bglA* or *bglS*.

Within the reading frame region, two potential CREs (CRE-1: nucleotides +124 to +136 and CRE-2: +247 to +259) were recognized and compared with other CRE sequences. As shown in Fig. 3, they are found to have high homology to the CRE's of the *B. subtilis amyE* [31], *bglS* [21], *xylA* [14], *gntR* [19], and *hutP* [33] genes, and the *bglA* [8] gene of *B. amyloliquefaciens*.

Hence, we speculated that the 10-fold glucose repression of the *xylA* gene described above might result from the same mechanism as that for the catabolite repression control in *B. subtilis*, a prototype of gram-positive bacteria catabolite repression (CR).

In a successive work, we will investigate in molecular details the catabolite repression of the *xylA* gene expression where the CRE-like sequences within the reading frame of the gene may play a critical role.

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