Catabolite Repression of the Xylanase Gene (xynA) Expression in Bacillus stearothermophilus No. 236 and B. subtilis

Sang-Goo Cho and Yong-Jin Choi†

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

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Catabolite repression of the Bacillus stearothermophilus No. 236 xynA gene, encoding an extracellular xylanase, was investigated in this work. Expression of the xynA gene in the B. stearothermophilus strain was found to be subject to glucose catabolite repression, and the level of repression was about 50-fold when the relative amounts of xynA transcript synthesized on different carbon sources were analyzed. The experiments with the B. subtilis MW15 strains carrying plasmids containing the xynA::aprA fusion gene showed that the cloned xynA gene did not require any specific carbon source for its induction. Nevertheless, the expression of the cloned gene was repressed by the presence of glucose. From the nucleotide sequence of the cloned xynA gene, we found two potential catabolite responsive elements (cre) within its reading frame region (cre-1: nucleotides +160 to +173 and cre-2: +173 to +186). Furthermore, by using various deletion derivatives of the xynA::aprA fusion plasmid (pMGW23), we suggested that only the cre-2 element might play a role in the glucose catabolite repression. Repression level of the xynA gene expression in the recombinant B. subtilis strain was estimated to be about 3-fold by analysis of the amounts of xynA transcript.

Key words: catabolite repression; xylanase; xynA; Bacillus stearothermophilus; Bacillus subtilis

β-Xylanase (1,4-β-d-xylan xylanohydrolase; EC 3.2.1.8) is a key enzyme in hemicellulose degradation, which hydrolyzes the internal β-1,4 bonds of xylan, a complex polymer comprising as much as 35% of the dry weight of higher plants. Thus the enzyme has many potential applications.1)

Although many reports on xylanase from a variety of microorganisms are available, little is known about the mechanism by which expression of xylanase genes is regulated in microbial cells. In most microorganisms, use of polymeric substrates is regulated by the availability of more easily metabolizable carbon sources, even though the mechanism of carbon catabolism might be different depending on the organisms.2)

In our previous work, it was found that synthesis of xylanase in Bacillus stearothermophilus No. 236 isolated from soil was induced by xylan, but repressed about 70-fold in the presence of glucose.3) However, the mechanisms involved in the regulation of the xylanase gene (xynA) expression have not been studied in detail. Catabolite repression (CR) in E. coli, a representative Gram-negative bacterium is a well understood paradigm of a global control of gene expression. It is known to involve the enzyme IIα of the phosphotransferase system (PTS) as the central regulatory protein that controls the intracellular cAMP level.4) However, in the Gram-positive bacteria, cAMP does not play a central role, and CR in these organisms is mediated by a negative regulatory mechanism.5) In Bacillus subtilis, a representative Gram-positive bacterium, three components, namely, the cis-acting catabolite responsive element (cre: TG8/AANCGTNATA/CA), CcpA (catabolite control protein A), and/or CcpB (catabolite control protein B), and the heat-stable protein HPr, have been identified as being involved in CR.6,7) Recently, there was also an article describing a function of NADP as a corepressor of CcpA.8)

In this study, as a first step to discover the mechanism(s) for CR of xynA gene expression in B. stearothermophilus No. 236, we assessed the CR level of xynA by measuring the relative amount of xynA transcript, and identified the potential cre sequence by doing deletion analysis of the cloned xynA gene.

Materials and Methods

Bacterial Strains and Plasmids. Bacillus stearothermophilus No. 236, a strong xylan degrader that produced xylanase A and other xylanolytic enzymes, was isolated from soil and kept in our lab.9) As host strains for the plasmids used in this work, E. coli JM109 (recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB) F′ [traD36 proAB+ lacUVZ ΔM15]) and B. subtilis MW15 (his nprR2 nprE18 ΔaprA3 ΔeglS102 ΔbglT bgISRV ΔxynA CmR) which was a mutant deficient in extracellular alkaline and neutral proteases, carboxymethyl cellulase, β-1,3 (4)-glucanase, and xylanase,10) were used.

A promoter-probe plasmid, pWP18, which had been derived from plasmid pSB,11) contained a promoterless subtilisin gene (aprA) preceded by the pUC18 poly linker.

Culture and Growth Conditions. Bacillus stearothermophilus No. 236 was grown at 45°C under vigorous aeration in the basal medium (BM; 10.6 g K2HPO4, 6.1 g Na2HPO4⋅2H2O, 2 g (NH4)2SO4, 0.5 g MgSO4, and 3.5 g yeast extract per liter)13) with appropriate carbon

† To whom correspondence should be addressed. Prof. Yong-Jin Choi; Phone: 82-2-3290-3417; Fax: 82-2-923-9932; E-mail: choiyj@kuccex.korea.ac.kr
sources as indicated in the text.

The *B. subtilis* MW15 strains bearing plasmids were grown at 37°C in Schaeffer's medium (2 × SG) with various carbon sources and 10 μg/ml kanamycin. The *E. coli* JM109 cells carrying plasmids were propagated at 37°C in Luria broth with 50 μg/ml ampicillin.

**General methods.** Standard methods of molecular biology were used unless otherwise specified. Most enzymes used in this work were purchased from New England Biolabs, Promega, United States of Biochemicals, or Boehringer Mannheim, and other chemicals were from Sigma Co.

Plasmid DNA was transferred to competent cells of *B. subtilis* MW15 prepared by the procedure of Spizizen. A DNA sample (0.1 μg) and the competent cell suspension (0.2 ml) were mixed and incubated for 90 min at 37°C. The transformation mixture was plated on Schaeffer's sporulation agar plates containing 10 μg/ml kanamycin and 1% skim milk.

Transformation into *E. coli* JM109 was done as described earlier.

**Construction of deletion derivatives.** Plasmids pMGWP23, pMGWP11, pMGWP6, and pMGWP62 were constructed as follows; Single-stranded plasmid pMG119B26 was deleted by the method of Dale et al. as described earlier. Deletion derivatives of pMG119B26 obtained were fractionated by gel electrophoresis and those with desirable sizes were selected and sequenced. Next, the HindIII-EcoRI fragments of the deletion derivatives were ligated with the HindIII-EcoRI digest of pBluescript to obtain pMGB23, pMGB11, pMGB6, pMGB61, and pMGB62.

To construct pMGWP series plasmids, the pBluescript derivatives listed above were double digested with KpnI and BamH1 and the resulting respective KpnI-BamH1 fragments containing part of the *xynA* gene were ligated with the KpnI-BamH1 arm of pWP18.

**Measurements of Xylanase and subtilisin activities.** Xylanase activity was assayed as described earlier. For the subtilisin assay, the recombinant *B. subtilis* MW15 strains were grown at 37°C in 2 × SG media containing 10 μg/ml of kanamycin in the presence or absence of carbon sources as described in the text. A sample of the culture (0.5 ml) was harvested when the OD600 of the culture broth reached 0.6 and then was centrifuged for 5 min at 16,000 × g at 4°C. Then, subtilisin was assayed by using the supernatant obtained above as the crude enzyme solution according to the method of Millet with some modifications.

One unit of subtilisin activity was defined as the amount of enzyme that produced a soluble dye giving an A595 of 1 in 1 ml of the reaction mixture.

**RNA preparation.** Total cellular RNAs were isolated by the procedure of Barry et al. from the cells of *B. stearothermophilus* No. 236 and *B. subtilis* MW15, which had been grown to OD600 = 0.6 in various culture media. To remove contaminating DNA, the RNA preparations were treated with RNase-free DNase I, extracted with phenol-chloroform, precipitated with ethanol, and finally dissolved in DEPC-treated water.

**Slot-blot hybridization.** The *xynA* mRNA was measured by the following protocol: 1, 5, and 20 μg of total RNA were dissolved in 500 ul of ice-cold 10 mm NaOH solution containing 1 mm EDTA, and then the RNA solutions obtained were 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 done using a DNA labeling and detection kit (Boehringer Mannheim). The DNA probe, the HindIII-HaeIII fragment (nucleotides -40 to +176) of the insert DNA of pMG119B6, was labeled with digoxigenin dUTP, and used in the hybridization reaction as recommended by the manufacturer. The intensities of the hybridization signals were compared using the Scanner CS-9000 (Shimadzu, Japan).

**Results**

**Effects of glucose on the *xynA* expression in *B. stearothermophilus* No. 236**

*B. stearothermophilus* No. 236, producing multiple xylanases, was grown for 12 hrs at 45°C in BM with each or a mixture of the various carbon sources including glucose, fructose, galactose, xylose, arabinoose, and xylan, and the total extracellular xylanase activities were assayed. The highest xylanase activity was obtained in the medium with xylan as the sole carbon source. However, when a mixture of xylan and glucose was used

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Growth (A600)</th>
<th>Xylanase activity (units/ml)</th>
<th>Relative amount of mRNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xyn</td>
<td>Xyn + Glc</td>
<td>Glc</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.058</td>
<td>0.058</td>
<td>0.058</td>
</tr>
<tr>
<td>4</td>
<td>0.251</td>
<td>0.283</td>
<td>0.243</td>
</tr>
<tr>
<td>6</td>
<td>0.593</td>
<td>0.723</td>
<td>0.576</td>
</tr>
<tr>
<td>8</td>
<td>0.918</td>
<td>1.135</td>
<td>0.895</td>
</tr>
<tr>
<td>12</td>
<td>1.423</td>
<td>1.621</td>
<td>1.342</td>
</tr>
</tbody>
</table>

* The cells were grown in the BM containing either 0.5% xylan or glucose, or both carbon sources at 45°C. The values shown are the averages of three independent experiments.
Catabolite Repression of Xylanase (xynA) Expression

B. amylovorans

B. subtilis

B. subtilis

B. subtilis

B. subtilis

B. steatothermophilus

Consensus sequence

Optimal sequence

Fig. 1. Comparison of 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,40 bgIS,20 gmr,11 hutP,1,16 xylA,20 B. amylovorans bgIA,25 B. steatothermophilus xylA,20 and xynA.17 (W=A or T, N=any base, #=gap). The underlined numbers are estimated positions from the possible transcription initiation sites of bgIA or bgIS.

as carbon source, the enzyme activity was only about 1.4% of the activity obtained by xylan alone. This indicates that the xylanase synthesis in B. steatothermophilus No. 236 was inhibited about 70-fold, probably by glucose-mediated catabolite repression.

To further analyze the influence of glucose on synthesis of xylanase, the bacterial cells were cultured in BM containing 0.5% xylan, 0.5% glucose, or a mixture of both as the carbon sources, and cell growth, xylanase activity and xynA transcript level were monitored from the beginning to the early stationary phase of the culture (12 b) (Table 1). Cell growth was observed to be almost the same in all the cultures but the xylanase activity and the xynA mRNA level were detectable only in the medium containing xylan as a sole carbon source, and increased at almost the same rate as that of cell growth.

Next, we examined the effects of glucose concentrations on expression of the xynA in the culture media containing 0.5% xylan and/or various concentrations of glucose from 0.1% to 2.0%. Nearly full CR was observed even at 0.25% glucose concentration when measured by both xylanase activity and the levels of xynA mRNA synthesized (data not shown).

From these results, we could assess that glucose repressed the xynA gene expression about 50-fold at the level of transcription.

cre sequence in the xynA gene

Catabolite repression acting on expression of the B. steatothermophilus xynA gene was thought to be mediated by the same mechanism as that in B. subtilis, so, we searched for a cre sequence from the nucleotide sequence of the xynA gene,17 the transcription initiation nucleotide of which was mapped by S1 nuclease analysis and assigned as +1, and within the reading frame region, recognized two potential cre sequences (cre-1:

nucleotides +160 to +173 and cre-2: +173 to +186) with high similarity to cre sequences of many other genes of B. subtilis,21,24 B. amylovorans,25 and B. steatothermophilus25 (Fig. 1).

Hence, we suspected that either one or both of the cre-like sequences should have been implicated in the 50-fold glucose repression in the synthesis of XynA mentioned above.

CR of XynA synthesis in B. subtilis MW15

To investigate CR of XynA synthesis in Bacillus species, we constructed a recombinant plasmid, pMGW23, as described in Materials and Methods (Fig. 2), and transferred the construct to B. subtilis MW15, a mutant strain deficient in alkaline protease (subtilisin: aprA) and xylanase. The B. subtilis strain carrying pMGW23 was grown in 2 x SG media with the carbon sources indicated in Table 2, and the extracellular xylanase activities were measured after the culture.

Xylanase activity of the culture contained xylan as a sole carbon source was 3-fold higher than that of the culture contained glucose, however, the same level of the activity was obtained from the cultures with either glucose or the mixture of glucose and xylan.

This demonstrated that glucose also mediated CR of
the xynA gene expression in the B. subtilis strain. In addition, we found that a low level of the total xylanase was constitutively synthesized which was not controlled by catabolite repression.

Then, in order to identify whether the potential cis-acting sequences were actually involved in the CR of the xynA gene expression in the B. subtilis strains, we constructed a series of deletion derivatives of pMGWP23 to make several xynA::aprA fusions as described in Materials and Methods (Fig. 2). The B. subtilis strains carrying the pMGWP23 derivatives were grown in 2×SG medium containing 10 μg/ml kanamycin and a carbon source presented in Table 3, and xylanase activity of the reporter protein (AprA) was measured after culture.

The MW15 strain carrying pMGWP23 produced nearly the same levels of subtilisin activity when the cells were grown on xylan, arabinose, galactose, xylose, or xylitol. But when glucose and some other rapidly metabolizable carbon sources such as fructose, sucrose, and maltose were used as an energy source, about half of the full activity (the activity shown when xylan was used as a sole carbon source) was measured. As expected, the same pattern of carbon source influence on the subtilisin production was observed with the strains harboring pMGWP11 and pMGWP6 (both plasmids have an insert containing intact cre-1 and cre-2 elements), although the total levels of enzyme activity were slightly lower than those observed with the MW15 strain carrying pMGWP23.

However, in contrast to the B. subtilis strains mentioned above, B. subtilis harboring pMGWP61 (contains only cre-1) or pMGWP62 (contains none of the cre-like sequences) showed almost the same levels of subtilisin activity regardless of the kinds of carbon sources added.

Based on these results, we concluded that CR control on the xynA gene expression in B. subtilis could be effectively removed by deleting the cre-2 element as shown in the case of the plasmid pMGWP61, while deletion of the cre-1 (pMGWP62) had little effect on the CR control.

**Measurement of the level of CR**

To analyze the levels of CR acting on expression of the xynA, subtilisin activity was measured in the B. subtilis strains carrying derivatives of pMGWP23 cultured in the presence or absence of glucose. As shown in Table

<p>| Table 2. Effects of Glucose on Expression of the Cloned xynA Gene in B. subtilis MW151a |
|----------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Plasmid in Strain MW15</th>
<th>Xylanase activity (units/ml)</th>
<th>Ratio (Xyn/Xyn+Glc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PWP18</td>
<td>0.061</td>
<td>1.02</td>
</tr>
<tr>
<td>PMGWGWP23</td>
<td>3.142</td>
<td>2.82</td>
</tr>
<tr>
<td>PMGWGWP11</td>
<td>0.063</td>
<td>1.00</td>
</tr>
</tbody>
</table>

1a Cells bearing each plasmid were grown in the 2×SG medium containing either 0.5% xylan or glucose, or both carbon sources at 37°C. The values shown are the averages of three independent experiments.

<p>| Table 3. Catabolite Repression of Subtilisin Synthesis by Various Sugars in B. subtilis MW15 Bearing pMGWP23 or Its Deletion Derivative1a |
|----------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Plasmid in strain MW15</th>
<th>Subtilisin activity level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1c</td>
<td>Fru</td>
</tr>
<tr>
<td>PWP18</td>
<td>2.4</td>
</tr>
<tr>
<td>PMGWGWP23</td>
<td>41.5</td>
</tr>
<tr>
<td>PMGWGWP11</td>
<td>32.1</td>
</tr>
<tr>
<td>PMGWGWP61</td>
<td>37.6</td>
</tr>
<tr>
<td>PMGWGWP62</td>
<td>38.9</td>
</tr>
</tbody>
</table>

1a Subtilisin activities obtained with various sugars are given as percentages of the value from the strain pMGWP23/MW15 grown in 2×SG medium with xylan.

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.

<p>| Table 4. Catabolite Repression of Subtilisin and xynA Transcripts Syntheses in B. subtilis MW15 Bearing pMGWP23 or Its Deletion Derivative1a |
|----------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Plasmid in strain MW15</th>
<th>Subtilisin activity (units × 103)</th>
<th>Ratio (Xyn/Glc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1c</td>
<td>Xyn</td>
<td>G1c</td>
</tr>
<tr>
<td>pWP18</td>
<td>4 ± 0.5</td>
<td>5 ± 0.4</td>
</tr>
<tr>
<td>pMGWP23</td>
<td>204 ± 14</td>
<td>85 ± 8</td>
</tr>
<tr>
<td>pMGWP11</td>
<td>144 ± 9</td>
<td>65 ± 3</td>
</tr>
<tr>
<td>pMGWP6</td>
<td>161 ± 8</td>
<td>78 ± 3</td>
</tr>
<tr>
<td>pMGWP61</td>
<td>83 ± 8</td>
<td>79 ± 8</td>
</tr>
<tr>
<td>pMGWP62</td>
<td>67 ± 6</td>
<td>63 ± 5</td>
</tr>
</tbody>
</table>

1a The values shown are the averages (± standard deviation) of three independent experiments.

1b Cells bearing each plasmid were grown with or without 1% glucose, and then subtilisin in the medium was assayed as described in Materials and Methods.

1c The integrated values (area) of densities for each blotting from multiple independent experiments were obtained using a chromatocan (CS-9000: Shimadzu, Kyoto). The xynA transcript levels presented are relative to that of strain MW15 containing pMGWP23, which was set at 100%.
4, the repression ratio was estimated to be about 2 for the strain carrying the plasmids that contained both cre-1 and cre-2 elements (pMGWP23, pMGWP11, and pMGWP6), while the Bacillus cells carrying the plasmids lacking cre-2 element (pMGWP61 and pMGWP62) revealed a repression ratio of around 1.

This indicates that the carbon catabolite repression was completely abolished in the strains harboring pMGWP61 or pMGWP62.

On the other hand, when the repression ratios were measured by the amounts of xynA transcript synthesized, the bacterial strains harboring pMGWP23, pMGWP11, or pMGWP6 gave repression ratios ranging from 2.9 to 3.3 when glucose was added to the cultures as a sole carbon source. In the levels of xynA transcript in the strains carrying pMGWP61 or pMGWP62, glucose addition did not cause any reduction in the levels of xynA transcript (Table 4).

**Discussion**

The results ofzymogram experiments done in our previous study showed that *B. stearothermophilus* No. 236 produced multiple xylanases including xylanase A, a member of the class G xylanases. In another work, we found that only xylan among the various carbon sources tested could fully induce xylanase synthesis, and glucose mediated about 70-fold repression of xylanase production in the *B. stearothermophilus* strain. In addition, the synthesis of β-xylanosidase and α-arabinofuranosidase were also assessed to be repressed about 40-fold and 2-fold, respectively, in the presence of glucose.

We also reported that the level of glucose repression of the xynA gene encoding β-xylanosidase was calculated to be about 10-fold by measurement of the relative amounts of xynA transcript synthesized in *B. stearothermophilus* No. 236. Moreover, in this work, carbon catabolite repression of the xynA gene at the level of transcription was analyzed to be about 50-fold in the presence of glucose.

From the results described above, we confirmed that carbon catabolite repression acting on synthesis of xylanolytic enzymes was a global regulatory mechanism functioning in *B. stearothermophilus* No. 236 even though the levels of repression could be different from genes to genes.

In this work, we recognized two potential cre sequences (cre-1: nucleotides +160 to +173 and cre-2: +173 to +186) within the coding region of the xynA gene of *B. stearothermophilus* No. 236, and suggested that only the cre-2 element might take part in the repression control of the xynA gene. Recently, Zalickas et al. reported that most 14-bp cre sequences were flanked by A+T-rich sequences and higher levels of CR occurred at cre sites bordered by A+T nucleotides than at the sites flanked by G+C rich sequences. In the case of the xynA gene, the cre-2 site was flanked by A+T rich sequence (TTAA and ATTA) but the cre-1 was bordered by ATGG and GGGC. This result supports the idea that the cre-2 site may be a true cre sequence of xynA gene.

Linder et al. reported that in *B. subtilis*, xylanase was synthesized constitutively while synthesis of β-xylanosidase was induced by xylose or xylan, and strongly repressed (about 100-fold) in the presence of glucose, and the glucose catabolite repression was completely abolished in the ccpA mutant of the *B. subtilis* strain. In contrast with the case of *B. subtilis*, the synthesis of both xylanase and β-xylanosidase in *B. stearothermophilus* No. 236 was induced by xylan, and their catabolic pathways were repressed by glucose. However, as seen in Table 3, the experiments done with *B. subtilis* MW15 carrying the xynA::aprA fusion plasmids showed that the cloned xynA gene did not require any specific carbon source for its induction while its expression was repressed about 2-3 fold by the presence of glucose. The level of repression shown in the cloned xynA gene expression is much lower than those for the same xynA gene expressed in *B. stearothermophilus* No. 236 (50-fold) and also for many other catabolite genes of *B. subtilis*.

The reason why CR in the xynA gene was underestimated in the *B. subtilis* MW15 strains may be partly due to the differences in the binding affinity of CcpA·HPγ complex to the cre elements in different species of microorganisms (compare data in Table 1 and those in Table 2).

**References**


