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Carbon Catabolite Repression (CCR) of Expression of the XylanaseA Gene of *Bacillus stearothermophilus* No. 236

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Abstract Previous work has identified that only the catabolite responsive element A (*cre*A; previously called cre-2) out of two potential *cre* sequences (cre-1: nucleotides +160 to +173 and cre-2: +173 to +186), recognized within the coding region of the xylanaseA gene (*xynA)* of *Bacillus stearothermophilus* No. 236, was actually involved in the carbon catabolite repression (CCR) of *xynA* expression in *B. subtilis*. However, the level of CCR of *xynA* expression was significantly lower than that for *xynA* expression in the original *B. stearothermophilus* No. 236 strain (70-fold repression). Therefore, to search for an additional *cre* element in the promoter region, the upstream region of the *xynA* gene was subcloned by chromosome walking, and as a result, another potential *cre* element (nucleotide -124~-137; designated *cre*B) was recognized in this region. The *cre*-like sequence revealed a high homology to the *cre* consensus sequence. The xylanase activity of *B. subtilis* MW15 bearing pWPBR14 (containing *cre*A and *cre*B) cultured in a medium containing xylose as the sole carbon source was about 7.7 times higher than that observed for the same culture containing glucose. *B. subtilis* MW15 bearing pWPBR23 (containing only *cre*A) produced an activity about 2.4 times higher. This pattern of CCR was confirmed using derivatives of *xynA::aprA* fusion plasmids. Furthermore, a measurement of the amounts of the *xynA* transcript showed a similar pattern as that for the production of xylanase. In addition, the synthesis of xylanase in *B. subtilis* QB7115 [a catabolite control protein A (*ccpA)* mutant strain] carrying pWPBR14 was almost completely relieved from glucose repression. Together, these results lead to a conclusion that the CCR of the expresssion of the *xynA* gene is mediated by CcpA binding at *cre*A and *cre*B sites in *B. subtilis*.

Key words: Carbon catabolite repression, xylanaseA, *Bacillus stearothermophilus*

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Xylan, the primary component of the cell walls of all land plants, consists of a backbone of β-1,4-linked D-xylopyranosyl residues and side chains of acetic acid, arabinose, and glucuronic acid or methylglucuronic acid [23]. The complete enzymatic degradation of xylan requires the cooperative action of several xylanolytic enzymes, including xylanase, β-xylosidase, α-arabinofuranosidase, acetyl xylan esterase, and α-glucuronidase.

The current authors previously isolated a strain of *Bacillus stearothermophilus* capable of synthesizing high levels of the xylanolytic enzymes described above, and also cloned genes encoding xylan-degrading enzymes from the genomic DNA of *B. stearothermophilus* No. 236 [3, 15, 19]. The *B. stearothermophilus* strain was also found to be subjected to carbon catabolite repression (CCR) [11] during the gene expression of xylanolytic enzymes to preferentially use energetically favorable carbon sources [1, 2]. As in cases of other Gram-positive bacteria with a low GC content in *B. stearothermophilus* No. 236, two proteins, the catabolite control protein A (CcpA) [9, 11, 21, 22] and the heat-stable protein (HPr) of the phosphoenol pyruvate-sugar phosphotransferase system (PTS) [4, 6, 16, 22], and a catabolite responsive element (*cre*) [5, 12, 24, 26] would appear to play a central role in CCR. In previous work, the xylanaseA gene (*xynA*) of the *B. stearothermophilus* strain was recognized to have two *cre*-like sequences within the coding region, yet only *cre*A (previously designated as *cre*-2; nucleotide +173 to +186) was confirmed to function as a *cis*-acting element in carbon repression control [2]. However, the level of repression of the *xynA* gene expression in a recombinant *Bacillus subtilis* strain (about 2.4-fold) was substantially lower than that observed for the same gene expession in *B. stearothermophilus* No. 236 (about 70-fold).

Therefore, to understand the precise mechanism for the CCR of the *xynA* gene of *B. stearothermophilus* No. 236 and elucidate the reason(s) for the underestimated repression levels in the *B. subtilis* strain, the 5' upsteam region of the

xynA gene was subcloned and the overall effect of the upstream sequence on the expression of the *xynA* gene was examined.

MATERIALS AND METHODS

Bacteria and Culture Media

B. stearothermophilus No. 236, a strong xylan degrader producing high levels of the xylanolytic enzymes, was isolated from soil [19]. The host strain used for the cloning experiments was *E. coli* DH5α. *B. subtilis* MW15 (*his nprR2 nprE18 ΔaprA3 ΔeglS102 ΔbglT bglSRV ΔxynA* CmR) and *B. subtilis* QB7115 (*trpC2 ccpA::spc amyE::(PBΔ levD-lacZ cat)*) [7] were used in the experiments related to the CCR of *xynA* gene expression.

B. stearothermophilus No. 236 was grown in a basal medium [10] supplemented with 0.75% xylose at 50°C on a rotary shaker (200 rpm). The *B. subtilis* MW15 and QB7115 strains carrying recombinant plasmids were grown in Schaeffer's medium (2× SG) [13] supplemented with carbon sources indicated in the text with 20 μg/ml kanamycin at 37°C. *E. coli* DH5α cells bearing recombinant plasmids were propagated in LB containing 100 μg/ml ampicillin at 37°C.

General Methods

The DNA manipulations and standard molecular biological methods used in this work were carried out as described by Sambrook *et al.* [18]. The transformation of *E. coli* DH5α and *B. subtilis* QB7115 was carried out by electroporation with a Gene Pulser (BioRad, Richmond, U.S.A.) and the transformation of *B. subtilis* MW15 was performed with competent cells prepared according to the procedure of Spizizen [20]. The genomic DNA of *B. stearothermophilus* was isolated by the method described previously [15].

Cloning of the Upstream Region of the *xynA* Gene of *B. stearothermophilus* No. 236

To subclone the upstream region of the *xynA* gene of *B. stearothermophilus* No. 236, the chromosomal DNA was digested with *Sph*I, and Southern blotting was performed by using a probe consisting of the 0.4 kb *Hin*dIII and *Pvu*II fragment between the *xynA* promoter and the coding region derived from the recombinant plasmid pMG15 [3]. A genomic DNA fragment of about 5.0 kb was responded when hybridized with the probe DNA. A genomic library was constructed with *E.coli* DH5α and pUC19 using the chromosomal fragments obtained as described above. The white colonies on an LB plate containing 100 μg/ml ampicillin, X-gal, and isopropyl β-D-thiogalactopyranoside (IPTG) were then screened by taking advantage of the ability of the recombinant carrying the *xynA* gene to form a clear zone on an agar plate containing 1.0% xylan. The recombinants selected were confirmed using the Southern hybridization technique.

Southern and Northern Blotting

The labeling and detection of the DNA probe were carried out according to the direction of the manufacturer using a Digoxigenin DNA Labeling and Detection kit (Boehringer GmbH, Mannheim, Germany). The DNA fragments on the 0.8% agarose gel were transferred to Hybond-N+ membranes (Amersham Pharmacia Biotech. Inc., Piscataway, U.S.A.). The reactions for the Southern blotting were done in a solution consisting of 5× SSC (0.75 M NaCl, 0.075 M sodium citrate), 1% blocking reagent, 0.1% N-lauroylsarcosin, and 0.02% SDS at 68°C for 12 h.

The total RNA of *B. subtilis* QB7115 grown to the exponential phase (OD_{600}: 0.8) in Schaeffer's medium supplemented with 0.5% glucose or xylose was prepared using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) and the RNA concentrations of each sample were determined spectrophotometrically. The RNA (10 μg) was separated in 1% agarose-formaldehyde gels, and transferred to Hybond-N+ membranes. The hybridization for the Northern blotting was carried out with a DIG-labeled DNA probe in a solution containing 5× SSC (0.75 M NaCl, 0.075 M sodium citrate), 50% formamide, 2% blocking reagent, 0.1% N-lauroylsarcosin, and 0.02% SDS at 68°C for 12 h. The hybridized probe was detected with an anti-DIG-alkaline phophatase conjugate and CDP-Star (Boehringer GmbH, Germany) as the substrate. The intensities of the hybridization signals were quantified using a Tina 2.10e (Raytest Isotopenmessgeraete GmbH, U.S.A.).

Nucleotide Sequencing and Analysis

The nucleotide sequencing was performed with an ABI PRISMTM Dye Terminator Cycle Sequencing kit using universal primers and analyzed by an ABI PRISM 310 automatic sequencer (Perkin Elmer Co., U.S.A.). A computer analysis of the DNA was done using the DNASIS (Hitachi Software Engineering Co., Japan) and PC/GENE (IntelliGenetics Inc.,U.S.A.) programs.

Construction of Plasmids Used in this Work

pWPBR14 was constructed by cloning the 1.4 kb DNA fragment from pHINC5.5 into pWPBR18. The DNA fragment was obtained by the double digestion of pHINC5.5 with *Sac*I and *Eco*RV and contained the *cre*-like elements A and B, plus the coding region of the *xynA* gene *of B. stearothermophilus* No. 236. pWPBR18 [2] was an *E. coli* and *B. subtilus* shuttle vector containing a promoter-less subtilisin gene (*aprA*) preceded by the pUC18 polylinker, as illustrated in Fig. 4. To subclone the DNA fragments around the *xynA* promoter region, four PCRs were done using pWPBR14 as the template and the following oligonucleotides as primers: creF1 (5'-CGCGGTACCCA-

AGTGAAATGTATGCGCTTACCTA-3'), creF2 (5'-GCAG-GTACCTTTTCTAGCAAAGGAGGTGATAGT-3'), creR1 (5'-ATAGGATCCATTCACCATCCCGCCGCCATCCGT-3'), and creR2 (5'-TATGGATCCACTGTAATTAGCCTCCG-GGCCCATT-3'). The forward and reverse primers described above contained the restriction sites for *Kpn*I and *Bam*HI, respectively. The *Bam*HI and *Kpn*I double-digested PCR products were ligated into pWPBR which had been cut with the same restriction enzymes. The resulting recombinant plasmids carrying the four distinct report genes were designated as pCR11WPBR18, pCR12WPBR18, pCR21WPBR18, and pCR22WPBR18 (Fig. 4).

Measurement of Xylanase and Subtilisin Activities

Birchwood xylan was suspended in phosphate buffer (50 mM, pH 7.0) at a concentration of 1% and used as the substrate for xylanase. The xylanase activity was measured by incubating 0.9 ml of the substrate suspension with 0.1 ml of the enzyme solution (the supernatant of the cultured broth) for 20 min at 55°C [3]. One unit of xylanase activity was defined as the amount of the enzyme which produced 1 μmole of xylose equivalents per min.

For the subtilisin assay, an aliquot of the culture (0.5 ml) was harvested when the OD_{600} of the culture reached to 0.6 and then centrifuged at 4°C for 5 min at 16,000 ×g. Thereafter, the subtilisin was assayed using the supernatant as the crude enzyme solution according to the method of Millet [14]. One unit of subtilisin activity was defined as the amount of enzyme that produced a soluble dye giving an A_{595} of 1 in 1 ml of the reaction mixture.

RESULTS

Subcloning of the Upsteam DNA of the *xynA* Gene of *B. stearothermophilus* No. 236

The level of the CCR in the *xynA* expression in the *B. subtilis* MW15 strain was measured as being substantially lower than that for the same *xynA* gene expressed in *B. stearothermophilus* No. 236 (70 times) [2]. Accordingly, it appeared that another *cre* may be involved in the expression of the *xynA* gene, probably located in the upsteam region of the *xynA* gene. Therefore, the upstream region of the *xynA* was subcloned to search for an additional *cis*-acting element (*cre*) by performing chromosome walking.

Indeed, a potential *cre* (designated *cre*B) was detected in the upstream region of the *xynA* gene (nucleotide -124~-137) (Fig. 1). As shown in Table 1, the *cre*B element revealed a high homology to the consensus of a *cre* element. As previously reported in all cases of *B. subtilus cre*s, the *cre*B had A+T rich flanking sequences [26]. Interestingly, however the *cre*B was located much further from the transcriptional start site than most other *cre*s of *B. subtilis*.

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AAG TTC GCA TAA ACA GAA GCA CAG AAG CAC GAC CTA AAG CAC TCA ATG GCT TGA AGG CAC      60

CTG AGC GGA GTT CAG GTG TCT TTT TTT TGC ATA TAA ACA TTT TGG GCG ATT TAT AAA TTT     120

CTA GAG TCG ACC ATC TTT TTT CTA AAA AAA ACA AGA TAA GAC CTT TAA TTT TTG AAG TGA     180
      HincII

AAT GTA TGC GCT TAC CTA CTA TAA TTT TCT AGC AAA GGA GGT GAT AGT AAA TAT GTT CCA     240
   creB (-137~-124)

AAA AGA GGT TCA AAG GTA GGT GAT GCC CCG GCA AAA CGA AGC TTG ACC ACG GCA AGA AGC     300
                                                    -35

AGG GAG CAA GAA TCA TTT TGG GTA GTC AAA TCT ATT TCT GAG GAG GAA CAT TTG ATG AAG     360
         -10            +1                              SD              M

TTA AAG AAG AAG ATG CTT ACT CTA CTC CTG ACG GCT TCG ATG AGT TTC GGT TTA TTT GGG     420

GCA ACC TCA AGT GCA GCA ACG GAT TAT TGG CAA TAT TGG ACG GAT GGC GGC GGG ATG GTG     480

AAT GCG GTT AAT GGG CCC GGA GGC AAT TAC AGT GTT ACC TGG CAA AAT ACC GGG AAC TTC     540
               creA (+173~+186)

GTG GTC GGC AAA GGC TGG ACG GTT GGA TCG CCG AAT CGG GTG ATC AAC TAC AAT GCG GGC     600
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Fig. 1. Nucleotide sequence of the *xynA* gene and the putative *cre* sequence.
The promoter region (-10 and -35 regions), Shine-Dalgarno sequence, and translational initiation codon are underlined. The transcriptional initiation nucleotide is assigned as +1. The location of each *cre* is given as a position relative to the transcriptional start site.

Table 1. Sequence alignment and positions of established carbon repression elements[a].

Gene or operon	DNA sequence[b]	Position (bp)[c]
Lev	TAACAA TGAAAACGCTTAAC ACAACT	-45.5
Gnt (cre_{up})	TAGAAA TGAAAGTGTTTGCA TAAAAG	-37.5
BglPH	CAAAAA TGAAAGCGTTGACA TCTCAC	-36.5
Acu	CATTGT TGAAAACGCTTTAT AATTTG	-26.5
amyE	TTTAAA TGTAAGCGTTAACA AAATTC	+4.5
mmg	AGAAAT TGTAAGCGCTGTCT ATCTTC	+21.5
acsA	TGAACT TGAAAGCGTTACCA GCAATA	+44.5
xyl	CTATTT TGGAAGCGTAAACA AAGTGG	+140.5
gnt (cre_{down})	TCTGAT TGAAAGCGGTACCA TTTTAT	+147.5
hut	CGCAAT TGAAACCGCTTCCA AAAAGA	+209.5
xynA (*creA*)	GGTTAA TGGGCCCGGAGGCA ATTACA	**+179.5**
xynA (*creB*)	GTGAAA TGTATGCGCTTACC TACTAT	**-130.5**
Consensus	WWWW TGWAARCGYTWNCW WWWW	

[a]The *cre* of this table, except for the *cre* of *xynA*, refers to Zalieckas *et al.* [26].

[b]Symbols for ambiguous nucleotides in the consensus sequence are as follows: W represents A or T; R represents A or G; Y represents C or T; and N represents A, C, G, or T.

[c]The location of each *cre* is given as a center position relative to the transcriptional start site.

CCR of *xynA* Gene Expression in *B. subtilis* MW15

To investigate whether the *cre*B sequence actually functions as a *cis*-acting element in the CCR of XynA synthesis, a recombinant plasmid pWPBR14 was constructed, as described in Materials and Methods, and *B. subtilis* MW15 (a mutant strain deficient in alkaline protease and xylanase) was then transformed with pWPBR14. The *B. subtilis* MW15/pWPBR14 strain was grown in a 2× SG medium supplemented with the carbon sources as indicated in Fig. 2, and the extracellular xylanase activities were determined after culturing for 12 h. The xylanase activity from the culture of *B. subtilis* MW15/pWPBR14 in the presence of xylose as the sole carbon source was about 7.7-fold higher than that from the culture containing glucose (Fig. 2). The CCR ratio estimated in *B. subtilis* MW15 bearing pWPBR23 (containing only *cre*A) was only about 2.4-fold. These results confirm that the *cre*B sequence is another functional *cis*-acting element in the CCR of the *xynA* gene expression.

Next, to explore the effect of glucose on the synthesis of the *xynA* transcript, the total RNA from the *B. subtilis* MW15 cells bearing the relevant recombinant plasmids was isolated as described above, and the amounts of the *xynA* transcript were determined by Northern blotting (Fig. 3A). In the presence of glucose, the transcription of the *xynA* gene was inhibited in both of the recombinant *B. subtilis* MW15 strains carrying pWPBR14 and pWPBR23, although *B. subtilis* MW15/pWPBR14 showed a significantly higher level of repression (Fig. 3B). In contrast, in the medium containing xylose as the carbon source, the two recombinant strains produced almost the same large amounts of the *xynA* transcript (Fig. 3A, Lanes 3 and 6).

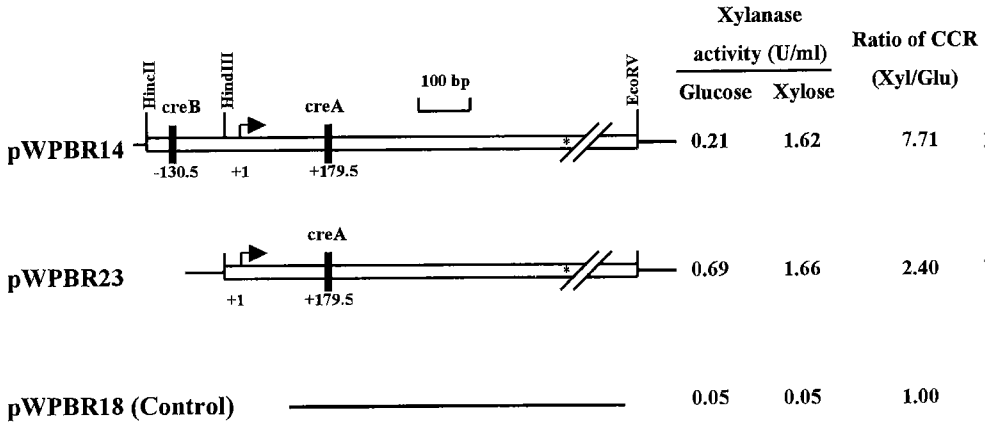

Fig. 2. Carbon catabolite repression of the xylanaseA gene in *B. subtilis* MW15.

Cells bearing each plasmid were grown in a 2× SG medium containing either 0.5% glucose or xylose at 37°C. pWPBR18, an *E. coli* and *B. subtilis* shuttle vector, was constructed as described previously [6]. pWPBR14 contains *cre*A and *cre*B, and pWPBR23 contains only *cre*A. The translational termination codon of the *xynA* gene is indicated by an asterisk (*). The box represents the insert DNA, and the line is for the pWPBR18 vector DNA. The xylanase activity was measured as described in Materials and Methods. The data shown is the mean values of duplicate experimental results.

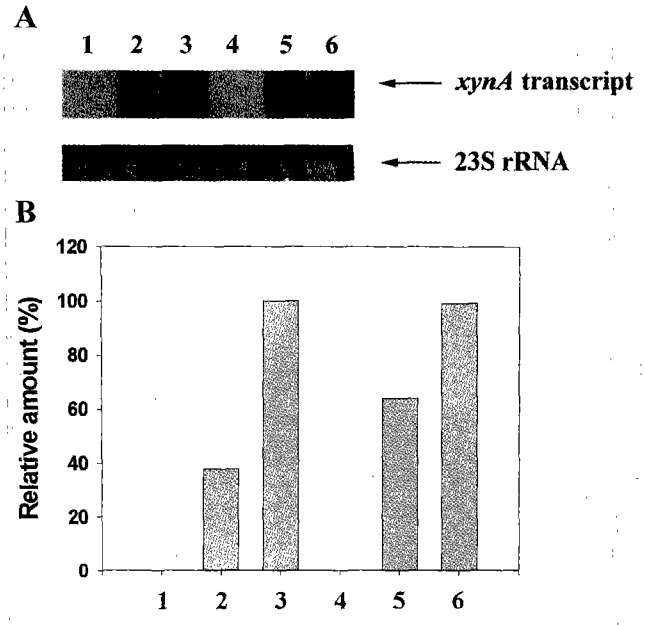

Fig. 3. Nothern blotting for CCR of the *xynA* gene at the level of transcription.

The total RNA of *B. subtilis* MW15 was prepared from cells grown to the exponential phase (OD_{600}: 0.8) in Shaeffer's medium supplemented with 0.5% glucose or xylose, and 10 μg of the RNA sample was loaded onto a 1% agarose-formaldehyde gel. Nothern blotting was carried out as described in Materials and Methods. (A) Lane 1, Total RNA prepared from *B. subtilis* MW15/pWPBR18 grown in the presence of glucose; Lane 2, *B. subtilis* MW15/pWPBR14 grown in glucose; Lane 3, *B. subtilis* MW15/pWPBR14 grown in xylose; Lane 4, *B. subtilis* MW15/pWPBR18 grown in xylose; Lane 5, *B. subtilis* MW15/pWPBR23 grown in glucose; Lane 6, *B. subtilis* MW15/pWPBR23 grown in xylose. (B) Quantative analysis of the Nothern blot.

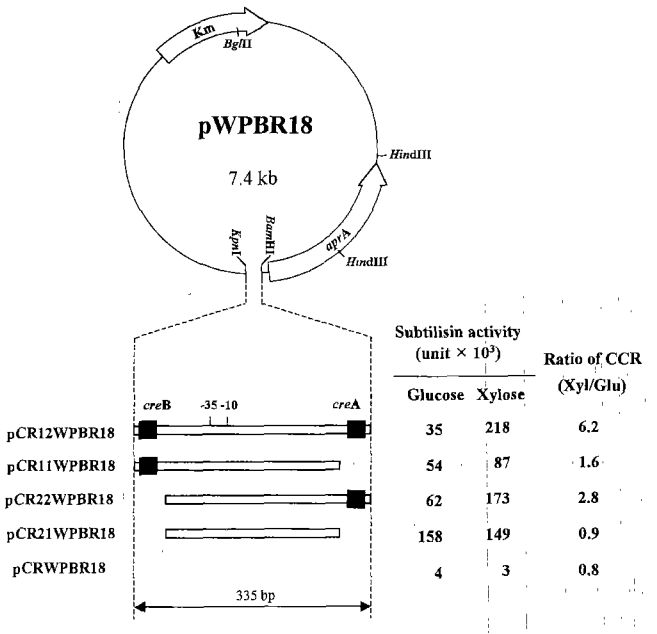

Fig. 4. Carbon catabolite repression of the subtilisin gene of *B. subtilis* MW15.

The plasmid pWPBR18 contains a promoterless subtilisin gene (*aprA*). Km denotes the gene encoded for kanamycin nucleotidyltransferase. pCR12WPBR18 contains *cre*A and *cre*B; pCR11WPBR18 contains only *cre*B; pCR22WPBR18 contains only *cre*A; pCR21WPBR18 contains only the *xynA* core promoter. The subtilisin activity was measured as described in Materials and Methods. The data shown is the mean values of duplicate experimental results.

The results from the Northern blot experiments were consistant with the CCR ratios assessed by measuring the xylanase activity produced by the same recombinant *B. subtilis* MW15 strains (Fig. 2). Together, these data indicate that the *cre*-like sequences *cre*A and *cre*B are both involved in the catabolite repression of *xynA* expression at the transcription level.

In addition, to define more precisely the effects of the two *cre* elements on the CCR of *xynA* expression, a series of pWPBR18 derivatives carrying different *xynA::aprA* fusions in respect to the *cre* elements was constructed as described in Material and Methods (Fig. 4). The *B. subtilis* strain harboring pCR21WPBR18 (containing no *cre* element) showed nearly the same level of subtilisin activity regardless of the carbon sources added to the culture medium. In contrast, the synthesis of subtilisin in *B. subtilis* MW15/pCR12WPBR18 (containing both *cre* elements) was severely repressed in the presence of glucose, as was in the case of xylanase production by *B. subtilis* MW15/pWPBR14. Again, based on the results of the experiments using the *xynA::aprA* fusion genes, it was confirmed that the two *cre* elements, *cre*A and *cre*B, function as *cis*-acting elements in the CCR of *xynA* expression, with *cre*A showing a slightly higher activity.

CCR of XynA Synthesis in *B. subtilis* QB7115

The mediation of CCR at *cre* sites in *B. subtilis* has been abolished to be relieved when the *ccpA* gene encoding the catabolite control repressor is inactivated [9, 26]. Therefore, it was examined whether CCR in XynA synthesis was abolished in *B. subtilis* QB7115 with a defect in the *ccpA* gene. However, the *B. subtilis* QB7115 strain was found to be highly intractable for transformation. No transformants were obtained using the normal procedures reported for the transformation of *B. subtilis*. Accordingly, an attempt was made to transfer pWPBR14 into the mutant strain using

Table 2. Carbon catabolite repression of the xylanaseA gene in the *ccpA* mutant *B. subtilis* QB7115[a].

Plasmid in Strain QB7115	Xylanase activity (U/ml)		Ratio of CCR (Xyl/Glu)
	Glucose	Xylose	
pWPBR18	0.39±0.10	0.43±0.12	1.10
pWPBR14	1.02±0.33	1.55±0.48	1.52

[a]Cells bearing each plasmid were grown in a 2× SG medium containing either 0.5% glucose or xylose at 37°C. The values shown are the averages ± the standard deviation of triplicate samples.

the electroporation technique described by Ha *et al.* [10] with some modifications. Nevertheless, transformants were obtained with a very low efficiency. As expected, the xylanase expression was almost completely relieved from glucose repression (Table 2). This result confirms that *xynA* expresssion is mediated by the CcpA binding at the *cre*A and *cre*B sites.

DISCUSSION

In our previous work, two potential catabolite responsive elements (*cre*-1: nucleotides +160 to +173 and *cre*-2: +173 to +186) were recognized within the coding region of the *xynA* gene of *B. stearothermophilus* No. 236, yet only *cre*A (previously called *cre*-2) was proved to function as a *cis*-acting element in the CCR of *xynA* expression [2]. However, the level of the CCR ratio of *xynA* expression in the recombinant *Bacillus subtilis* MW15/pWPBR23 was estimated to be about 2.4, which was substantially lower than that observed for *xynA* expression in the original *B. stearothermophilus* strain (70-fold repression). Accordingly, to search for an additional *cre* element within the upsteam region of the *xynA* gene, a 2.5-kb DNA fragment upstream of the *xynA* core promoter was subcloned, and another potential *cre* element (named *cre*B) flanked by A+T rich sequences which was centered at position -130.5 was identified (Fig. 1). Recently, Zalieckas *et al.* reported that the most 14-bp *cre* sequences are flanked by A+T rich sequences, and higher levels of CCR are observed at *cre* sites bordered by A+T nucleotides than at sites flanked by G+C rich sequences.

The level of CCR in the expression of the *xynA* gene was estimated to be about 7.7 for the *B. subtilis* MW15/pWPBR14 strain containing both *cre* elements. Nevertheless, this value is still significantly lower than that shown in the original *B. stearothermophilus* No. 236 strain. In Gram-positive bacteria with a low-GC content represented by *B. subtilis*, two proteins play a central role in CCR, namely the catabolite control protein CcpA and HPr of the phosphoenol-pyruvate-sugar phosphotransferase system [11]. In particular, HPr is also known to inhibit the uptake of alternative carbon sources (inducer exclusion) and efflux of free sugars (inducer expulsion) [15, 17], as well as mediating CCR by forming a CcpA-HPr repressor complex. Therefore, the functional difference in the inducer exclusion and/or expulsion between the two *Bacillus* spp. could be the cause for the low level of CCR observed in the *B. subtilis* MW15 strain. Also, the substantial difference in the CCR levels in the *xynA* expression between the two *Bacillus* spp. may be partly explained by a difference in the binding affinity of the CcpA-HPr repressor complexes in the two strains. Gosseringer *et al.* [8] reported that the expression of the xylose utilization operon of *B. megaterium* is subject to CCR mediated by CcpA, and HPr-Ser-P promotes the non-cooperative binding of CcpA to *cre* sites, while Glc-6-P triggers the cooperative binding of CcpA to a downstream *xyl cre* and auxiliary *cre* site located within the *xyl* promoter region. In addition, they suggested a DNA looping mechanism in the cooperative binding event of CcpA to *cre*s by Glc-6-P, and that this mechanism may be a feasible explanation of CCR mediated by two distantly located *cre* sites. The large distance (300 bp) between the *cre*A and *cre*B located in the *xynA* gene of *B. stearothermophilus* No. 236 could also be explained by a DNA looping mechanism with cooperative CcpA binding to the *cre* sites. Moreover, this cooperativity of CcpA binding could explain the synergistic repression effect revealed by the *cre*A and *cre*B elements in *xynA* expression in the recombinant *B. subtilis* MW15/pWPBR14 strain (Fig. 4).

In conclusion, the fact that the CCR of *xynA* expression is mediated by the CcpA binding at the *cre*A and *cre*B sites was confirmed by measuring the relative xylanase activity in the presence and absence of glucose (Fig. 2), and the amounts of *xynA* transcript synthesized (Fig. 3) along with the relief of the CCR of the *xynA* expression in the *ccpA* mutant strain (Table 2). Moreover, the *ccpA* gene from the genome of *B. stearothermophilus* No. 236 was recently cloned and its characterization is now in progress.

Acknowledgments

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