Transcriptional Regulation of the Bacillus ohbensis Cyclodextrin Glucanotransferase Gene in B. subtilis

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The expression of the cyclodextrin glucanotransferase (CGTase) gene (cgt) of Bacillus ohbensis, when introduced into an α-amylose-defective strain of B. subtilis on a multicopy plasmid, pH300PLK, was induced in the presence of starch and was subject to catabolite repression by glucose as well as in the original strain, B. ohbensis. We constructed a cgt::lacZ translational fusion to study the expression in B. subtilis, and this construct was confirmed to be subject to both starch induction and catabolite repression. In order to define the region involved in the regulation of the cgt gene, a series of cgt::lacZ gene with various lengths of deletion in the promoter region was constructed on pH300PLK. DNA regions responsible for starch induction and catabolite repression were found to be located in the deletion experiment. Primer extension analysis showed that the catabolite repression gene was controlled at the initiation of transcription, while the starch induction is likely to be controlled by a transcriptional termination-antitermination mechanism.

Key words: cyclodextrin glucanotransferase; gene regulation; starch induction; catabolite repression; Bacillus subtilis

In several microorganisms belonging to eubacteria, Archea, and fungi, synthesis of amylolytic enzymes that catalyze hydrolysis of α-1,4-glucosidic bonds, such as α-amylase, glucoamylase, and α-glucosidase, is often subject to starch induction and glucose repression. It is known that the features of starch induction are similar in these organisms, the molecular basis of these regulations is not known in detail except that of Taka-amyolase A of Aspergillus oryzae. In our previous study on the synthesis of cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) of an alkalophilic bacterium, Bacillus ohbensis, we found that the starch induction and the catabolite repression of the CGTase synthesis are regulated at the posttranscriptional and transcriptional level, respectively. The gene expression controlled at a posttranscriptional level should be a unique case for an extracellular enzyme.

To understand the regulation mechanisms involved in starch induction and catabolite repression, genetic studies are indispensable. Unfortunately, however, there is no report so far of transformation of B. ohbensis and the only transformation system reported for an alkalophilic Bacillus, that of sp. C-125, proved not applicable to B. ohbensis. We also tried in vain to apply to B. ohbensis the protoplast transformation and electroporation methods used for B. subtilis. Therefore, it is currently not possible either to analyze genetically the expression of the cgt gene in B. ohbensis with a reporter gene or to identify the genes encoding regulatory factors.

An alternative way to examine the regulation of the cloned cgt gene-expression is to use another species of the genus Bacillus as a host, e.g., B. subtilis, in which a transformation system was established, if the expression of the cgt gene is apparently regulated in the same way. Previously, we reported the production of the CGTase using the expression and secretion system of B. subtilis, where the original B. ohbensis cgt promoter was replaced with that of the B. subtilis cellulase gene. In this study, we examined the expression of the cgt gene with its own promoter region within B. subtilis and confirmed both starch induction and catabolite repression. But the expression analysis with a cgt::lacZ fusion gene suggested that starch induction in B. subtilis is controlled differently from that in B. ohbensis, which should lead us to notice a novel gene regulation system of B. subtilis.

Materials and Methods

Bacterial strains and growth conditions. B. subtilis strain RM125 arg-15 leuB8 hsrM hsmM was transformed with chromosomal DNA of 207-21 m8 hsrM amyE07 aroI906 metB5 leuB8 lys2, and an amylase-defective transformant was selected by screening for the halo-negative phenotype on Tryptose Blood Agar Base (Difco) plates containing 1% corn starch. The strain thus selected was designated RM125A1 arg-15 leuB8 amyE07 hsrM hsmM, and used as a host for the expression of the cgt gene. Escherichia coli K12 JM109 recA endA1 gyrA96 thi hsdR17 supE44 relA1 Δ (lac-proAB) (F’ traD36 proA+ lacIq lacZD15r) was used for plasmid constructions. E. coli cells were grown at 37°C in L medium (1% tryptone, 0.5% NaCl, 0.5% yeast extract, pH 7.0), with ampicillin at 75 μg/ml when needed. B. subtilis transformants were grown at 37°C in L medium, LS medium (L medium plus 1% soluble starch) and LSG medium (L medium plus 1% soluble starch and 1% glucose) with tetracycline at 12.5 μg/ml.

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Transformation of *E. coli* and protoplasts of *B. subtilis* was done as described by Sambrook *et al.* and by Chang and Cohen, respectively.

**Plasmid construction.** Plasmids pUC118 and pHY300PLK were used as the cloning vectors for *E. coli* and *B. subtilis*, respectively. Plasmid pUC19CGT is a pUC19-derivative carrying a 4.7-kb SacI-EcoRI fragment containing the *cgt* gene. Plasmid pMC1871 was used as the source of the *E. coli lacZ* gene-cartridge to construct the *cgt*::*lacZ* fusion. To introduce the *cgt* gene into *B. subtilis*, the 2.7-kb *ApaI*-SphI fragment, containing the *cgt* open reading frame with 451 bp and 160 bp of the 5'- and 3'-untranslated regions, respectively, was isolated from pUC19CGT. The *ApaI* and *SphI* ends were converted to *BglII* and *BamHI* sites with linkers, respectively, and ligated into *BglII*-*BamHI* sites of pHY300PLK, giving rise to plasmid pHC300S.

For the construction of the *cgt*::*lacZ* translational fusion, pUC19CGT was digested with *SphI*, which has a cleavage site in the middle of the coding region of the *cgt* gene, and treated with BAL31 nuclease and the Klenow fragment (Takara Shuzo Co., Ltd., Japan). The resultant DNA was digested with *SacI*, and 1.0-1.2 kb fragments containing the 1.1-kb 5'-upstream region and various lengths of the NH2-terminal coding region of *cgt* were isolated by agarose gel electrophoresis and ligated into the *SacI*-*SmaI* sites of pUC118. Of the plasmids thus obtained, one with a deletion up to the eighth NH2-terminal codon was selected by DNA sequencing. The *BamHI*-*lacZ* cartridge from pMC1871 was then inserted into the *BamHI* site of this plasmid. To introduce the *cgt*::*lacZ* fusion gene into *B. subtilis*, the 3.4-kb *VspI*-*XbaI* fragment containing the whole fusion with the 353-bp 5'-untranslated region of the *cgt* gene was inserted into the *XbaI* site of pHY300PLK, after converting the *VspI* site to an *XbaI* site with Klenow fragment and an *XbaI* linker. The resultant plasmid was designated pC1101A.

For the deletion of the 5'-upstream region of the *cgt*::*lacZ* fusion, the double-strand nested-deletion method was done with plasmid pC1101A, using *SmaI* and *SalI* sites of the vector located upstream of the *cgt*::*lacZ* gene, after converting the *SmaI* site to a *KpnI* site with a *KpnI* linker. The deletions in the 5'-upstream region were confirmed by DNA sequencing.

**Primer extension analysis.** Total RNA was isolated from *B. subtilis* cells containing pC1101A grown in L, LS or LSG medium by the hot-phenol extraction method as previously described, and used for the primer extension experiments. The first primer extension experiment (see Fig. 3) was done using a 17-mer oligonucleotide (5'-GTTCGTGGGGGTTGATCTGGTAC-3'; primer A), which is complementary to the DNA sequence within the *lacZ* coding region, from position +209 to +225 with respect to the transcriptional start site of the *cgt*::*lacZ* fusion. The second primer extension experiment (Fig. 5) was done with two oligonucleotides, 5'-AGGATGCGGCGTGGTATGGAG-3' (primer B) and 5'-AACACCCCTTCCTATCCAATA-3' (primer C), complementary to the DNA sequences from position +23 to +41 and +97 to +116, respectively, with respect to the transcriptional start site of the *cgt*::*lacZ* fusion. These oligonucleotides were synthesized with Applied Biosystems model 392 DNA synthesizer, and 5'-end labeled with [γ-32P]ATP (110 TBoq/mmole; DuPont) and T4 polynucleotide kinase (Takara Shuzo Co., Ltd., Japan). Fifty μg of total RNA was annealed with the primers and used for primer extension reaction with avian myeloblastosis virus reverse transcriptase. Primer extension products were resolved on an 8% polyacrylamide-8 M urea sequencing gel.

**Enzyme assays.** The CGTase activity in the culture supernatant was measured as starch-degrading activity by the blue value method. The β-galactosidase assay was done by the method of Ferrari *et al.*, and the specific activity was expressed in Miller units. Protein contents were measured by Bradford method with bovine serum albumin as the standard.

**Results**

**Regulation of the cgt expression in B. subtilis**

In our previous attempt to introduce the *cgt* gene into *B. subtilis*, we used a pUB110-derived plasmid containing the whole *cgt* gene and more than 1 kb of extra fragments in both the 5' and 3' regions. However, it was unsuccessful because each transformant had a plasmid with a large deletion reaching the *cgt*-coding region. We suspected that the long extra regions might somehow cause this deletion, and eliminated most of the extra

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**Fig. 1.** Course of Extracellular CGTase Production in *B. subtilis* Carrying Plasmid pHC300S.

*B. subtilis* RM125A1 harboring pHC300S was grown in L (squares), LS (circles), and LSG medium (triangles). The open and closed symbols show cell growth, monitored by measuring the optical density at 660 nm, and the CGTase activity in the culture supernatant, respectively.
regions; the 2.7-kb *Apa*LI-SpI fragment was recloned into a multicopy plasmid, pHY300PLK, giving rise to plasmid pHC300S. This plasmid could be successfully introduced and stably maintained in a *B. subtilis* strain.

When *B. subtilis* RM125A1, an α-amylase defective strain, harboring pHC300S was cultured in L medium, we detected a low but significant CGTase activity in the culture supernatant after the exponential phase of growth (Fig. 1). The addition of 1% starch, referred to as LS medium, increased the production of the enzyme about four-fold. On the contrary, the addition of 1% glucose to LS medium completely repressed the CGTase production. These results indicate that the expression of the *cgt* gene in *B. subtilis* is induced by starch and subject to catabolite repression by glucose. This pattern of the CGTase production in *B. subtilis* closely resembled that in *B. ohbensis*, except a relatively high CGTase activity in *B. subtilis* in the absence of starch (L medium).

**Regulation of cgt ′::lacZ expression in B. subtilis**

To examine the regulation of the *cgt* expression in *B. subtilis* in more detail, a translational *cgt* ′::lacZ fusion was constructed on the multicopy plasmid. To avoid a possible negative effect of the signal peptide encoded by the *cgt* gene on the expression of the *cgt* ′::lacZ fusion, most of the signal sequence was removed, as described in Materials and Methods.

*B. subtilis* RM125A1 was transformed with the plasmid pCL101A containing the *cgt* ′::lacZ fusion and the β-galactosidase activity was examined (Fig. 2). During exponential growth, the β-galactosidase activity was very low, irrespective of the culture conditions. After the end of exponential growth, the β-galactosidase activity began to increase in both L medium and LS medium. The activity in LS medium further continued to increase and reaching three times as high as that in L medium at the end of the cultivation. In contrast, the activity remained almost undetectable in the presence of both starch and glucose. A similar pattern of regulation, *i.e.*, starch induction and catabolite repression, was also observed when a transcriptional *cgt-lacZ* fusion gene was used, in which the *E. coli lacZ* gene was connected out of frame with the *cgt* gene at the NH2-terminal portion (data not shown). This result clearly shows that the expression of the *cgt* ′::lacZ gene in *B. subtilis* was stimulated by starch, and repressed by glucose, possibly at the transcriptional level.

**Mapping of the cgt promoter in B. subtilis**

The 5'-end of the *cgt* mRNA in *B. ohbensis* was previously identified as a nucleotide 162 bp upstream of the transcriptional start site,[13] and the promoter sequence was similar to the consensus sequence for *B. subtilis* σ70 RNA polymerase.[30] To find whether this promoter is also functional in *B. subtilis*, we did a primer extension analy-
sis. Total RNA was isolated from *B. subtilis* RM125A1 (pCL101A) cultured in L, LS, or LSG medium at the exponential phase of growth (4 h) and the stationary phase of growth (8 h). The RNA was annealed with primer A and the complementary strand was extended with avian myeloblastosis virus reverse transcriptase. A single transcriptional start site was identified as the A at 162-bp upstream of the translational start site (Fig. 3). This shows that the original promoter of the cgt gene is also functional in *B. subtilis*. The amount of primer extension products from the culture grown in LS medium was much higher than that from the culture grown in L medium. We could not detect any primer extension products with RNA from the cells cultured in LSG medium. These results suggest that both starch induction and catabolite repression by glucose are regulated at the transcriptional level.

**Deletion analysis of the 5′ upstream region of the cgt promoter**

In order to identify any promoter regions responsible for the regulation of the cgt expression, including starch induction and catabolite repression, we constructed a series of deletions in the 5′-upstream region of the cgt::lacZ fusion on plasmid pHY300PLK. *B. subtilis* RM125A1 harboring those plasmids was cultivated in L, LS, and LSG medium for 12 h, and the β-galactosidase activities were compared (Fig. 4).

The strain harboring pCL101A containing up to −189 bp from the transcriptional start site showed
Fig. 5. Primer Extension Analysis of cgt'::lacZ Transcription with the Upstream and Downstream Primers of the Inverted Repeat Structure.

Total RNA extracted from B. subtilis harboring pCL101A grown under the conditions shown for four, eight, or 12 h was used for the reverse transcriptase reaction with Primer B (A) or Primer C (B). A, G, C and T indicate the products of sequencing reactions with the same primers. In (C), the nucleotide and deduced amino acid sequences of the 5' region of the cgt'::lacZ gene are shown. The nucleotide and amino acid sequences of lacZ gene from pMC1871 are shown in Italicics. The putative promoter elements (−35 and −10) and ribosome binding site (S D) are overlined. The transcriptional start site is shown by a thick arrow. The sequences complementary to the primers used in the reverse transcriptase reactions are shown by thick horizontal arrows. The two sets of facing arrows with solid and dotted lines show the palindromic sequences which could give rise to a transcriptional terminator and an antiterminator, respectively (see Discussion).

about four-fold more β-galactosidase activity in LS medium than in L medium, and the β-galactosidase activity in LSG medium was about half of that in L medium, showing the presence of starch induction and catabolite repression. Deletion of bp −189 to −156 resulted in almost the same activities in the three media as those of pCL101A, except a relatively high β-galactosidase activity in LS medium. A marked reduction of the cgt'::lacZ expression in all the three media was observed by further deletion from −155 bp to −104 bp, suggesting that a regulatory element for the high level expression of cgt' gene was located in this region. Strains harboring pCL86D and pCL77D showed almost the same activities as that harboring pCL103D. However, a deletion down to −52 bp caused a further reduced cgt'::lacZ expression in the three media.

Comparison of the activities in LS medium with those in L medium showed the presence of starch induction in all the constructs, although the level of induction was reduced according to the successive deletions. Therefore, we assumed that an element responsible for the starch induction might be downstream from the promoter. On the other hand, by comparison of the activities in LSG medium with those in L medium, we could not detect any response to glucose when the deletions went further than −103 bp. In a separate experiment, when cultured in L medium plus 1% glucose, the strains harboring pCL101A, and pCL155D showed reduced β-galactosidase activities compared with those in L medium, however, the strains harboring pCL103D, pCL86D, pCL77D, and pCL51D showed no response to glucose (data not shown). Therefore we concluded that an element responsible for catabolite repression is between −155 and −103 bp.
Functional analysis of the inverted repeat sequence in the 5' non-coding region

The results described above suggested that a regulatory region for starch induction is downstream of the promoter. In the 5' non-coding sequence, we found an inverted repeat structure followed by a short T-tract (Fig. 5C), and this structure was supposed to function as a transcriptional terminator. To investigate this, we isolated total RNA from *B. subtilis* RM125A1 harboring pCIL101A cultured in L, LS, and LSG medium, and analyzed the amount of cgt'::lacZ mRNA by primer extension using two primers, B and C, which are complementary to the upstream and downstream region of the possible stem-loop structure, respectively. As shown in Fig. 5A, the amount of primer extension product with Primer B in the absence of starch was almost equal to, or more than that in the presence of starch. On the other hand, four-fold more products of primer extension with Primer C were observed in the presence of starch than in its absence at the point of 12 h (Fig. 5B). These observations indicate that starch induction is not regulated at the initiation of transcription, and that at least in L medium without starch, termination of transcription has occurred at a position between Primers B and C. The result with Primer C was in good agreement with that observed in Fig. 3, and also that of β-galactosidase assays (Fig. 2). These results strongly suggest that the inverted repeat structure contributes to the starch induction, possibly by a transcriptional termination-antitermination mechanism.

Discussion

We have shown that the production of *B. ohbensis* CGTase in *B. subtilis* is regulated with the same pattern as in *B. ohbensis*. The production was induced by starch, and repressed by glucose in both the organisms, but the starch induction was regulated by a quite different mechanism. In *B. subtilis*, when the inducer molecule, starch, was absent, most of the transcription seemed to be terminated at the inverted repeat structure located in front of the cgt open reading frame. In its presence, the transcription seemed to continue through the inverted repeat structure to produce the full-length mRNA. On the other hand, in *B. ohbensis*, we detected almost the same amount of the full-length mRNA irrespective of the presence or the absence of starch, however, the CGTase protein in the cultural broth was scarce when starch was not present. Therefore we concluded that the starch induction is controlled by a transcriptional termination-antitermination in *B. subtilis*, but at a posttranscriptional level in *B. ohbensis*. The physiological or mechanistic reason for this discrepancy is not known. Also, the inverted repeat structure, which is probably the key regulatory element in *B. subtilis*, seems to have no function in *B. ohbensis*, at least as a transcriptional terminator.

Contrary to the starch induction, the catabolite repression of cgt expression seemed to occur at the initiation of transcription in both organisms. From the results in Fig. 4, the region between −155 and −103 bp relative to the transcriptional start site is obviously important for the repression. As we noted in our previous paper, there are two sequences similar to the catabolite responsive element (CRE) proposed by Hueck et al., at −91 to −78 bp and +184 to +197 bp, relative to the transcriptional start site, of which the latter one was deleted through the construction of the cgt'::lacZ fusion. The CRE element located upstream of a promoter region was reported to act as a catabolite activator element, and the strain harboring pCIL103D did not respond to glucose, positively or negatively, in spite of the presence of the upstream CRE-like element. Therefore it is not likely that the upstream CRE-like element in the cgt gene functions as a cis-acting regulatory element for catabolite response, especially for catabolite repression, mediated by CcpA and Hpr proteins. Further investigation is needed to clarify this.

The deletion analysis revealed the presence of a region responsible for a high level expression of the cgt gene in the three media, at bp −155 to −103. This region may contain an element for binding a transcriptional regulator. It should be noted that there is no similar sequence in this region to the consensus sequence for the DegU binding, which is known to activate the transcription of the genes for many extracellular enzymes in *B. subtilis*.

From the results in Fig. 5, starch induction of the cgt expression in *B. subtilis* is obviously regulated by a transcriptional termination-antitermination mechanism. Such a regulation is observed in several catabolic operons in *B. subtilis*, such as the sac, lic, and bgl operons, the gpl regulon and the hui operon, and also in other bacteria. In some of the cases, such as the *E. coli* bgl operon and the *B. subtilis* sac regulon, an alternative inverted repeat structure, an antiterminator, can be directed from a region partially overlapping the inverted repeat structure of the terminator. In the case of the cgt gene, an alternative, rather unstable inverted repeat was also found in the appropriate position (facing arrows with dotted lines in Fig. 5C), which may act as an antiterminator.

In each regulon controlled by the transcriptional termination-antitermination mechanism, a specific antiterminator protein was encoded by the same regulon. It is not likely that the cloned fragment in pCIL101A encodes an antiterminator protein besides containing a promoter for the cgt gene, because the length of the fragment is only 377 bp, which is too short to encode an antiterminator protein. This suggests that an endogenous protein from the *B. subtilis* host functions as an antiterminator protein for starch induction of cgt gene.

What is the nature of the inducer molecule for this regulation? As the host strain used for the cgt'::lacZ expression, RM125A1, showed no amylolytic activity including CGTase activity, it seems possible that starch remained intact throughout the cultivation. Since starch itself seems too large to enter a cell, the cell may sense the presence of starch at the cell surface and transfer a signal into the cell to express the cgt'::lacZ fusion. Alternatively, the smaller maltooligosaccharides mixed with the starch, or those produced from starch by the action of endogenous α-glucosidases, may act as inducers.
Interestingly, when we used an α-amylase-producing strain as a host, the cgt′::lacZ expression in LS medium was decreased to the level of that in L medium (data not shown). It is possible that the α-amylase hydrolyzed the inducer molecule so that the expression was not induced, or the expression was partially repressed by glucose produced by the α-amylase from starch.

Although we could not obtain useful information on the starch induction mechanism in B. ohbensis, the results presented here gave us a notion of a novel mechanism for the starch induction in B. subtilis. At present, there is only one gene identified in B. subtilis, mall, encoding an maltose-inducible α-glucosidase (suclase-isomaltase-maltase), the expression of which is induced by starch.  

In this case, starch induction did not occur in an α-amylase-defective strain, showing that maltose produced by the α-amylase from starch is the actual inducer.  

The requirement of the α-amylase activity for starch induction of mall expression is strictly different from that of cgt expression. The expression of the α-amylase gene of B. subtilis is also not induced by starch. Therefore further investigation on this regulation will discover a novel mechanism for starch induction in B. subtilis.

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References
Expression of \textit{B. ohbensis} CGTase Gene in \textit{B. subtilis}


