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Regulation of Cycloinulooligosaccharide Fructanotransferase Synthesis in *Bacillus macerans* and *Bacillus subtilis*

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Abstract Cycloinulooligosaccharide fructanotransferase (CFTase) converts inulin into cyclooligosaccharides consisting of six to eight molecules of β-(2→1)-linked cyclic D-fructofuranose through intramolecular transfructosylation. We have examined the regulation of CFTase synthesis in *Bacillus macerans* and *Bacillus subtilis*. Synthesis of the CFTase was induced by inulin and it was subject to carbon catabolite repression (CCR) by glucose in both microorganisms. The DNA sequence upstream of the promoter of the CFTase gene was not involved in the inulin induction and glucose repression of the CFTase gene expression in *B. subtilis*. This suggests that the DNA element(s) responsible for the inulin induction and glucose repression is located downstream of the promoter region. Unexpectedly, the CCR of the expression of CFTase gene was observed not to be dependent on CcpA protein in *B. subtilis*.

Key words: Catabolite repression, cycloinulooligosaccharide, fructanotransferase, inulin induction

Cycloinulooligosaccharide fructanotransferase (CFTase) converts inulin into cyclooligosaccharides (cyclofructans) consisting of 6 to 8 molecules of β-(2→1)-linked cyclic D-fructofuranose through intramolecular transfructosylation [7]. Cyclofructan has a characteristic crown ether in the central part of the molecule that can bind cationic molecules via charge-dipole electrostatic interactions [16]. Accordingly, cyclofructan is considered to have potential capacities as a novel host molecule in bioorganic chemistry, an ionophore, and an effective stabilizer for liposomes.

Previously, we isolated a strain of *B. macerans* CFC1 from soil which produced an extracellular CFTase [9], and subsequently the extracellular enzyme was purified and characterized [10]. Recently, we cloned and characterized the CFTase gene (*cft*, GenBank accession no. AF222787) of *B. macerans* CFC1 (unpublished data). The *cft* gene consists of an ORF of 4,002 nucleotides which encodes a polypeptide of 1,333 amino acids including a signal peptide of 31 amino acids in the N-terminus with a calculated M_r of 149,563. The CFTase with a mass of 150 kDa (CFT150) was found to be processed (between Ser389 and Phe390 residue) to form a 107 kDa protein (CFT107) in the *B. macerans* CFC1 cells. The CFT107 enzyme was produced in the *B. macerans* CFC1, but it was not detected from the recombinant *Escherichia coli* cells which carried the *cft* gene, indicating that the processing event occurs in a host-specific manner. The analysis of biochemical properties of both CFT150 and CFT107 showed that the N-terminal 358 residues region of CFT150 played a role in increasing the enzyme's affinity to the inulin substrate.

To date, three CFTases have been isolated and characterized from *B. circulans* OKUMZ31B [8], *B. circulans* MCI-2554 [6, 12], and *B. macerans* CFC1 [10]. However, no information is available in regards to the molecular basis of the regulation of CFTase synthesis. In our previous work [9], extracellular production of the CFTase by *B. macerans* CFC1 was detected only when the cells were grown in the presence of inulin. In this study, we examined the effects of carbon sources on the CFTase synthesis in *B. macerans* and *B. subtilis*.

Regulation of CFTase Synthesis in *B. macerans* CFC1

In the preliminary experiment, we found that the synthesis of CFTase in *B. macerans* initiated at the stationary phase in the presence of inulin. However, CFTase synthesis was detected at the log phase. To have some insight into the regulation mechanism(s) for CFTase synthesis, the effect of carbon sources on the synthesis of CFTase in *B. macerans* CFC1 was examined. Cells were grown for 24 h in the basal mediun (BM) consisted of 1.0% of carbon sources, 0.5% of peptone, 0.4% of KH_2PO_4, 0.05% of $MgSO_4$, and 0.05% KCl (pH 7.5). The extracellular production of CFTase was induced only in the presence of inulin.

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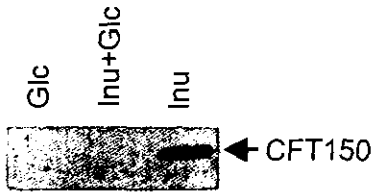

Fig. 1. Western blot analysis for CFTase synthesis in *B. macerans* CFC1.
The intracellular-soluble proteins of cells grown in the BM containing carbon sources indicated in the Figure for 24 h were probed with a polyclonal anti-CFT150 antibody. Inu, inulin; Glc, glucose.

When cultured in the presence of inulin plus glucose for 24 h (at this time point, the cells were at the second log phase which was caused by inulin consumption), neither CF6 nor CFTase activities were detected in the culture fluid of *B. macerans* CFC1 (data not shown). In addition, Western blot analysis revealed that the CFTase was not synthesized in the presence of either glucose or inulin plus glucose as carbon sources (Fig. 1). This demonstrated that the CFTase synthesis in the *B. macerans* cells was subject to both inulin induction and glucose repression.

Then, what is the actual inducer molecule for inducing the expression of *cft* gene? Since inulin is thought to be too large to pass through the cell membrane, the *B. macerans* cells may sense the presence of inulin at the cell surface and transfer a signal inside the cell to induce expression of the *cft* gene. Alternatively, the smaller inulooligosaccharides produced by the action of an endogenous inulinase may act as an inducer. The culture domain for the initiation of the CFTase synthesis in *B. macerans*, the stationary phase supports the hypothesis that an inulooligosaccharide, but not inulin, may be an inducer for the CFTase synthesis. Further studies for identifying the inducer and understanding the inulin induction mechanism will be needed.

Effect of Glucose on the Expression of *cft* Gene in *B. subtilis*

To examine the regulation of *cft* gene expression in *B. subtilis*, we first constructed a recombinant plasmid pWCFHis which was designed to express a *cft* allele encoding the C-terminal six His-tagged CFTase (CFTaseHis6). The plasmid pWCFHis was obtained by subcloning the 4.5 kb *Sal*I-*Eco*RI fragment of the plasmid pCFMHis into the aprA deleted pWP18 [pWP18 is a derivative of Pub101 which contains a promoterless subtilisin gene (*aprA*) preceded by the Puc18 polylinker]. Plasmid pCFMHis was previously constructed in our Laboratory and contained the *cft* allele encoding CFTaseHis6 [10]. The *B. subtilis* DB104 cells carrying pWCFHis grown in the LB medium produced most of CFTase extracellularly (>90% of total activity).

B. subtilis DB104/pWCFHis cells were grown in the BM containing 1% glucose, 1% inulin or a mixture of two carbon sources at 37°C for 24 h. Then, the extracellular

Table 1. Effects of glucose on the synthesis of CFTase in *B. subtilis*/pWCFHis cells.

Carbon source (1%, w/v)	Cell growth (A_{600})	Extracellular activity (mU/ml of culture broth)
Inulin	1.58	25.4
Glucose	0.72	0
Inulin+Glucose	2.53	15.9
None	0.44	0

Cells were grown in the BM containing 1% carbon sources at 37°C for 24 h.

CFTase activities were measured to understand the influence of glucose on the synthesis of CFTase in *B. subtilis*. The synthesis of CFTase was induced by inulin and repressed strongly by glucose as well (Table 1). Whereas, when *B. subtilis* DB104/pWCFHis cells were grown in the presence of 1% inulin plus 1% glucose, the level of CFTase activity was about 60% of the activity obtained from the recombinant cells which were cultured in the presence of inulin. Furthermore, the cell growth in the presence of 1% inulin plus 1% glucose showed a typical diauxy curve in which the cells reached the first stationary phase after the 8 h culture, and thereafter, the cells were in a lag phase about for 4 h (data not shown). Therefore, the kinetics of the CFTase synthesis in the presence of inulin and glucose mixture for 24 h cultivation time can be explained by the fact that the cells metabolized inulin only after complete consumption of glucose was made. These results indicate that the expression of the *cft* gene in *B. subtilis* was induced by inulin and subject to catabolite repression by glucose.

Regulation of the CFTase Synthesis in *B. subtilis*

In many gram-positive bacteria of low G+C content including *B. subtilis*, CCR of many genes is mediated at cis-acting catabolite responsive elements (*cre*) by the catabolite repressor protein (CcpA) [5, 14, 15]. The catabolite responsive element [1, 3, 11, 17-19] is known to consist of a consensus of 14 bp palindromic sequence, TGT/AANCGNTNA/TCA (where underlined letters represent the most critical bases, and N is any base) [5]. In the 5' non-coding region of the *cft* gene, we found two CRE-like sequences (CRE-like#1; TGAAAAGCCTGACA, nt -310 to -297 and CRE-like#2; TGGAAACGTTTCA, nt -117 to -105, relative to the translation start site of the *cft* gene) with high homology to the consensus sequence. In the preliminary experiment, we found that a 86 bp upstream sequence of the translation start site is sufficient for transcription of the *cft* gene in *E. coli*.

To examine whether the CRE-like sequences described above actually function as a cis-acting element in the glucose repression of the *cft* gene expression, we constructed two derivatives of pWCFHis by using the PCR cloning technique [pWCFA; deleted from nt -450 to -190 (deleted CRE-like#1), pWCFB; deleted from nt -450 to -86 (deleted both CRE-like#1 and CRE-like#2), see Fig. 2]. The recombinant

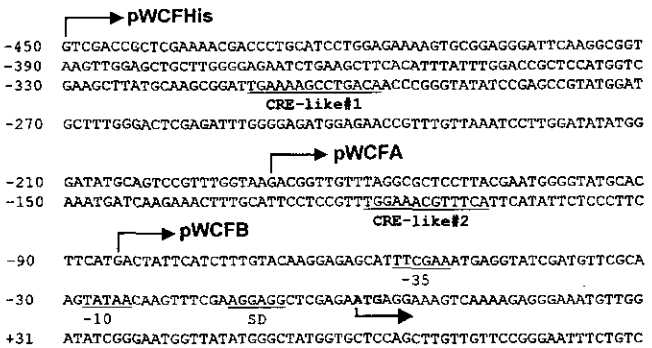

Fig. 2. Nucleotide sequence around the promoter region of the *cft* gene.
The indicated position of the nucleotide was relative to the translation start site. The translation start site was indicated by an arrow. The full nucleotide sequence of the *cft* gene was assigned as GenBank accession no. AF222787.

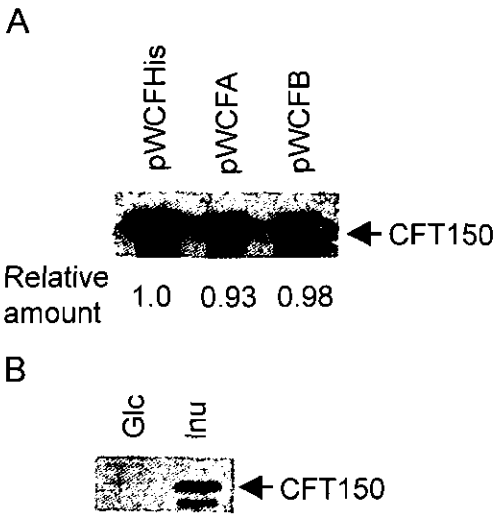

Fig. 3. Western blot analysis for expression of the *cft* gene in *B. subtilis*.
The intracellular-soluble protein samples were probed with the polyclonal anti-CFT150 antibody. Equal amounts of proteins were subject to 8% SDS-PAGE. (A) Expression of the *cft* gene in the presence of inulin. The DB104 cells carrying *cft* gene were grown in the presence of 1% inulin for 22 h. The intensities of the reacted signals were compared by the Scanner CS-9000 (Shimadzu, Japan). (B) CCR of expression of the *cft* gene was not dependent on CcpA protein. The SF13CDH (CcpA-deficient mutant) cells carrying pWCFHis were grown in the presence of 1% inulin or 1% glucose for 22 h. Inu, inulin; Glc, glucose.

B. subtilis DB104 cells carrying the plasmid pWCFA or pWCFB produced similar levels of CFTase activity to that for the *B. subtilis* strain carrying the plasmid pWCFHis when cultured in the LB medium at 37°C for 16 h. Next, the DB104 cells carrying each of the above recombinant plasmids were cultured in the BM containing 1% glucose or 1% inulin, and the extracellular CFTase activities were assayed. As shown in Table 2, in the presence of inulin the plasmids pWCFA and pWCFB gave a similar level of the enzyme activity compared to the pWCFHis. Whereas, in the presence of glucose, all the DB104 strains carrying pWCFHis, pWCFA, or pWCFB showed no CFTase activity. This result implied that none of the CRE-like#1 and #2 elements was implicated in the glucose repression. Therefore, we suspect that the DNA element responsible for the glucose repression may be located in the downstream region of the promoter. The expression level of the *cft* gene in the presence of inulin was also assayed by using the Western blot. As shown in Fig. 3A, the level of inulin induction determined with all the constructs was similar, suggesting that the element(s) responsible for the inulin induction might also be downstream of the cft promoter.

CCR mediated at *cre* sites in *B. subtilis* was reported to be relieved by inactivation of the *ccpA* gene [2-4, 13, 18]. Therefore, we examined whether the glucose repression of the *cft* gene expression was eliminated in a CcpA-deficient *Bacillus subtilis* mutant, SF13CDH [18]. When cultured in the presence of 1% inulin for 22 h, the SF13CDH carrying the pWCFHis produced extracellularly 25 mU/ml CFTase activity similar to that for the wild type strain. Unexpectedly, in the presence of 1% glucose, CFTase activity was not detected from the culture fluid of the SF13CDH/pWCFHis strain and the CFTase protein band from the intracellular fraction was also not detected by the Western blot (Fig. 3B). This result indicates that CCR of the *cft* gene expression may be not depended on the CcpA of *B. subtilis*. At present, we can not explain the precise mechanism for the CCR of the *cft* gene expression in *B. subtilis*. However, it is also possible that other *trans*-acting factor(s) may regulate expression of the *cft* gene in response to carbon availability [19].

In conclusion, we examined regulation of the CFTase synthesis in *B. macerans* and *B. subtilis*. The synthesis of CFTase was induced by inulin, and repressed by glucose in both organisms. The DNA region upstream of the *cft* promoter region was observed not to affect the inulin induction and glucose repression of the *cft* gene expression in *B. subtilis*. Therefore, we suspect that the DNA element(s) responsible for the glucose repression and inulin induction may be located in the downstream region of the promoter sequence. In addition, we also observed that the CCR of the *cft* gene was not dependent on CcpA in *B. subtilis*.

Table 2. Effects of glucose on expression of the *cft* gene in *B. subtilis* DB104 bearing pWCFHis or its upstream deletion derivatives.

Plasmid	Extracelluar CFTase activity (mU/ml)	
	Inulin	Glucose
PWCFHis	25.3	0.0
PWCFA	24.2	0.0
PWCFB	26.4	0.0

Cells were grown in BM containing 1% inulin or 1% glucose for 22 h.

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