J. Microbiol Biotechnol. (2000), 10(2), 275–280

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

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Cloning and Expression of Inulin Fructotransferase Gene of *Arthrobacter* sp. A-6 in *Escherichia coli* and *Bacillus subtilis*

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Received: January 4, 2000

Abstract The inulin fructotransferase (depolymerizing) (IFTase, EC 2.4.1.93) gene of *Arthrobacter* sp. A-6 was cloned and expressed in *Escherichia coli* and *Bacillus subtilis*. The IFTase gene consisted of an ORF of 1,311 nucleotides encoding a polypeptide of 436 amino acids containing a signal peptide of 31 amino acids in the N-terminus. The molecular mass of the IFTase based on the nucleotide sequence was calculated to be 46,116 Da. The recombinant *E. coli* DH5α cells expressing the *Arthrobacter* sp. A-6 IFTase gene produced most of the IFTase intracellularly. In contrast, the recombinant *B. subtilis* DB104 carrying the IFTase gene on a *B. subtilis-E. coli* expression vector secreted the IFTase into the culture fluid efficiently.

Key words: Inulin fructotransferase, *Bacillus* expression, difructofuranose, DFA, *Arthrobacter*

Inulin is a polyfructan consisting of a linear β-(2→1)-linked polyfructose chain with a terminal glucose residue, and serves as a carbohydrate reservoir in various plants such as chicory, dahlia, and Jerusalem artichoke. Inulin fructotransferase (depolymerizing) (IFTase, EC 2.4.1.93) converts inulin into di-D-fructofuranose 1,2':2,3' dianhydride (DFA III) and small amounts of oligosaccharides through intramolecular transfructosylation. Since the initial discovery of IFTase from *Arthrobacter ureafacious* by Tanaka *et al.* in 1972 [20], several other IFTases have been isolated and characterized from *Arthrobacter* species [6, 8, 21, 25] and *Bacillus* sp. snu-7 [7]. However, until now, only two genes encoding DFA-producing enzymes have been cloned: the IFTase gene of *Arthrobacter* sp. H65-7 [14] and the gene coding for inulin fructotransferase (DFA I-producing) (EC 2.4.1.200) of *Arthrobacter globiformis* S14-3 [5]. DFA I, di-D-fructofuranose 1,2':2,1' dianhydride, is another type of DFA produced from inulin [16].

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DFA III is a low-calorie sweetener with about half the sweetness of sucrose. Moreover, this sweetener has some important physiological benefits such as promotion of *Bifidobacterium* growth, prevention of tooth decay [10], and stimulation of calcium absorption in both the small and large intestines [19]. Therefore, DFA III could be used by various food industries when it could be mass-produced at a relatively low cost.

Previously, we have isolated a bacterial strain of *Arthrobacter* from soil which produced an extracellular IFTase [11], and subsequently this enzyme was purified and characterized [9]. The present paper describes the molecular cloning of the IFTase gene (*ift*) of *Arthrobacter* sp. A-6 and the expression of this gene in *Escherichia coli*. In addition, we made an attempt to produce the IFTase extracellularly using a *Bacillus subtilis* strain.

General Methods

The DNA manipulations and standard molecular biological methods used in this work were as described in Sambrook *et al.* [15]. The transformations of *E. coli* DH5α and *B. subtilis* DB104 (*his nprE aprE*) were carried out by electroporation using a Gene Pulser (BioRad, U.S.A.) and the procedure of Spizizen [18], respectively. The genomic DNA of *Arthrobacter* sp. A-6 was isolated based on the method developed by Doi [3].

Cloning of *ift* Gene from *Arthrobacter* sp. A-6

Based on the N-terminal sequence (ADNPDGSN) of the purified IFTase from *Arthrobacter* sp. A-6 [9] and the sequence (MGFVYLEHA, 205 to 213 residues) of the internal region of the IFTase from *Arthrobacter* sp. H65-7 [14], primers D1 [5' GCI GA(T/C) AA(T/C) CCI GA(T/C) GGI TCI AA 3', I; inosine] and D3 [5' GC (A/G)TG (T/C)TC IAG (A/G)TA IAC (A/G)AA ICC CAT 3'] were synthesized. Using these primers (20 pmol each) and 100 ng of the genomic DNA as a template, a PCR was performed using an AccuPower™ PCR PreMix reaction kit (Bioneer, Korea) and DeltaCycler I™ System (Ericomp Co., U.S.A.). The PCR reactions were carried out for 30

cycles (94°C for 50 sec, 49–55°C for 50 sec, and 72°C for 50 sec). A 0.5 kb DNA fragment was amplified in PCR at an annealing temperature of 50°C. The amplified 0.5 kb DNA was then subcloned into pUC118, resulting in the plasmid pDA. The subcloned DNA fragment in the plasmid pDA was confirmed to be a part of the *ift* gene based on its nucleotide sequence and a comparison with the reported IFTase gene of *Arthrobacter* sp. H65-7 [14].

To clone the entire *ift* gene of *Arthrobacter* sp. A-6, the chromosomal DNA was digested with *Kpn*I, and Southern blotting was performed using the 230 bp *Bst*EII-*Xho*I fragment of the PCR product from the pDA plasmid. A genomic DNA fragment with a size of about 4.2 kb was detected as hybridized with the probe DNA. A genomic library was constructed in *E. coli* DH5α using the chromosomal DNA fragments treated with *Kpn*I (from 3.6 to 4.3 kb) and the pUC118 vector. The white colonies on a Luria-Bertani (LB) agar containing 50 μg/ml ampicillin plus X-gal and isopropyl β-D-thiogalactopyranoside were selected, and then screened by colony hybridization. Out of 1300 white colonies on the selective medium, 5 clones showed a positive response. All the positive clones had the same length and same restriction pattern as the insert DNA (4.2 kb). One clone was named as pDFA, and another clone in which the insert DNA was oriented in the reverse direction of pDFA as pDFAR.

Nucleotide Sequence of *ift* Gene

The complete nucleotide sequence of the 4.2 kb *Kpn*I insert fragment was determined using ABI PRISM 310

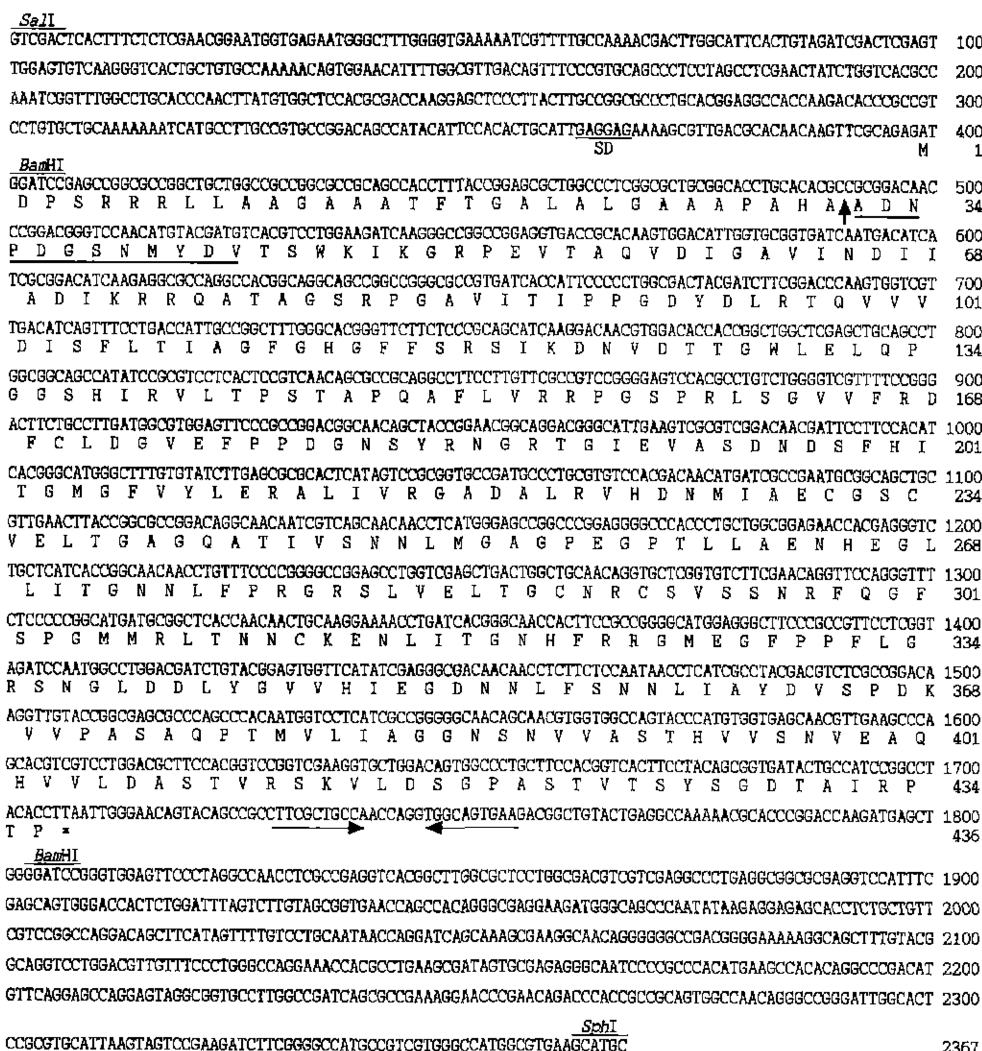

Fig. 1. Nucleotide and deduced amino acid sequences of *ift* gene of *Arthrobacter* sp. A-6.

The amino acid sequences of the purified IFTase from *Arthrobacter* sp. A-6 determined by N-terminal sequencing are underlined. The vertical arrow indicates the cleavage site of the signal peptide The putative Shine-Dalgarno (SD) sequence is underlined. The putative transcription terminator (inverted repeat sequence) is indicated by horizontal arrows. The '+1' position of the nucleotide shown in the Figure corresponds to the 947 position of the 4 179 bp *Kpn*I insert. The nucleotide sequence data has been deposited in the GenBank database and assigned accession no AF124980.

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A   M-DPSRRRLLAAGAAATFTGALALGAAAPAHAADNPDGSNMYDVTSWKIKGRPEVTAQVDIGAVINDIIADIKRRQATAG  79
H   MMDPSRRRLLGAGAVATLTGALALGAAAPAQAADSTEETNRYDVTSWKIKGRPEVTAQIDIGAVINDIIADVKKRQTTAK  80
S   M------------------------------------ANTVYDVTTW---SGATISPYVDIGAVINQIIADIKANQTSQA  41
    *                                    . .****.*   . .....  .*******.****.* .*...

A   SRPGAVITIPPGDYDLRTQVVVDISFLTIAGFGHGFFSRSIKDNVDTTGWLELQPGGSHIRVLTPSTAPQAFLVRRPGSP 159
H   ARPGAVITIPPGDYDLRTQVVVDVSYLTIAGFGHGFFSRSIKDNVDTSGWLELQPGGSHIRVLTPSTAPQAFLVRRAGSP 160
S   ARPGAVIYIPPGHYDLLTRVVVDVSFLQIKGSGHGFLSEAIRDESSTGSWVETQPGASHIRVKNTDGNREAFLVSRSGDP 121
    .****** ****.*** *.****.*.* * * ****.* .* *  .*..*.* ***.*****  . ..  .****.*.*.*

A   ----RLSGVVFRDFCLDGVEFPPDGNSYRNG--RTGIEVASDNDSFHITGMGFVYLERALIVRGADALRVHDNMIAECGS 233
H   ----RLSGVVFRDFCLDGVEFPPDGNSYRNG--RTGIEVASDNDSFHITGMGFVYLEHALIVRGADALRVHDNMIAECGN 234
S   NVVGRLNSIEFKGFCLDGVT---DSKPYSPGNSKIGISVQSDNDSFHVEGMGFVYLEHAIIVKGADAPNITNNFIAECGS 198
        **... *..******.   *. .*. *  ..** * ******* .********.*.**.****  ..   *.*****.

A   CVELTGAGQATIVSNNLMGAGPEGPTLLAENHEGLLITGNNLFPRGRSLVELTGCNRCSVSSNRFQGFSPGMMRLTNNCK 313
H   CVELTGAGQATIVSNNLMGAGPEGATLLAENHEGLLITGNNLFPRGRSLVELTGCNRCSVTSNRFQGFFPGIMRLINNCK 314
S   CIELTGASQVAKITNNFLISAWAGYSIYAENAEGPLITGNSLL-WAAN-ITLSDCNRVSISSNKLLSNFPSMVALLGNCS 276
    *.*****.*.. ..**  .   .  * ..  *** ** ***** *. ..   .*..*** *..**.. .  *..  * .**.

A   ENLITGNHFRRGMEGFPPFLGRSNGLDDLYGVVHIEGDNNLFSNNLIAYDVSPDKVVPASAQPTMVLIAGGNSNVVASTH 393
H   ENLITGNHFRRGMEGFPPFLGTSNGLDDLYGVVHIAGDNNFFANNLIAYDVSPDRIVPPNAQPTMILVAGGDSNVVATNH 394
S   ENLIAANHFRRVSGD-----GTSTRFDDLFGLVHIEGNNNTVTGNMFSFNVPASSISPSGATPTIILVKSGDSNYLATNN 351
    ****..*****  ..      * *. .***.*.***.*.**  ..*.....*....  * .* **..*. .*.** .*...

A   VVSNVEAQHVVLDASTVRSKVLDSGPASTVTSYSGDTAIRPTP 436
H   VVSNVETQHVVLDASTVRSKVLDSGPASKVTSYSADTAIRPTP 437
S   IVSNVSAM-VVLDGSTTATRIIYSAKNSQLNAYTTSYTLVPTP 393
    .****..  ****.**. .... *. .*  ..*... .. ***
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Fig. 2. Comparison of the deduced amino acid sequence of *Arthrobacter* sp. A-6 IFTase (A) with those of *Arthrobacter* sp. H65-7 IFTase (H) and *Arthrobacter globiformis* S14-3 inulin fructotransferase (DFA I-producing) (S).
The identical residues and similar residues are indicated by asterisks and dots, respectively

automated sequencing (Perkin Elmer Co., U.S.A.). The insert DNA consisted of 4,179 bp, and the *ift* gene was found to reside in the 2,367 bp *Sal*I-*Sph*I fragment of the insert as shown in Fig. 1. In the above *Sal*I-*Sph*I fragment, a single open reading frame was found whose twelve amino acid sequences (ADNPDGSNMYDV, 32 to 43 residues) were a perfect match with the previously determined N-terminal sequences of the purified IFTase [9]. The *ift* gene consisted of 1,311 nucleotides and encoded a polypeptide of 436 amino acids including a signal peptide of 31 amino acids in the N-terminus. The molecular mass and pI of the IFTase were calculated to be 46,116 Da and 5.9, respectively. The deduced molecular mass of the IFTase described above agreed well with that of the purified enzyme (49,000 Da by SDS-PAGE). The G+C content of the *ift* gene was 63.2% with a strong bias at the third codon position (79.8%). The deduced amino acid sequence of the IFTase showed a 90.1% identity with that of the *Arthrobacter* sp. H65-7 enzyme [14], and a 57.3% identity with that of the inulin fructotransferase (DFA I-producing) of *Arthrobacter globiformis* S14-3 [5] (Fig. 2).

Expression of *ift* Gene in *E. coli*

To confirm the expression of the cloned *ift* gene and cellular localization of the gene product in *E. coli* DH5α cells, the extracellular and intracellular IFTase activities of the recombinant *E. coli* strains containing pDFA or pDFAR were measured. As shown in Table 1, irrespective of the orientation of the insert DNA, both recombinant *E. coli* strains produced the IFTase, indicating that the promoter of the *ift* gene was recognized by the transcriptional apparatus of *E. coli*. This is an interesting difference with the *ift* gene of *Arthrobacter* sp. H65-7 that reportedly is not expressed in *E. coli* by its own promoter [14]. However, most of the enzyme activities (>90%) synthesized by *E. coli* strains were detected within the cells, thereby indicating that the signal peptide of *Arthrobacter* sp. A-6 IFTase did not function effectively in *E. coli*.

Table 1. Cellular localization of IFTase in *E. coli* DH5α.

Plasmid	Enzyme activity (U/ml of culture broth)	
	Intracellular	Extracellular
pDFA	13.5±0.7	1.52±0.08
pDFAR	1.33±0.05	0.23±0.02
pUC118	0.0	0.0

The recombinant *E. coli* DH5α cells were grown in a 20 ml LB broth containing 50 μg/ml ampicillin in a 250-ml flask at 37°C for 8 h with shaking. Thereafter, 0.2 ml of the pre-culture broth was inoculated into 20 ml of the same medium. Next, the cells were cultured at 37°C for 16 h with shaking. The *E. coli* DH5α cells containing the different plasmids showed nearly the same rate of growth under these culture conditions. The culture broth was centrifuged at 3,000 ×g for 10 min at 4°C. The supernatant obtained was used as the extracellular crude enzyme source. The collected cells were washed three times with a 50 mM phosphate buffer (pH 6.0) and then resuspended in the same buffer. The cell extract obtained after the ultrasonic treatment (6 cycles of 20 sec at a 50 W output with cooling in an ice bath) of the cell suspension was used as the intracellular crude enzyme. The assay mixture for determining the IFTase activity consisted of 0.5 ml 5% (w/v) inulin in a 50 mM phosphate buffer (pH 6.0) and 0.05–0.5 ml of the crude enzyme solution. The reaction mixture was adjusted to 1.0 ml by adding a 50 mM phosphate buffer (pH 6.0). The reaction was carried out at 60°C for 10 min. The reaction product, DFA III, was determined by HPLC as described previously [9]. One unit of the enzyme activity was defined as the amount of enzyme producing 1 μmol DFA III per min under these assay conditions. The results are the means (±standard deviation) of three independent experiments

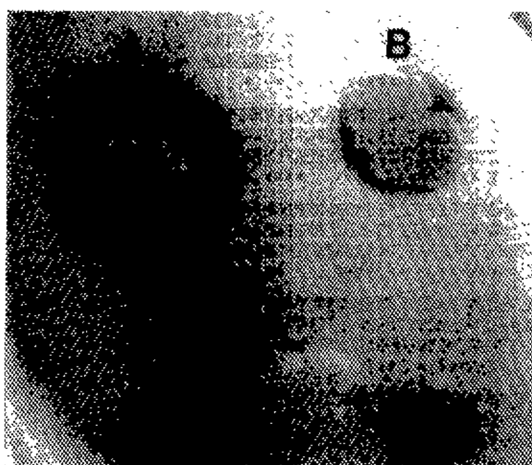

Fig. 3. Clear-zone formation of recombinant *E. coli* DH5α expressing *ift* gene from *Arthrobacter* sp. A-6.
The cells were incubated at 37°C for 2 days in an LB agar medium containing 50 µg/ml ampicillin and partially solubilized inulin. A, *E. coli* DH5α/pDFA. B, *E. coli* DH5α/pDFAR; C, *E. coli* DH5α/pUC118.

Interestingly, the plasmid pDFA, in which the *ift* gene was oriented in the same direction as the *lac* promoter of pUC118 and where its translation start codon was located about 1.35 kb downstream of the *lac* promoter, showed a 10 times higher level of IFTase activity than that shown by pDFAR in which the *ift* gene was positioned in the reverse orientation. It was also confirmed that *E. coli* carrying pDFA produced larger amounts of IFTase than *E. coli*/pDFAR, based on detecting the clear-zones formed around the strain's colony on the LB agar containing inulin. As shown in Fig. 3, *E. coli*/pDFA formed a larger clear-zone around its colony than *E. coli*/pDFAR in accordance with the results of the enzyme assay (Table 1). Taken together, these results suggest that the expression of the *ift* gene on the plasmid pDFA is strongly directed by the upstream *lac* promoter of the pUC118 vector.

Construction of Recombinant Plasmids

Plasmid pDFB was constructed by subcloning a 2,367 bp *Sal*I-*Sph*I fragment (GenBank accession no. AF124980) containing the *Arthrobacter* sp. A-6 *ift* gene into pUC118. Plasmid pWDF was constructed by subcloning a 2,394 bp *Kpn*I-*Hin*dIII fragment containing the *ift* gene from pDFB into the same restriction sites of pWPBR18 [2], a *B. subtilis*-*E. coli* shuttle vector. Plasmid pEBDF was constructed by subcloning a 1,408 bp *Bam*HI fragment from pDFB into the *Bam*HI site of pEBP313 [12], a *B. subtilis*-*E. coli* expression vector. The *ift* gene on the plasmid pEBDF encodes the IFTase fused from its second amino acid (Asp) with N-terminal 7 amino acids (MNSSVPG) from the EB promoter region, a 319 bp fragment isolated from the *B. subtilis* chromosomal DNA of the pEBP313 vector (see Fig. 4).

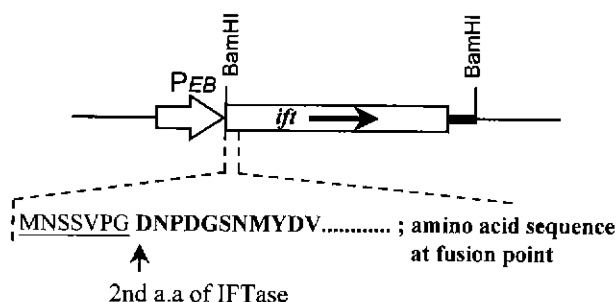

Fig. 4. Description of pEBDF plasmid.
The *ift* structural gene is represented as an open box. The seven amino acid residues of the EB promoter (P_{EB}) region at the point of fusion are underlined.

Secretory Expression of *ift* Gene in *B. subtilis*

Arthrobacter sp. A-6 is a gram-positive bacterium that secretes nearly all the synthesized IFTase into the culture fluid [11]. However, the recombinant *E. coli* cells expressing the *Arthrobacter* sp. A-6 *ift* gene produced most of the IFTase intracellularly. This can be explained primarily by the differences in the protein secretion mechanism(s) between the Gram-positive and the Gram-negative bacteria [13, 17]. Therefore, an attempt was made to secrete the IFTase directly into the growth medium using the *B. subtilis* strain, which greatly facilitates its downstream purification step. IFTase activity was not detectable in the recombinant *B. subtilis* DB104 carrying pWDF in which the *ift* gene had been designed to be transcribed by its own promoter. Accordingly, using a *B. subtilis*-*E. coli* expression vector, pEBP313 [12], the plasmid pEBDF was constructed in which the *ift* gene was expressed under the control of an EB promoter isolated from the *B. subtilis* chromosomal DNA. As shown in Table 2, nearly all the recombinant IFTase produced by the *B. subtilis*/pEBDF cells was secreted into the culture fluid.

The total level of IFTase activity produced by *B. subtilis* DB104/pEBDF was 5 times lower than that produced by

Table 2. Recombinant *B. subtilis* cell produced IFTase in extracellular form.

Host	Plasmid	Enzyme activity (U/ml of culture broth)	
		Intracellular	Extracellular
E. coli DH5α	pWDF	0.34±0.05	n.d[a]
	pEBDF	4.26±0.22	0.13±0.01
B. subtilis DB104	pWDF	n.d	n.d
	pEBDF	n.d	0.83±0.12

The recombinant *E. coli* DH5α and *B. subtilis* DB104 cells were grown in a 30 ml LB broth containing 50 µg/ml ampicillin and 30 µg/ml kanamycin, respectively, at 37°C for 18 h. The optical densities at 600 nm for the recombinant *E. coli* DH5α and *B. subtilis* DB104 cells were 5.2±0.3 and 3.3±0.2, respectively. The enzyme assay was performed as described for Table 1. The results are the means (±standard deviation) of three independent experiments. [a]n.d, not detectable, in this case, the enzyme reaction was carried out for 2 h under standard assay conditions.

E. coli DH5α carrying the same plasmid when both strains were cultured with the same LB medium and conditions. *B. subtilis* is a useful host for the production and secretion of heterologous proteins [4, 22-24]. However, several bottlenecks for heterologous protein secretion in *B. subtilis* have been documented in recent years: insoluble aggregates of heterologous proteins in the cytoplasm, the removal of signal peptide by signal peptidase during or shortly after the translocation of the preprotein, a cell wall barrier, and the proteolytic degradation of the secreted proteins [1]. The EB promoter used in this work, originating from *B. subtilis*, is known to exert its effect during the vegetative growth phase [12]. In the case of the production of the *B. thuringiensis* subsp. *kurstaki* HD-73 crystal protein under the control of the EB promoter, both *B. subtilis* and *E. coli*, each carrying the same plasmid, produce similar amounts of the crystal protein intracellularly with overnight cultivation in LB medium [12]. At present, the reasons for the lower IFTase activity shown in *B. subtilis* DB104 compared with that in *E. coli* DH5α are unclear. When considering the similar promoter strength of the EB promoter in both *B. subtilis* and *E. coli*, as reported previously [12], the expression level of the *ift* gene on the pEBDF plasmid in *B. subtilis* DB104 may be similar to that in an *E. coli* DH5α cell. Therefore, it is likely that the lower IFTase activity in *B. subtilis* DB104 may result from the proteolytic degradation of the secreted IFTase, although the *B. subtilis* DB104 strain lacks two major secreted proteases, subtilisin (AprE) and neutral protease (NprE).

Acknowledgments

We are grateful to Dr. Seung Hwan Park from Korea Institute of Bioscience and Biotechnology for supplying the pEBP313 vector. This work was supported by a grant (HMP-97-F-5-0023) from the Ministry of Health and Welfare, Republic of Korea.

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