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Novel *Bifidobacterium* Promoters Selected Through Microarray Analysis Lead to Constitutive High-Level Gene Expression

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For the development of a food-grade expression system for *Bifidobacterium*, a strong promoter leading to high-level expression of cloned gene is a prerequisite. For this purpose, a promoter screening host-vector system for *Bifidobacterium* has been established using β-glucosidase from *Bifidobacterium lactis* as a reporter and *Bifidobacterium bifidum* BGN4 as a host, which is β-glucosidase negative strain. Seven putative promoters showing constitutive high-level expression were selected through microarray analysis based on the genome sequence of *B. bifidum* BGN4. They were cloned into upstream of β-glucosidase gene and transformed into *Escherichia coli* DH5α and *B. bifidum* BGN4. Promoter activities were analyzed both in *E. coli* and *B. bifidum* BGN4 by measuring β-glucosidase activity. β-Glucosidase activities in all of the transformants showed growth-associated characteristics. Among them, P919 was the strongest in *B. bifidum* BGN4 and showed maximum activity at 18 h, while P895 was the strongest in *E. coli* DH5α at 7 h. This study shows that novel strong promoters such as P919 can be used for high-level expression of foreign genes in *Bifidobacterium* and will be useful for the construction of an efficient food-grade expression system.

Keywords: *Bifidobacterium*, promoter, expression, vector

Introduction

Bifidobacterium is a strictly anaerobic Gram-positive bacterium with high guanine and cytosine content and often Y-shaped or clubbed morphology. They all contain fructose-6-phosphate phosphoketolase, which can be used as a differential marker for the genus *Bifidobacterium* (Bezkorovainy and Miller-Catchpole, 1989; Mitsuoka, 1990). *Bifidobacterium* is a major commensal bacterium in the intestines of humans and animals. It is considered to play a beneficial role in the maintenance of the balance of normal intestinal flora in humans (Park *et al.*, 2008).

Ever since the first report on the existence of plasmids in the genus *Bifidobacterium* (Sgorbati *et al.*, 1982), continuous progress has been made in the development of cloning vectors for *Bifidobacterium* to improve its probiotic characteristics (Kullen and Klaenhammer, 2000). A high-level expression system requires a strong promoter to express foreign genes in a host. Many *Escherichia coli* promoters have been reported to contain a -10 region similar to those of *Lactobacillus acidophilus* promoters (Kullen and Klaenhammer, 2000), but they cannot be used in foods because of their non-food-grade nature. Therefore, the development of food-grade expression systems has been pursued in various studies using genetic elements derived from food-grade microorganisms (Kim *et al.*, 2009). For this purpose, the screening of strong promoters from food-grade microorganisms is an important research area (Kim *et al.*, 2009). However, only a few studies have reported the characterization of promoters from the genus *Bifidobacterium*. Park *et al.* (2008) constructed an expression vector using a 16S rRNA promoter of *Bifidobacterium longum* and expressed cholesterol oxidase from *Streptomyces* in *B. longum*. Klijn *et al.* (2006) used the *gusA* gene of *E. coli* as a reporter and characterized 3 promoters from *B. longum* using microarray analysis.

Reporter genes are generally used to select recombinant plasmids and characterize promoter strength in various bacterial strains (Sirard *et al.*, 1995; Gibson and Tabita, 1996; Rist and Kertesz, 1998).

In this report, we constructed a promoter screening host-vector system for *Bifidobacterium*. The structural gene of β-glucosidase from *Bifidobacterium lactis* AD011 was cloned into pBES2, a *Bifidobacterium–E. coli* shuttle vector, and β-glucosidase–free *Bifidobacterium bifidum* BGN4 was used as a host. Seven putative promoters selected on the basis of microarray analysis of *B. bifidum* BGN4 were then cloned and tested for their promoter activities.

Materials and Methods

Bacterial strains, media, and plasmids

E. coli DH5α was grown in Luria-Bertani broth (Difco, USA) at 37°C with vigorous shaking. *B. bifidum* BGN4 was grown in MRS medium (Difco) supplemented with 0.05% (w/v) L-cysteine·HCl at 37°C. Ampicillin and chloramphenicol (Sigma, USA) were used at concentrations of 50 and 3.6 μg/ml, respectively, for transformant selection. The bacterial strains and plasmids used in this work are listed in Table 1.

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Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli DH5α	Cloning host	Hanahan (1983)
B. bifidum BGN4	β-glucosidase negative source of promoters	
Plasmids		
pBES2	Cm^r, Amp^r: *E. coli-Bifidobacterium* shuttle vector	Park *et al.* (2003)
pBES2-16pG	pBES2 derivative containing 16S rRNA promoter from *Bifidobacterium longum* upstream of β-glucosidase	Park *et al.* (2008)
pBES2-G	Promoter screening vector for *Bifidobacterium* containing promoterless β-glucosidase gene	This work (Fig. 1)
pGEM-T easy	Amp^r, M13ori pBR322ori, linear T-overhangs vector	Promega
pBES2-632G	pBES2 derivative containing the P632 promoter upstream of β-glucosidase	This work
pBES2-644G	pBES2 derivative containing the P644 promoter upstream of β-glucosidase	This work
pBES2-895G	pBES2 derivative containing the P895 promoter upstream of β-glucosidase	This work
pBES2-834G	pBES2 derivative containing the P834 promoter upstream of β-glucosidase	This work
pBES2-888G	pBES2 derivative containing the P888 promoter upstream of β-glucosidase	This work
pBES2-919G	pBES2 derivative containing the P919 promoter upstream of β-glucosidase	This work
pBES2-1527G	pBES2 derivative containing the P1527 promoter upstream of β-glucosidase	This work

General cloning techniques and vector construction

The chromosomal DNA of *B. bifidum* BGN4 was isolated using a PureLink™ Genomic DNA Kit (Invitrogen, USA) according to the manufacturer's instructions and used as a template for polymerase chain reaction (PCR) amplification. Seven genomic DNA regions predicted to have promoter activity were amplified using each primer set containing *Xba*I and *Bam*HI restriction sites (Table 2). PCR products were ligated into a pGEM-T Easy vector (Promega, USA) and transformed into *E. coli* DH5α using the $CaCl_2$ method (Sambrook *et al.*, 1989). Plasmid DNA was isolated from *E. coli* DH5α using a Plasmid Purification Mini Kit (Nucleogen, Korea) according to the manufacturer's instructions. DNA fragments were purified from the agarose gel using a gel extraction kit (QIAGEN Korea Ltd., Korea). All restriction enzymes were purchased from Promega.

Microarray analysis

A total of 1,000 probes were designed based on the genome sequence data of *B. bifidum* BGN4 (not published), and Combimatrix customized chips for these genes were designed and manufactured by Macrogen Inc. (Korea). *B. bifidum* BGN4 was grown in MRS medium supplemented with 0.05% (w/v) L-cysteine·HCl with or without controlling the pH at 5.0 with 4 N NaOH. A 5-L jar fermenter (Hanil Inc., Korea) was used to cultivate the *B. bifidum* BGN4. During cultivation, 1.5 ml of culture broth was harvested at the indicated time points and the total RNA was extracted using a RiboPure™ Bacteria Kit (Ambion Inc., USA) according to the manufacturer's instructions. Microarray analysis was performed at Macrogen Inc.

Transformation of *Bifidobacterium*

Expression vectors were prepared from *E. coli* DH5α using an Axyprep Midi Kit (Axygen Biosciences, USA) and methylated using GpC methyltransferase (M.CviPI; NEB, USA) according to the manufacturer's instructions. After methylation, the expression vectors were transformed into *B. bifidum* BGN4 by electroporation according to Kim *et al.* (2010). The fructose-6-phosphate phosphoketolase test and plasmid preparation was conducted to confirm the correct transformants among the developed colonies.

Sequence analysis of the putative promoter

DNA sequence analysis was performed using the Applied Biosystems 3730 DNA Analyzer at the Genome Research Facility of Seoul National University.

The promoter regions and transcription starting points of 7 putative promoters were predicted using the program at http://www.fruitfly.org/seq_tools/promoter.html. The minimum promoter score was fixed at 0.7.

Promoter activity assay

The β-glucosidase activities of recombinant *E. coli* DH5α and *B. bifidum* BGN4 were analyzed using *p*-nitrophenyl-β-D-glucopyranoside as a substrate to compare the promoter

Table 2. Primers for amplifying the putative promoters from *B. bifidum* BGN4

Primer names	Sequences of primers	
	Forward	Reverse
P632	5′-tctagaACGAATAGGCAGGCGTTGCTG -3′	5′-ggatccGCTGGCTCCTTTGTTTGCGTA-3′
P644	5′-tctagaCACGCGCAGCTGTTTTGAAG-3′	5′-ggatccGTAATTGTCCTCCTGGACGT-3′
P895	5′-tctagaATCGTACAGCACGAAAACCGT-3′	5′-ggatccTGCCGTCCGCCCTTTCTAGCG-3′
P834	5′-tctagaTTTTCGGCGGGTCTTCGGTCC-3′	5′-ggatccCATCGAGGCATGATTGTAGCA-3′
P888	5′-tctagaTTGCCGCTGCCGGTCGTATCG-3′	5′-ggatccAGCCTTACAGTCCATTCCTTG-3′
P919	5′-tctagaTGAAGTGTGTCGTGTGGCGT-3′	5′-ggatccTGGTGTACCTTTTCTTGCTT-3′
P1527	5′-tctagaGCCTCGATGGCGGCTTCGGG-3′	5′-ggatccAATGGCTCTCCTTGTAATAC-3′

Forward and reverse primers were designed to contain *Xba*I (tctaga) and *Bam*HI (ggatcc) sites, respectively

Table 3. List of genes with high-level median signal values in microarray data for *B. bifidum* BGN4

Gene names	Gene Comments[a]	Median signal values[b]							
		With pH control				Without pH control			
		8[c]	9	10	11	8	9	10	11
644	Tuf	65535	65535	65535	65535	65535	65535	65535	65535
1527	rpsP	65535	65535	65535	65535	65535	65535	65535	65535
919	rplM	9911.5	16664.5	15641	15974.5	17120	16380	25741	16061.5
888	rpmJ	53734	54808.5	50175.5	45308.5	61536	65535	63081	59946
632	ybhL	58367.5	56733.5	53298	59489.5	55920	60428.5	65535	57233.5
834	cell wall	51138	41770.5	54758	48567.5	52433	57057	65535	58731
895	rplR	18737	20172.5	10552.5	15758.5	17589.5	16451.5	19626	9422.5

[a] Tuf, peptide elongation factor Tu; rps, ribosomal protein small; rpl, ribosomal protein large; rpm, ribosomal protein medium; ybh, putative inner membrane protein; cell wall, cell wall biogenesis
[b] The maximal median signal value was set at 65535 and the bigger signals were also indicated as 65535
[c] 8, 10 min before NaOH input; 9, 40 min after NaOH input; 10, 70 min after NaOH input; 11, 160 min after NaOH input

strengths.

B. bifidum BGN4 and *B. lactis* AD011 were cultured in MRS medium supplemented with 0.05% (w/v) L-cysteine·HCl at 37°C. Nine *B. bifidum* BGN4 transformants harboring pBES2-632G, pBES2-644G, pBES2-895G, pBES2-834G, pBES2-888G, pBES2-919G, pBES2-1527G, pBES2-G, and pBES2-16pG were cultivated in MRS medium supplemented with 0.05% (w/v) L-cysteine·HCl and 3.6 μg/ml of chloramphenicol at 37°C.

E. coli DH5α transformants (pBES2-632G, pBES2-644G, pBES2-895G, pBES2-834G, pBES2-888G, pBES2-919G, pBES2-1527G, pBES2-G, and pBES2-16pG) and *E. coli* DH5α were cultured in LB medium at 37°C with vigorous shaking, and ampicillin was used at 50 μg/ml as necessary.

During cultivation, 1.0 ml of culture broth was harvested at indicated times and centrifuged at 10,000×*g* for 2 min. The cell pellet was washed twice with 200 μl of ice-cold 20 mM phosphate buffer (pH 5.0), disrupted by sonication in 500 μl of the same buffer, and centrifuged at 10,000×*g* for 2 min at 4°C to obtain the supernatant as crude enzyme. Fifteen microliters of enzyme solution was mixed with 10 μl of 5 mM *p*-nitrophenyl-β-D-glucopyranoside and incubated at 37°C for 20 min. The reaction was stopped by the addition of 100 μl of 1 M Na_2CO_3. The released *p*-nitrophenol (*p*NP) was measured at 405 nm using *p*NP (Sigma) as a standard. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol (*p*NP) per min at 37°C.

Results

Microarray analysis of *B. bifidum* BGN4

When *B. bifidum* BGN4 was grown in MRS medium, the pH decreased and reached 5.0 after 8 h, 10 min. Subsequently, the pH of the one reactor was controlled at 5.0 by 4 N NaOH, while the other was not. The pH controlled reactor showed better growth and reached higher optical density after 10 h, 50 min. In this study, we attempted to obtain promoters of *Bifidobacterium* showing constitutive high-level expression. Microarray data analysis revealed 7 genes showing constitutively high-level expression in both culture conditions (Table 3). Among them, 644 and 1527 were the strongest, 919 and 895 were the weakest, and 888, 632, and

Table 4. Nucleotide sequences of the putative promoters from *B. bifidum* BGN4

Promoter (size)	Promoter sequences
P632 (102 bp)	Acgaataggcaggcgttgctggtgtgatgctcaaggctgaacattaatgatttgttcagccttctccgttacga***TG***aagc**G**tacgcaaacaaaggagccagc (0.74)
P834 (180 bp)	Ttttcggcgggtcttcggtccccgccagtgagattcatctctattattctcccgggagtgtcgcgccggtcgtgtgaagattgccggttttcccgtgatgt***TG***ccga**T**atggcgtgatttcctagcgtgagtgttttcaggcgaaagatgcatgcctgtgtgctacaatcatgcctcgatg (0.73)
P895 (526 bp)	Atcgtacagcacgaaaaccgtgcgcggggccgtttggctactgcctttcccgatagcccattgaaaatcgtgtacggataggtgtatcggcgcactgagaggcgagaataatgggctatctgcatttcgaggggttgatggcagagtagaggcgctccgggaaccgcatgattccaacaaccgtagggcagtgataccgaggggatcgtgctctgacaacctcacgaaacgcagatagcgccgaggggcggcagccctgaggggatatgacaggattcgggcgtgcgacacgcccgaattccctataggtacccctggtgctttaattaacaagttgcctgttttgggctcgcaaattggaa***T***caaaa**A**agcgagcgtggaacaaggggtccgtttcgacgggcttcttgcatgtctacgcactgatgtggttatggccgcgaaaagtattttcgtgtgtcatgctcgccggtgccctttagcaggatggtaatagtcaacgaatcgctagaaaggggcggacggca (0.72)
P644 (173 bp)	Cacgcgcagctgttttgaagccgtaagtgtctccagtcagcggtaaaatcatataccgctgactgggttctggctccaaagtggcaagaaacccagaaacccagtacaa***TG***tagc**G**agtgtcttgtgccgcaagcgcaagccaactacacgagacgtccaggaggacaattac (0.97)
919 (206 bp)	Tgaagtgtgtcgtggcgttgcgaaatgtcaaaggtggcgttatca***T***agaggA**A**ttgttgtgtttccctaaggggtccttcaaagcgctttcccactcgccatcgaggactttcagggagcccacggataatgaggccgaaaccgccaagcctgaccagcgcggcagtccaagcccggaacacaataagcaagaaaaggtacacca (0.81)
P1527 (576 bp)	Gcctcgatggcggcttcgggctggacagcgtccaagcaaacccgccgttatccgtatcgaaaccgcccgctgcgaccggccaaggtaccccatgtggccgtatctcgttttcaaggggttgatttcggctccgccggacatctatctgcgtttcaaggggcgccaaaacgcgatatccgaagtatgatcggcgaaataccaacggaatcatgcggttcttgggccacttatactttgggaccaacccctcgaaacgcagataggcattcggaacaacacgaatcaaccccgaaatgcagatagccccgaatcctccgccagtgaatgccgaccaggctttcaagcatcacgtcagacaccttgcgcgccgatgtttttgccacatcgaccccgacgatcgctcgccagctccagcgtgtcgggtatccaccccctatgcgttataatttatcggttattcgtgatgggtggagccctctcattctgcctttcatgaggaacacgtgggttctcggttgaggtgccccacacacaatcgaaaa***T***ccgca**A**acctagtattacaaggagagccatt (0.90)
P888 (164 bp)	Ttgccgctgccggtcgtatcgccggcaggagcaaggataaagcaagaaattataacgggtcatgcacgcctcggcgtgtcgtgacccgttatgatatattctttaaatttggtg**T**gtcttcggacatacaag***TG***taa**T**acacccaA**gga**atggactgt**aagg**ct (0.93, 0.78, 0.96)

Predicted promoter regions are underlined and transcription start points are in bold and capitalized. Putative TG-dinucleotide is indicated in bold italic and putative ribosome binding site are shown in gray boxes. The numbers in parentheses are predicted promoter scores.

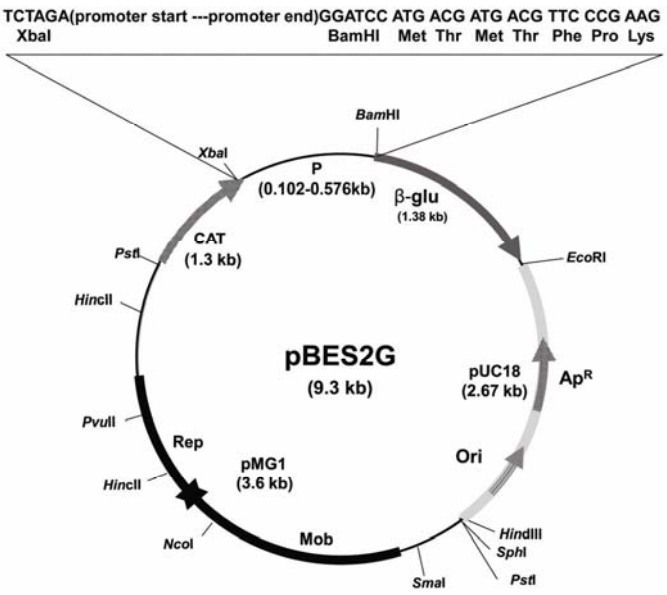

Fig. 1. Schematic map of the promoter screening vector pBES2G containing the *E. coli* origin of replication (Ori). The β-glucosidase reporter gene is in broken line, the chloramphenicol acetyl transferase gene (CAT) is light gray, and the *B. longum* replicon pMG1 that predicted replication proteins Rep is in black. The cloning sites at the start of the β-glucosidase reporter gene are expanded and annotated with restriction sites (*Xba*I and *Bam*HI) plus translation products above the plasmid.

834 were in between. They were predicted to be involved in ribosomal structure (644, 1527, 919, 888), translation (895), cell wall biogenesis (834), and putative inner membrane protein (632). Accordingly, we assumed that these 7 genes have their own promoters that do not need any induction.

Cloning and sequence analysis of the putative promoter genes

The putative promoter regions of the 7 genes were PCR

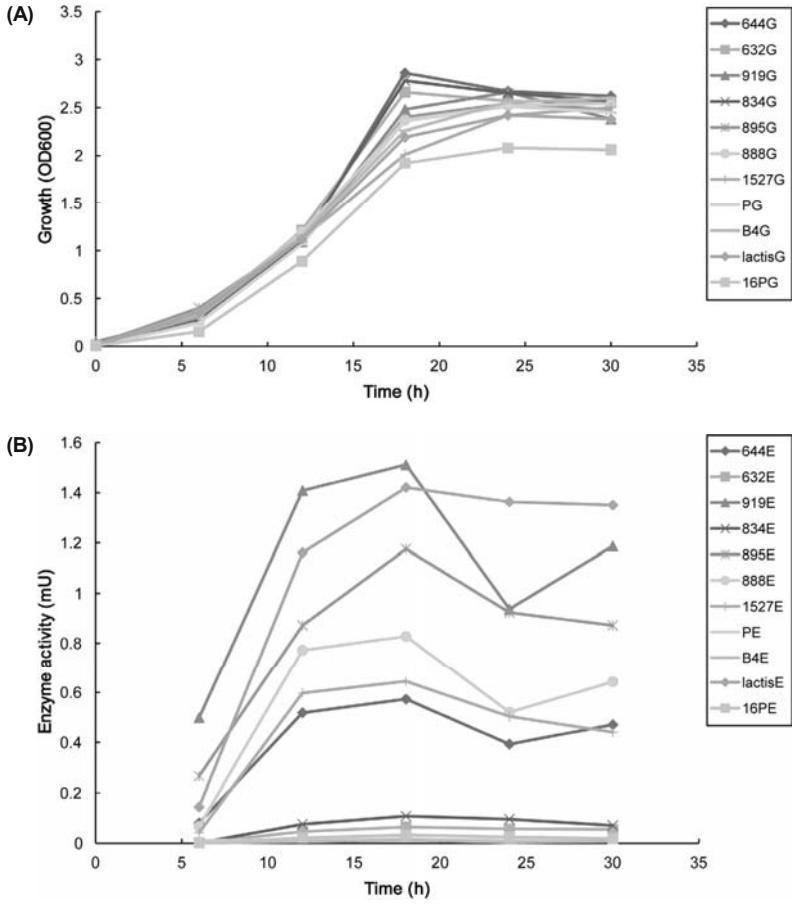

Fig. 2. Cell growth (A) and β-glucosidase activities (B) of recombinant *B. bifidum* BGN4 harboring each numbered promoter. In the legends, G and E mean growth (OD_{600}) and enzyme activity of each transformant, respectively. P means recombinant with pBES2G and 16P means recombinant with 16S rRNA promoter of *B. longum* MG1. B4 and lactis mean *B. bifidum* BGN4 and *B. lactis* AD011.

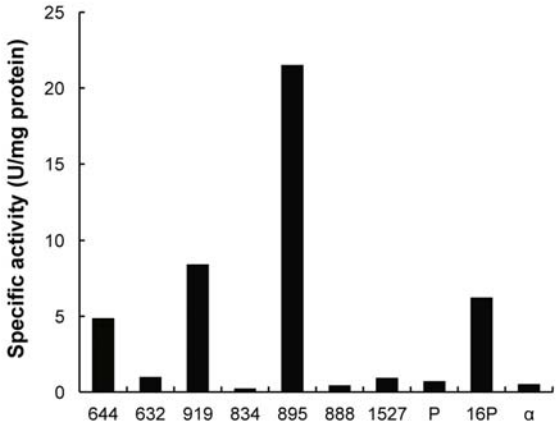

Fig. 3. Specific β-glucosidase activities of recombinant *E. coli* DH5α harboring various promoters grown in LB medium at 7 h. P, pBES2G; 16P, 16S rRNA promoter from *B. longum* MG1; α, *E. coli* DH5α

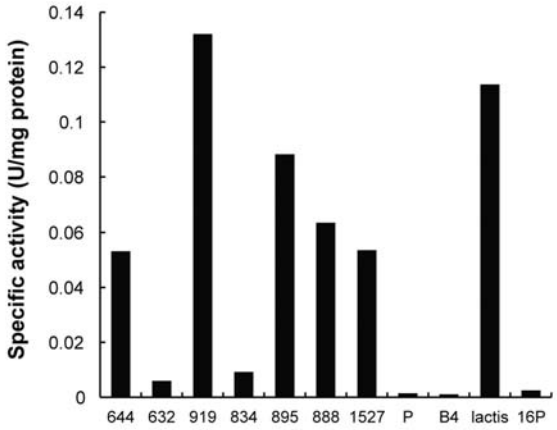

Fig. 4. Specific β-glucosidase activities of *B. lactis* AD011 and recombinant *B. bifidum* BGN4 with various promoters and in MRS at 18 h. The legends for each strain are the same as those of Fig. 2.

amplified and cloned as described in the 'Materials and Methods' section. The amplified fragments were 102–576 bp in size. The promoter prediction revealed one possible promoter region in P632, P834, P895, P919, P888, P1527, and three in P888. The -16 sequence and TG motif was observed (Table 4).

Construction of expression vectors using promoters

The β-glucosidase gene (YP_002469020 of CP001213) from *B. lactis* AD011 was cloned into *E. coli-Bifidobacterium* shuttle vector pBES2 to construct pBES2G (Fig. 1). β-Glucosidase was used as a reporter to evaluate each promoter's expression level. Each putative promoter region was inserted upstream of β-glucosidase gene in the pBES2G vector and transferred to *E. coli* DH5α (Fig. 1). The promoterless vector pBES2G was used as a negative control. Each recombinant expression vector was then purified from *E. coli* DH5α, methylated *in vitro*, and transformed into *B. bifidum* BGN4.

Cell growth and promoter activity assay

To examine the activity of each promoter, cells of all wild-type and recombinant strains were cultivated as described in the 'Materials and Methods' section. The promoter activities were analyzed by measuring β-glucosidase using *p*-nitrophenyl-β-glucopyranoside as a substrate. As expected, all promoters were shown to produce β-glucosidase when *B. bifidum* BGN4 was used as the expression host (Fig. 2), whereas promoterless vector and wild-type *B. bifidum* BGN4 did not show β-glucosidase activity (Fig. 2). The β-glucosidase activity increased with cell growth and decreased after the stationary phase. Maximal activity was observed at 18 h at the final stage of the exponential phase (Fig. 2).

The P919 promoter showed the strongest specific β-glucosidase activity among the promoters examined in *B. bifidum* BGN4 at 18 h, while the others were weaker than that of the original host *B. lactis* AD011.

The enzyme activities of recombinant *E. coli* DH5α were higher than their corresponding activities of recombinant *B. bifidum* BGN4 and the maximum activity was detected at 7 h (data not shown). Among them, P895 was the strongest in *E. coli* DH5α and P919 was the second. Recombination with P888, P834, P1527, or without promoter produced little activity as shown in wild-type *E. coli* DH5α (Fig. 3).

Discussion

Microarray analysis of total RNA from *B. bifidum* BGN4 revealed 7 genes showing relatively high-level expression among 1,000 genes during cultivation with or without pH control. However, these data represent promoter strength at the transcription level. The promoters should be analyzed at the translation level to determine their strength as well. For this reason, we established a promoter screening host-vector system using β-glucosidase as a reporter and β-glucosidase-negative *B. bifidum* BGN4 as a host. Seven putative promoters from the *B. bifidum* BGN4 were then cloned and their promoter activities were analyzed by the measurement of β-glucosidase activity in both *B. bifidum* BGN4 and *E. coli* DH5α. Based on these results, P919 and P895 yielded the strongest enzyme activities, while microarray showed that P644 and P1527 were stronger than P919 and P895 (Table 3). This result suggests that we have to consider the expression levels at both the translational and the transcriptional stages to screen strong promoters.

In our previous study, we constructed an expression vector using a 16S rRNA promoter from *B. longum* MG1 and successfully expressed cholesterol oxidase in both *E. coli* and *B. longum*. However, this promoter unexpectedly failed to produce β-glucosidase activity in *B. bifidum* BGN4 (Fig. 4), although it showed moderate enzyme activity in *E. coli* DH5α (Fig. 4). This might be explained by the absence of homology between the promoter region of the 16S rRNA of *B. bifidum* and that of *B. longum* (data not shown). This result indicates that each promoter has host specificity. These genes were predicted to encode ribosomal proteins (919, 895, 1527) and the peptide elongation factor Tu (644). Kim

et al. (2009) also cloned the tuf promoter as a strong promoter for *Lactococcus* according to microarray analysis of *Lactococcus lactis*.

Although one study reported that lactic acid bacteria promoters have the similar activities in *L. lactis* and *E. coli* (Jeong *et al.*, 2006), there is no such report for *Bifidobacterium*. In this study, the promoter activities in *E. coli* DH5α were higher than those in *B. bifidum* BGN4. This might be due to the host range of the respective promoters (Kim *et al.*, 2009). The copy number of the same shuttle vectors may affect their promoter activities when they are present in different hosts. The *E. coli–Bifidobacterium* shuttle vector pBES2 harbors *ori* for *E. coli* and *B. longum* MG1. The difference of the *Bifidobacterial* species can affect the activity of the promoter and *ori*. The growth rates of the recombinant *E. coli* DH5α at 7 h were also greater than those of recombinant *B. bifidum* BGN4 at 18 h.

The analysis of the activity patterns of the promoter in *B. bifidum* BGN4 suggested that promoter activity was growth-associated, revealing a general characteristic of constitutively expressed genes. There was no significant difference in growth patterns between *B. bifidum* BGN4 and recombinant *B. bifidum* BGN4 strains (Fig. 2). This result suggested that the strong promoters developed in this study did not inhibit the growth of *B. bifidum* BGN4.

Many studies have shown that the -10 and/or -35 hexamer sequences greatly affect promoter strength (McCracken *et al.*, 2000). Additionally, other factors such as TG motif (Helmann, 1995), spacers, UP elements (Estrem *et al.*, 1998), and promoters' 3-dimensional structures were reported to affect transcription efficiency (Jensen and Hammer, 1998). In many Gram-positive bacteria, the -16 region TG motif was observed 1 bp upstream of the -10 sequence (Voskuil and Chambliss, 1998). However, the -16 region TG motif was found -6 bp upstream of the -10 sequence in the 7 promoters of *B. bifidum* BGN4 (Table 4).

When the putative promoter sequences were analyzed using NCBI BlastN database, all of them showed homology only within other *B. bifidum* genomes except for P919. Interestingly, sequences that were highly homologous with P919 were found in *B. bifidum* (100%), *Bifidobacterium dentium* (90%), *B. longum* (95%), *Bifidobacterium adolescentis* (88%), and *Bifidobacterium animalis* (83%). Therefore, we suggest that promoter P919 may work in these *Bifidobacterium* species with strong activity. The presently characterized promoters will be useful for the development of food-grade expression systems for *Bifidobacterium*.

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