Experimental determination and characterization of the gap promoter of Bifidobacterium bifidum S17

Zhongke Sun¹, Christina Westermann¹, Jing Yuan², and Christian U Riedel¹*

¹Institute of Microbiology and Biotechnology; University of Ulm; Ulm, Germany; ²Institute of Disease Control and Prevention; Academy of Military Medical Sciences; Beijing, China

Keywords: bifidobacteria, probiotics, expression, promoter, constitutive

Introduction

Bifidobacteria are an ecologically, functionally, and numerically important bacterial group of the intestinal microbiota. In naturally delivered, breast-fed infants, bifidobacteria account for up to 95% of all bacteria in stool samples, making them by far the predominant component of the fecal microbiota in this group.¹-³ Despite a significant reduction in bifidobacterial numbers after weaning, bifidobacteria are still an important group of the fecal microbiota of adults.¹,² A number of health promoting effects have been attributed to the presence of bifidobacteria in the gastrointestinal tract. These positive effects include cholesterol reduction, improvement of lactose intolerance, alleviation of constipation, resistance against pathogen colonization, alleviation of intestinal inflammation, and prevention of colon cancer.⁴-⁶ Based on these properties bifidobacteria are widely used in functional foods as so-called probiotics, i.e., microbial food supplements, which confer a health benefit when administered in adequate amounts.⁷ Most data on health-promoting effects of probiotics in general and bifidobacteria in particular has been obtained in studies performed in vitro or in animal models. So far, the European Food Safety Authority has rejected all of the claims submitted for probiotics based on the fact that they have not been substantiated sufficiently in clinical trials. This stresses the need for a clear definition of target groups and relevant biomarkers and a more detailed analysis of the underlying molecular mechanisms.

The most widely used approaches to investigate the contribution of a given gene to an observed effect are either generation of defined mutants or overexpression of the gene in question. However, most bifidobacteria harbor several restriction-modification systems resulting in transformation efficiencies that are below levels required for targeted mutagenesis using suicide vector systems.⁸,⁹ In the absence of efficient systems for targeted mutagenesis, systems for efficient expression are indispensable for the analysis of single genes. Also, efficient expression systems are required to generate recombinant bifidobacteria with enhanced health-promoting properties of bifidobacteria, increased viability during gastrointestinal transit, or strains that can be used as live vaccines or gene delivery vectors for tumor therapy.⁸,¹⁰,¹¹

Constitutive or inducible promoters with high transcriptional activity are key to efficient protein expression. A number of promoters of various Bifidobacterium sp. strains have been determined experimentally. These promoters were shown to be

The DNA sequence upstream of the glyceraldehyde 3-phosphate dehydrogenase gene (gap) of various strains of bifidobacteria is used in a number of vector systems for homologous and heterologous expression in this group of bacteria. To date none of the bifidobacterial gap promoters (Pgap) have been verified experimentally. Here, we probe a range of putative bifidobacterial promoters hypothesized to show high constitutive transcriptional activity using a β-glucuronidase reporter system. In silico analysis revealed a predicted bacterial promoter upstream of the gap gene of Bifidobacterium bifidum S17. The corresponding DNA sequences was cloned into the promoter probe vector pMDY23 and yielded highest reporter activities among the promoter sequences tested confirming previous studies. Using rapid amplification of cDNA ends (5′-RACE), we identified the transcription start site (TSS) of Pgap of B. bifidum S17. The experimentally determined TSS and the associated -10 and -35 regions do not match with the promoter predicted in silico. Moreover, a potential ribosome-binding site (RBS) was identified upstream of the ATG start codon of the gap gene, which is complementary to the 3′-end of the 16S rRNA with only 1 mismatch suggesting efficient initiation of translation. Alignment of the Pgap sequences of a number of representative bifidobacteria showed a high level of conservation and the presence of -35 and -10 regions, which are similar but not identical to the consensus promoter sequences of house-keeping genes of Escherichia coli and Bacillus subtilis. Collectively, these results confirm the suitability of Pgap for high level, constitutive expression in bifidobacteria.

*Correspondence to: Christian U Riedel; Email: christian.riedel@uni-ulm.de
Submitted: 07/07/2014; Revised: 08/11/2014; Accepted: 08/11/2014
http://dx.doi.org/10.4161/bioe.34423
regulated by specific carbon sources, environmental stresses including acidic pH, bile, nutrient limitation, or host derived proteases. In most cases, the corresponding transcriptional regulators have been identified. Additionally, a number of sequences upstream of genes that are thought to be constitutively expressed were used for homologous or heterologous expression in bifidobacteria. These include the sequences up stream of the 16S rRNA, gap, and hup genes of different Bifidobacterium sp. However, none of these promoters were determined experimentally or analyzed in more detail.

In the present study, we analyzed a selection of promoters hypothesized to be constitutively active in bifidobacteria. Furthermore, we determined the promoter elements of the gap promoter of B. bifidum S17, which was recently used successfully for expression of fluorescent proteins in different Bifidobacterium sp.

Results and Discussion

Four different promoter regions were assayed for transcriptional activity using the promoter probe vector pMDY23. The range of promoters tested include the DNA sequences upstream of the gap gene encoding glyceraldehyde-3-phosphate dehydrogenase (Pgap) and the luxS gene for the methylthioadenosine/S-adenosyl-homocysteine (MTA/SAH) nucleosidase LuxS (PluxS) of B. bifidum S17. Furthermore, the sequences upstream of the gene for histone-like HU protein (P hup) and the 16S rRNA gene (P16S) of B. longum NCC2705 were included. Pgap and Phup were already used for protein expression in bifidobacteria by different groups. PluxS was assumed to show transcriptional activity based on the detection of autoinducer-2 activity in culture supernatants of B. longum NCC2705.

Promoter regions upstream of the genes were amplified by PCR and cloned upstream of the gusA reporter gene in pMDY23 (Fig. 1). The cloned plasmids were transformed into B. bifidum S17 and supernatants of late logarithmic cultures grown in MRSc were assayed for GusA reporter activity. Highest GusA activities were measured for B. bifidum S17/pMDY23-Pgap (Fig. 1). Markedly lower GusA activity was measured for Phup.

This is in line with a previous study by our group showing higher Pgap-driven reporter activity in B. bifidum S17 compared with other promoters in all growth phases tested. Furthermore, several other groups also reported high transcriptional activity of bifidobacterial gap promoters and these promoters have been used successfully for expression in bifidobacteria.

The enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) encoded by the gap gene catalyzes the conversion of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate. Although GAPDH is primarily known for its role in glycolysis, it is also required for formation of lactate from glyceraldehyde 3-phosphate and, in consequence, efficient energy conservation from substrates that are fermented via the bifidus shunt. Thus, it seems reasonable to hypothesize that the gap gene is constitutively expressed at high levels in a wide range of bacteria including bifidobacteria.

Since Pgap showed highest transcriptional activity in B. bifidum S17 compared with other promoters tested, we sought to analyze this promoter in more detail. The 5′-untranslated region upstream of the ATG start codon of gap contains a putative ribosome-binding site (RBS, Fig. 2A). During initiation of translation, the RBS interacts with a sequence motif in the 3′-end of the 16S rRNA termed anti-Shine-Dalgarno sequence (anti-SD). The anti-SD of B. bifidum S17 is identical to that of other Gram-positive bacteria including Bacillus subtilis and Lactococcus lactis. Moreover, the gap RBS and anti-SD of B. bifidum S17 are highly complementary with only one mismatched base pair to the anti-SD (Fig. 2A) indicative of efficient translation initiation.

In silico analysis suggested a putative bacterial promoter in the cloned gap promoter region with a predicted transcriptional start site (TSS) 114 base pairs upstream of the gap ATG start codon (Fig. 2B). Moreover, to confirm the transcription start site of the gap gene of B. bifidum S17 experimentally 5′-RACE was performed on RNA samples prepared from two independent cultures of B. bifidum S17 grown on MRSc to late exponential growth phase. For each RNA sample, inserts of two randomly selected E. coli clones containing pJet1.2-derivatives were sequenced. Alignment of all four inserts unequivocally
indicated a thymidine residue 62 bp upstream of the ATG start codon of the \textit{gap} open reading frame as TSS (Fig. 3), which is considerable different to the predicted TSS (Fig. 2B).

Upon visual analysis, no sequences motifs with obvious homology to the consensus -10 (TATAAT) or -35 (TTGACA) sequences of $\sigma^A$-dependent promoters of \textit{B. subtilis}, i.e., the prototype house-keeping promoter of Gram-positive bacteria, could be identified. In order to identify potential -35 and -10 regions, the $P_{\text{gap}}$ sequence of \textit{B. bifidum} S17 was aligned to the DNA sequences upstream of the \textit{gap} genes of five other \textit{Bifidobacterium} sp. The results suggest a -35 region with the sequence TTGCTC, a spacer of 17 bases, and a -10 region consisting of the nucleotides TACAGT for $P_{\text{gap}}$ of \textit{B. bifidum} S17 (Fig. 4A).

The consensus -35 and -10 sequences of the analyzed \textit{gap} promoters of bifidobacteria are TTGGCN and TANAGT, respectively, with a spacer of 17–19 bases (Fig. 4A). Compared with the canonical -35 (TTGACA) and -10 (TATAAT) regions of $\sigma^{70}$- and $\sigma^A$-dependent promoters of \textit{E. coli} and \textit{B. subtilis}, the bifidobacterial $P_{\text{gap}}$ consensus -35 and -10 sequences each differ in two nucleotides (Fig. 4B). However, the TTG motif in the -35 and the four nucleotides TANANT in the -10 region showing highest conservation in $\sigma^{70}$ or $\sigma^A$-dependent promoters of a wide range of other bacteria\textsuperscript{27-29} are present.

Using BLAST analysis, a gene (\textit{bbif}_1216) was identified in the genome of \textit{B. bifidum} S17, whose deduced amino acid sequence showed high homology to the prototype $\sigma^A$ of \textit{B. subtilis}.\textsuperscript{30} The protein contains Sigma-70 factor regions 1.2, 2, 3, and 4 (residues 205–240, 272–342, 351–427, and 440–493, respectively) with a helix-turn-helix motif for DNA binding and a motif for interaction with the RNA polymerase $\beta^\prime$ core subunit in region 2. Together with the high conservation of the -35 and -10 sequences of $P_{\text{gap}}$, this suggests that the gene product of \textit{bbif}_1216 is likely to be the house-keeping sigma factor $\sigma^A$ of \textit{B. bifidum} S17 directing the RNA polymerase to $P_{\text{gap}}$ to initiate transcription.

Collectively these results confirm high constitutive transcriptional activity of the $P_{\text{gap}}$ promoter of \textit{B. bifidum} S17 in line with a role of its gene product GAPDH in the central metabolism. The overall structure of the \textit{gap} promoter indicates that transcription of the \textit{gap} gene is dependent on the house-keeping $\sigma^A$ encoded by \textit{bbif}_1216 ($\textit{rpoD}$) and the high complementarity of the RBS to the Anti-SD suggests efficient translation. This makes
Materials and Methods

Bacterial strains, plasmids, and growth conditions

All strains and plasmids used in this study are listed in Table 1. E. coli DH10B was used as cloning host and was aerobically grown in Luria broth (LB) at 37 °C. B. bifidum S17 was cultivated in Lactobacilli MRS medium (BD DifcoTM) supplemented with 0.5 g/L L-cysteine (Sigma) at 37 °C. Anaerobic conditions were achieved by growth in sealed jars and removal of oxygen by AnaeroGenTM (Oxoid) sachets. Where appropriate 200 μg/ml spectinomycin was added for selection.

Cloning of promoter regions

Genome sequences of B. bifidum S17 (GeneBank ID: CP002220.1) and B. longum NCC2705 (GeneBank ID: AE014295.3) are publicly available. Promoter regions upstream of the gap (bbif_0612) and luxS genes (bbif_1299) of B. bifidum S17 and the hup (bl1798) and 16S rRNA (blr01) genes of B. longum NCC2705 were amplified from chromosomal DNA by PCR using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) and specific primer pairs (Table 2). PCR products were digested with restriction enzymes BglII and XhoI, ligated to BglII/XhoI restricted pMDY23,21 and ligation mixes were electroporated31 into E. coli DH10B. Following selection on LB agar containing 200 μg/ml spectinomycin, plasmids of positive clones were isolated and cloned DNA sequences were confirmed by restriction analysis and Sanger sequencing. Plasmids containing the correct promoter regions were transformed into B. bifidum S17 according to a published protocol.32

β-glucuronidase assay

Fresh overnight cultures of MRSc grown B. bifidum S17 strains harboring pMDY23 derivatives containing the cloned promoter regions upstream of the gusA gene were used to inoculate 10 ml MRSc to an initial optical density at 600 nm (OD600) of 0.1. Cultures were grown under standard conditions for 8 h, i.e., to late exponential growth phase, when cells were harvested for quantification of β-glucuronidase (GusA) reporter activity.

GusA activity was measured in crude extracts as described previously.20 Briefly, crude extract containing 1–5 μg total protein as determined using 2-D Quant kit (GE Healthcare Life Science) was brought to a volume of 100 μl reaction with GUS assay buffer (50 mM Na2HPO4 pH 7, 1 mM EDTA, 0.1% Triton X-100, 5 mM dithiothreitol) and transferred to the wells of a
standard 96-well microtiter plate. Reactions were started by adding 25 μl of a 5 mM 4-methylumbelliferyl-β-D-glucuronide solution and incubated for 30 min at 37 °C. Then 10 μl of the reaction mix were transferred into a fresh 96-well plate containing 190 μl stop buffer (0.2 M Na2CO3 in H2O, pH 9.5). The concentration of 4-methylumbelliferone produced from 4-methylumbelliferyl-β-D-glucuronide by GusA was quantified by measuring fluorescence using a Tecan infinite M200 plate reader with excitation at 388 nm and emission at 480 nm. The enzyme activity was defined as relative fluorescence units per mg protein (RFU/mg). For each promoter fragment, crude extracts of three independent cultures were analyzed in triplicate.

Determination of the Pgap TSS

The TSS of Pgap was experimentally determined by 5’0-RACE as described elsewhere. Total RNA was isolated from 10 ml of an MRSc-grown overnight culture of B. bifidum S17 as described previously. Genomic DNA was removed by DNase I digestion and samples were purified using RNeasy Mini Kit (Qiagen). To obtain gap cDNA transcripts, cDNA was synthesized from 2 μg total RNA using the gene-specific primer GSPR1 (Table 2) and the 2nd generation 5’0/3’0 RACE Kit (Roche) according to the manufacturer’s instructions. First strand cDNA transcripts were purified with High-Pure PCR Clean-up Kit (Roche) before appending a homopolymeric tail using terminal deoxyribonucleotidyl transferase and dATP. The tailed cDNA was directly amplified by PCR using the oligo dT-anchor primer and the gene specific primer GSPR3 (Table 2). To increase specificity, a second round of PCR was performed on the PCR products using the anchor primer and the gene specific primer GSPR4 (Table 2). PCR products were resolved by electrophoresis on a 1.5% (w/v) agarose gel and specific amplicon bands were excised.

Table 1. Bacterial strains and plasmids used in this study

| Strain | Characteristics | Source/Reference |
|--------|-----------------|-----------------|
| E. coli DH10B | cloning host | InvitrogenTM |
| Bifidobacterium sp. | intestinal isolate from a breast-fed infant |  |
| B. longum subsp. longum NCC2705 | type strain |  |
| B. bifidum S17/pMDY23 | B. bifidum S17 harboring pMDY23 |  |
| B. bifidum S17/pMDY23-Pgap | B. bifidum S17 harboring pMDY23-Pgap |  |
| B. bifidum S17/pMDY23-PgapS | B. bifidum S17 harboring pMDY23-PgapS | this work |
| B. bifidum S17/pMDY23-P16S | B. bifidum S17 harboring pMDY23-P16S | this work |

Plasmid

| Relevant characteristics |
|--------------------------|
| pMDY23 | E. coli-Bifidobacterium shuttle vector harboring the gusA reporter gene, template for amplification of spc gene, Spc’ |
| pMDY23-Pgap | expression of gusA driven by the Pgap promoter, Spc’ |
| pMDY23-PgapS | expression of gusA driven by the Pgap promoter, Spc’ |
| pMDY23-P16S | expression of gusA driven by the P16S promoter, Spc’ |
| pJET1.2 | Blunt-end cloning vector |

| Table 2. Sequence and other relevant information of oligonucleotides used in this study |
|--------------------------|
| **Oligonucleotide/Purpose** | **Sequence (5’ → 3’)** | **Source and/or Reference** |
|--------------------------|
| **Amplification of promoters** |
| Pgap-f_BglII | GAAGATCTGGCGAAATGCCTGCATGAATC | this work |
| Pgap-f_Fhol | GCCCTCGAGCTCCTGGATCGAGT | this work |
| Pgap-f_BglII | GAATCTACGAGGAGTACCGGTGGATCG | this work |
| Pgap-f_Fhol | GCCCTCGAGCTCCTGGATCGAGT | this work |
| Pgap-f_BglII | GAAGATCTACGAGGAGTACCGGTGGATCG | this work |
| Pgap-f_Fhol | GCCCTCGAGCTCCTGGATCGAGT | this work |
| Pgap-f_BglII | GAAGATCTACGAGGAGTACCGGTGGATCG | this work |
| Pgap-f_Fhol | GCCCTCGAGCTCCTGGATCGAGT | this work |
| Pgap-f_BglII | GAAGATCTACGAGGAGTACCGGTGGATCG | this work |
| Pgap-f_Fhol | GCCCTCGAGCTCCTGGATCGAGT | this work |
| **5’-RACE** |
| oligo dT anchor | GACCCCGGATATGCAGTGCTGCACTGTTTTTTTTTTTTTTTTT | Roche |
| PCR anchor | GACCCCGGATATGCAGTGCTGCACTGTTTTTTTTTTTTTTT | Roche |
| GSPR1 | GGAGGTGAGGAGGATGTTGTA | this work |
| GSPR3 | GTTCCTGACCCAGGGATG | this work |
| GSPR4 | CATCTCTCGGCGTAGACC | this work |

*recognition sequences of relevant restriction enzymes are underlined.*
and purified from the gel using NucleoSpin Extract II kit (Macherey-Nagel). Purified DNA fragments were ligated into blunt end cloning vector pET1.2 (Thermo Scientific) and ligations transformed into E. coli DH10B. Transformants were selected by plating on LB agar containing 100 μg/ml ampicillin and inserts of positive clones were sequenced by Sanger sequencing (GACT Biotech) using primer pET1_PF provided with pET1.2 kit.

Bioinformatic sequence analysis

Sequences upstream of the gap genes of different bifidobacteria were downloaded from the NCBI database. Bacterial promoter prediction was performed using the online BPROM software tool. Sequences obtained from 5′ and inserts of positive clones were sequenced by Sanger sequencing (Macherey-Nagel). Purified DNA fragments were ligated into and purified from the gel using NucleoSpin Extract II kit (Clontech).

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

Funding

This study was partially funded by the German Academic Exchange Service and the German Ministry of Education and Research (grant D/09/04778) to C.U.R. Z.S. was supported by a Ph.D. fellowship of the German Academic Exchange Service. C.W. received a Ph.D. fellowship of the Landesstiftung Baden-Württemberg (Grant No. 1215 LGGF-E).

References

1. Kurokawa K, Ikeh T, Kuwahara T, Oshika K, Toh H, Toyoda A, Takami H, Morita H, Sharma V, Sivritsaina TP, et al. Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. DNA Res 2007; 14:169-41; PMID:17916580; http://dx.doi.org/10.1093/dnares/dnm018
2. Yatsunenko T, Rey F, Manary MJ, Trehan I, Dlugosz-Bello MG, Cortesas M, Magrini M, Hidalgo G, Baldassano RN, Anshum AP, et al. Human gut microbiome viewed across age and geography. Nature 2012; 486:222-7; PMID:22696611
3. Turlron F, Peano C, Pass DA, Foroni E, Severgnini M, Claesson MJ, Kerr C, Hourihane J, Murray D, Fulgini F, et al. Diversity of bifidobacteria within the infant gut microbiota. PLoS One 2012; 7:e36957; PMID:22606315; http://dx.doi.org/10.1371/journal.pone.0036957
4. Lee JH, O’Sullivan DJ. Genomic insights into bifidobacteria. Microbiol Mol Biol Rev 2010; 74:378-416; PMID:20805404; http://dx.doi.org/10.1128/MMBR.00044-10
5. Leahy SC, Higgins DG, Fitzgerald GF, van Sinderen D. Getting better with bifidobacteria. J Appl Microbiol 2005; 98:1303-15; PMID:16167966; http://dx.doi.org/10.1111/j.1365-2672.2005.02620.x
6. Picard C, Fioretanto J, Francois A, Robinson T, Neant D, Getting better with bifidobacteria. J Biol 2005; 22587027; http://dx.doi.org/10.1128/AEM.01951-12
7. FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics. Guidelines for the Evaluation of Probiotics in Food. 2002.
8. Sun Z, Baur A, Zhurina D, Yuan J, Riedel CU. Expression of fluorescent protein lacZ in Bifidobacterium breve UCC2003. Microb Biotechnol 2014; 7:2351-60; PMID:24505453; http://dx.doi.org/10.1111/mbt.12186
9. Grimm V, Gleisner M, Neu C, Zhurina D, Riedel CU. Expression of a fluorescent protein in Bifidobacterium breve UCC2003 for analysis of host-microbe interactions. Appl Environ Microbiol 2014; 80:264-402; PMID:24584243; http://dx.doi.org/10.1128/AEM.04261-13
10. Pruin K, Neves AR, Zomer A, O’Connell-Motherway M, Clynes M, Mercenier A, Arigoni F, Pedrone RD. Construction of a reporter vector for the analysis of Bifidobacterium longum promoters. Appl Environ Microbiol 2006; 72:7401-5; PMID:16979785; http://dx.doi.org/10.1128/AEM.00130-14
11. Sun Z, He X, Brancaccio VF, Yuan J, Riedel CU. Bifidobacteria exhibit LuxS-dependent autocoider 2 activity and biofilm formation. PLoS One 2014; 9:e88260; PMID:24505453; http://dx.doi.org/10.1371/journal.pone.0088260
12. Khokhlova EV, Efimov BA, Kafarskaia II, Shkorpov AN. Heterologous expression of secreted biologically active human interleukin-10 in Bifidobacterium breve. Arch Microbiol 2010; 192:769-74; PMID:20631991; http://dx.doi.org/10.1007/s00203-010-0606-4
13. O’Connell KJ, Motherway MO, Liedtke A, Fitzgerald GF, van Sinderen D. Ribose utilization by the human commensal Bifidobacterium breve UCC2003. Appl Environ Microbiol 2014; 80:3604-14; PMID:24705323; http://dx.doi.org/10.1128/AEM.00130-14
14. Pokusaeva K, Neves AR, Zomer A, O’Connell-Motherway M, Macharry J, Curley P, Fitzgerald GF, van Sinderen D. Ribose utilization by the human commensal Bifidobacterium breve UCC2003. Microb Biotechnol 2010; 3:311-23; PMID:21255330; http://dx.doi.org/10.1111/j.1751-7915.2009.00152.x
15. Pokusaeva K, O’Connell-Motherway M, Zomer A, Macharry J, Fitzgerald GF, van Sinderen D. Cellodextrin utilization by bifidobacterium breve UCC2003. Appl Environ Microbiol 2011; 77:1661-79; PMID:21216899; http://dx.doi.org/10.1128/AEM.01786-10
16. Trindade MI, Abrav RT, Reid S. Induction of sucrose utilization genes from Bifidobacterium lactis by sucrose and raffinose. Appl Environ Microbiol 2003; 69:24-32; PMID:12513973; http://dx.doi.org/10.1128/AEM.70.1.101-123.2005
17. Roz LA, Huynh N, Carding SR. Defining the bacterioides ribosomal binding site. Appl Environ Microbiol 2013; 79:1980-9; PMID:23335775; http://dx.doi.org/10.1128/AEM.03086-12
18. Makrides SC. Strategies for achieving high-level expression of genes in Escherichia coli. Microbiol Rev 1996; 60:512-38; PMID:8840785
19. Huerta AM, Francino MP, Moret E, Collado-Vides J. Selection for unequal densities of sigma70 promoter-like signals in different regions of large bacterial genomes. PLoS Genet 2006; 2:e185; PMID:17065948; http://dx.doi.org/10.1371/journal.pgen.0020185
20. Helmann JD. Compilation and analysis of Bacillus subtilis promoters. Arch Microbiol 2005; 186:77-90; PMID:15755955; http://dx.doi.org/10.1007/s00203-004-0481-5
21. Petersen HU. Initiation of protein synthesis in bacteria. Microbiol Mol Biol Rev 2005; 69:101-23; PMID:15755955; http://dx.doi.org/10.1128/MMBR.69.1.101-123.2005
22. van Veen W, Hoorn N, Carding SR. Defining the bacterioides ribosomal binding site. Appl Environ Microbiol 2013; 79:1980-9; PMID:23335775; http://dx.doi.org/10.1128/AEM.03086-12
23. Makrides SC. Strategies for achieving high-level expression of genes in Escherichia coli. Microbiol Rev 1996; 60:512-38; PMID:8840785
24. Huerta AM, Francino MP, Moret E, Collado-Vides J. Selection for unequal densities of sigma70 promoter-like signals in different regions of large bacterial genomes. PLoS Genet 2006; 2:e185; PMID:17065948; http://dx.doi.org/10.1371/journal.pgen.0020185
25. Helmann JD. Compilation and analysis of Bacillus subtilis sigma A-dependent promoter sequences; evidence for extended contact between RNA polymerase and upstream promoter DNA. Nucleic Acids Res. 1995; 23:2351-60; PMID:7630711; http://dx.doi.org/10.1093/nar/23.13.2351
26. Lasser S, Margalit H. Compilation of E. coli mRNA promoter sequences. Nucleic Acids Res 1993; 21:1507-16. PMID:8479906; http://dx.doi.org/10.1093/nar/21.7.1507
27. Haldenwang WG. The sigma factors of Bacillus subtilis. Microbiol Rev 1995; 59:13-30; PMID:7780809
31. Sheng Y, Mancino V, Birren B. Transformation of Escherichia coli with large DNA molecules by electroporation. Nucleic Acids Res 1995; 23:1990-6; PMID:7596828; http://dx.doi.org/10.1093/nar/23.11.1990

32. MacConaill LE, Fitzgerald GF, Van Sinderen D. Investigation of protein export in Bifidobacterium breve UCC2003. Appl Environ Microbiol 2003; 69:6994-7001; PMID:14660341; http://dx.doi.org/10.1128/AEM.69.12.6994-7001.2003

33. Scotto-Lavino E, Du G, Frohman MA. 5’ end cDNA amplification using classic RACE. Nat Protoc 2006; 1:2555-62; PMID:17406509; http://dx.doi.org/10.1038/nprot.2006.480

34. Gleinser M, Grimm V, Zhurina D, Yuan J, Riedel CU. Improved adhesive properties of recombinant bifidobacteria expressing the Bifidobacterium bifidum-specific lipoprotein BopA. Microb Cell Fact 2012; 11:80; PMID:22694891; http://dx.doi.org/10.1186/1475-2859-11-80

35. Solovyev V, Salamov A. Automatic Annotation of Microbial Genomes and Metagenomic Sequences. In: Li RW, Ed. Metagenomics and its Applications in Agriculture, Biomedicine and Environmental Studies. Nova Science Publishers; 2011. page 61-78.

36. Marchler-Bauer A, Lu S, Anderson JB, Chisnau F, Derbyshire MK, DeWeese-Scott C, Fong JH, Geer LY, Greer RC, Gonzales NR, et al. CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res 2011; 39:D225-9; PMID:21109532; http://dx.doi.org/10.1093/nar/gkq1189

37. Zhurina D, Zomer A, Gleinser M, Brancaccio VF, Aukter M, Waidmann MS, Westermann C, van Sinderen D, Riedel CU. Complete genome sequence of Bifidobacterium bifidum 517. J Bacteriol 2011; 193:301-2; PMID:21037011; http://dx.doi.org/10.1128/JB.01180-10

38. Schell MA, Karmitzantrou M, Snel B, Vilanova D, Berger B, Pessi G, Zwahlen M-C, Desiere F, Bork P, Delley M, et al. The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci U S A 2002; 99:14422-7; PMID:12381787; http://dx.doi.org/10.1073/pnas.212527599