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Original article

Heterologous gene expression and secretion in *Bifidobacterium longum*

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Abstract – Strains of the genus *Bifidobacterium* are used in fermented dairy products where they are supposed to exert health-promoting effects on the consumers. To study *Bifidobacterium* sp. properties, to improve the strains or to use them for safe delivery of vaccinal or anticarcinogenic polypeptides into the human digestive tract, it is strategically important to develop a system for heterologous expression and secretion. For this purpose, we constructed pBESAF2, a secretion vector based on the α-amylase expression and secretion signals (ESS) isolated from *Bifidobacterium adolescentis*. The signal peptide was analyzed in silico and its cleavage site was experimentally determined by N-terminal sequencing of the mature amylase. To validate this secretion vector, the phytase structural gene from *Escherichia coli* MC4100 was cloned downstream of the ESS and the resulting plasmid was introduced into *Bifidobacterium longum* MG1. Recombinant cells expressed phytase (with activity up to 21.1 U) and more than 95% of the phytase activity was found in the culture medium, suggesting that *E. coli* phytase can be produced and properly secreted and folded when expressed in *B. longum*. This is the first report of the development of a secretion vector for bifidobacteria and its use in a heterologous protein export in *B. longum*. This work is the first step towards the production of other proteins of food or therapeutic interest in bifidobacteria.

***Bifidobacterium* / secretion / heterologous expression**

Résumé – Expression hétérologue et sécrétion chez *Bifidobacterium longum.* Certaines souches du genre *Bifidobacterium* sont utilisées dans des produits laitiers fermentés pour leurs potentiels effets bénéfiques sur la santé de l'hôte. Pour étudier les propriétés de *Bifidobacterium* sp., pour améliorer les souches ou pour les utiliser afin de délivrer des protéines vaccinales ou anticancéreuses dans le tube digestif de l'homme, il est stratégiquement primordial de développer un système d'expression et sécrétion hétérologue pour *Bifidobacterium* sp. À cette fin, nous avons construit pBESAF2, un vecteur de sécrétion utilisant des signaux d'expression et de sécrétion (SES) d'une amylase isolée de *Bifidobacterium adolescentis*. Le peptide signal a été analysé in silico et son site de clivage a été déterminé par séquençage amino-terminal de l'amylase mature. Pour valider ce vecteur de sécrétion, le gène spécifiant la phytase d'*Escherichia coli* MC1400 a été cloné en aval des SES et le plasmide résultant a été introduit dans *B. longum* MG1. Les bactéries recombinantes exprimaient la phytase (avec une activité allant jusqu'à 21,1 U) et plus de 95 % de l'activité phytase était détectée dans le surnageant de culture. Ce travail décrit la mise au point du premier vecteur de sécrétion spécifiquement développé pour *Bifidobacterium* ainsi que son utilisation chez *B. longum* pour exprimer et sécréter une forme active de la phytase d'*E. coli*. Ce résultat ouvre la voie à la production d'autres protéines d'intérêt agro-alimentaire ou thérapeutique.

***Bifidobacterium* / sécrétion / expression hétérologue**

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1. INTRODUCTION

Bifidobacterium species are non-pathogenic, Gram-positive, anaerobic bacteria, which inhabit the intestinal tract of humans and animals [13]. In breast-fed infants, bifidobacteria represent more than 90% of the gut bacterial population [6]. Bifidobacteria have been proven to exert health-promoting effects on the host by maintaining intestinal microflora balances, reducing lactose intolerance and serum cholesterol levels, increasing vitamin synthesis, and boosting the immune system and intrinsic anticarcinogenic activity of the host [14]. Reflecting these beneficial properties, *Bifidobacterium* spp. are commonly used in commercial, fermented dairy products [20]. However, since these beneficial effects are strain-dependent, ongoing research aimed at improving strain characteristics of *Bifidobacterium* is warranted. In this regard, the development of a vector system for *Bifidobacterium* is strategically important, not only for strain improvement, but also because *Bifidobacterium* is a promising delivery system into the human intestinal tract for other useful gene products, such as antigens, in live vaccine development.

In a previous study, we cloned and characterized an expression and secretion signal (ESS) from *Bifidobacterium* [9]. In the present report we applied the ESS element for the development of an expression-secretion system for the genus *Bifidobacterium*.

Phytases catalyze the hydrolysis of phytate, thereby releasing inorganic phosphate [19]. These enzymes are of interest for biotechnological applications, in particular for improving dietary phytate-phosphorus utilization by simple-stomached animals such as swine and poultry [4]. Similar to humans, these species have little endogenous phytase activity, thus most of their ingested phytate is excreted. Supplemental phytase in pig diets is effective at improving the bioavailability of phytate-phosphorus, thereby decreasing phosphorus pollution [4].

In *Escherichia coli*, the *appA* gene codes for a bifunctional enzyme that exhibits both acid phosphatase and phytase activities. These activities have a pH optimum of 4.5 and are stable at pH values from 2 to 10, while their temperature optimum is 60 °C [18]. The mature AppA protein (45 $kg \cdot mol^{-1}$) is localized in the periplasmic protein of *E. coli,* while the AppA precursor contains a N-terminal signal peptide of 22 amino acids [5]. Manner et al. [12] studied the effectiveness of an *E. coli* phytase, in comparison with a commercially available *Aspergillus* phytase, at improving the bioavailability of phosphorus in broilers, layers and young pigs. They observed that *E. coli* consistently enhanced the availability of phytate in the same range or slightly more effectively than *Aspergillus* phytase, although the magnitude of this effect was quite small in layers and pigs.

In this report, we constructed an expression and secretion system using the genus *Bifidobacterium* and used it to express and secrete the *E. coli* AppA phytase in *B. longum* MG1.

2. MATERIALS AND METHODS

2.1. Bacterial strains, media and plasmids

The bacterial strains and plasmids used in this study are listed in Table I. *E. coli* DH5α was cultured at 37 °C in Luria broth with vigorous shaking. Ampicillin was used at a concentration of 50 $\mu g \cdot mL^{-1}$ and chloramphenicol at 3 $\mu g \cdot mL^{-1}$ for the selection of transformed bacteria. *Bifidobacterium* strains were grown in Brain Heart Infusion (BHI) broth (Difco, Detroit, USA) and MRS medium (Difco, Detroit, USA) supplemented with 0.05% (w/v) L-cysteine HCl at 37 °C.

2.2. General cloning techniques and sequence analysis

Plasmid DNA preparation from *E. coli*, restriction enzyme digestion, ligation and transformation of *E. coli* were carried out according to the procedure described by Sambrook et al. [21]. Plasmid DNA from

Table I. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Source or reference
Bifidobacterium strains		
B. adolescentis INT57	Source of ESS	[8]
B. longum MG1	Transformation host	[16]
E. coli strains		
DH5α	Cloning host	[7]
MC4100	Source of phytase gene (*appA*)	[3]
Plasmids		
pBES2	Cm^r, Amp^r; *E. coli-Bifidobacterium* shuttle vector	[16]
pYBamy59	Cm^r, Amp^r; pBES2 derivative containing a functional promoter and the structural gene for ESS	[9]
pBESAF2	Cm^r, Amp^r; pBES2 derivative containing a functional promoter and the ESS	This study
pBESAF2-appA	Cm^r, Amp^r; pBES2 derivative containing a functional promoter and ESS and *E. coli* phytase gene (*appA*)	This study

Bifidobacterium strains was prepared as described by Park et al. [15]. Nucleotide sequences were determined using the BigDye terminator and the ABI377 system (PE Applied Biosystems, Foster City, CA, USA). All restriction and modifying enzymes were purchased from Promega (Madison, WI, USA). Sequence analysis of the cloned gene was also performed using the BigDye terminator and the ABI377 system (PE Applied Biosystems, CA, USA). Signal sequence analysis was performed using the SignalP software program held at http://www.cbs.dtu.dk/Services/SignalP/ [2].

2.3. PCR amplification of DNA

In a previous study, a 2477 bp DNA fragment containing *amyB,* an amylase gene, was isolated from *B. adolescentis* INT57 and analyzed [9]. It was cloned into pBES2 to construct pYBamy59 and used to transform *B. longum* MG1, resulting in MG1 (amy59). The ESS was amplified from pYBamy59 using primers ESS-F and ESS-R (Tab. II). For amplification of the *appA* gene, genomic DNA was prepared from *E. coli* MC4100 using GeneReleaser® (Bioventure Inc., Murfreesboro, TN, USA) and primers appA-F and appA-R (Tab. II) were used. For the design of the primer set, the nucleotide sequence of the a*ppA* gene was obtained from GenBank under the accession number M58708. The PCR reaction was performed as follows: 25 ng of DNA in a final volume of 50 μL containing deoxyribonucleoside triphosphate (0.25 to 0.5 $mmol \cdot L^{-1}$ each), oligonucleotides (50 $pmol \cdot L^{-1}$) (Tab. II), and 1.0 to 3.0 U of LA-Taq polymerase (TAKARA, BIO Inc., Shiga, Japan). Amplification was performed on a PROGENE (Techne, Cambridge, UK) with 30 cycles of denaturation at 95 °C for 30 sec (5 min in the first cycle), annealing at 60 °C for 60 s, and elongation at 72 °C for 90 s (10 min in the last cycle).

2.4. Transformation of *Bifidobacterium*

Electrocompetent cells of *B. longum* MG1 were prepared according to the method of Argnani et al. [1] with minor modifications. *B. longum* MG1, grown in MRS-cysteine medium overnight, was inoculated into

Table II. Oligonucleotides used for PCR amplification.

Primer	Sequence*
ESS-F	5'-CCGAAA**T**C**TAG**A^{241}**GAAATACCGCAATGC-3'
ESS-R	5'-CTCGCCCAT**GGATCC**C^{879}GCCAGTCCGTAATGCTTGCG-3'
AppA-F	5'-GTCT**GGATCC**ATGC^{250}AGAGTGAGCCGGAGCTGAAG-3'
AppA-R	5'-GTCATC**GAATT**^{1524}**C**AGAGCATTCAGGTAACT-3'

* Underlined nucleotides represent modifications with regard to the original gene and the bold letters represent restriction enzyme recognition sites.
** Nucleotide number of original sequence of each gene.

fresh MRS-cysteine medium containing 0.2 mol·L^{-1} sucrose and cultivated anaerobically at 37 °C. When the culture reached OD_{600} = 0.45, cells were harvested by centrifugation (8000 *g*) for 10 min and washed twice using 0.2 mol·L^{-1} sucrose solution. The pellet was resuspended in 1/320 of the initial volume of 0.2 mol·L^{-1} sucrose. One microgram of DNA was thoroughly mixed with 80 µL of competent cells and transferred to a pre-chilled cuvette for application of an electric pulse using a Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA), which was set at 12.5 kV cm^{-1}, 200 Ω and 40 µF. The transformed cells were selected on MRS medium containing 3 µg·mL^{-1} of chloramphenicol and 0.2 mol·L^{-1} sucrose.

2.5. Analysis of intracellular and extracellular phytase activity

Phytase activity was measured using sodium phytate as a substrate [17]. One phytase unit was defined as the activity that releases one µmole of inorganic phosphorus from sodium phytate per minute at 37 °C. *B. longum* MG1 strain with pBES2 was used as a control because it showed no apparent phytase activity. The transformed cells were grown in a modified MRS medium (glucose 2%, Tween 80 0.1%, yeast extract 0.5%, ammonium citrate 0.2%, peptone 0.5%, sodium acetate 0.5%, $CaCl_2$ 0.05%, magnesium sulfate 0.01%, maleic acid 0.01%, sodium carbonate 0.2%, L-cysteine·HCl 0.05%, and manganese sulfate 0.005%). Aliquots of 1 mL of culture broth were taken at various time points. Cells were collected by centrifugation at 8000 *g* for 5 min in a microfuge and 50 µL-aliquots of the broths were prepared. In addition, the harvested cells were washed twice with 1 mL of 0.1 mol·L^{-1} sodium acetate buffer (pH 5.4) and disrupted by sonication. The cell debris was precipitated by centrifugation at 10000 *g* for 15 min at 4 °C. To each sample, 350 µL of 0.1 mol·L^{-1} sodium acetate buffer (pH 5.4) and 4 µL of 0.1 mol·L^{-1} sodium phytate were added and incubated at 58 °C for 30 min. Fifty µL of the reaction sample were transferred into a fresh tube and 50 µL of 1 mol·L^{-1} citric acid and 400 µL of AAM solution [100 µL of 10 mmol·L^{-1} ammonium molybdate, 100 µL of 5 N H_2SO_4 and 200 µL of acetone] were added and mixed. The absorbance was measured at 450 nm.

2.6. Protein purification and N-terminal sequencing

To purify α-amylase from the supernatant of MG 1 containing pYBamy59, cells were grown anaerobically in 1000 mL starch-BHI liquid media containing 0.05% L-cysteine·HCl (w/v) and chloramphenicol (3 µg·mL^{-1}) at 37 °C. The supernatant was separated from the cell pellet by centrifugation at 8000 *g* for 10 min at 4 °C. Proteins in the supernatant were precipitated with 75% ammonium sulfate and centrifuged at 3000 *g* for 30 min at 4 °C. The pellet was dissolved in 100 mmol·L^{-1} sodium acetate buffer (pH 5.5). To remove ammonium sulfate from the concentrated protein solution, dialysis was carried out in 20 mmol·L^{-1} sodium acetate buffer (pH 5.5) using the

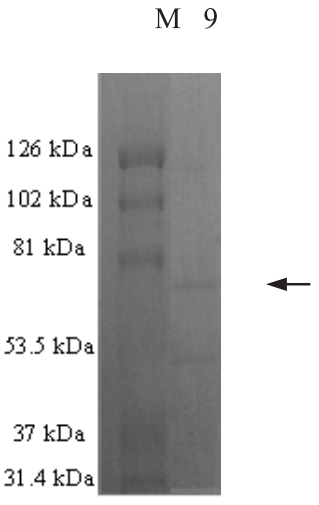

Figure 1. Purification of amylase from culture broth of MG1 (amy59). M, size marker; 9, fraction number of FPLC elutes; ← amylase band confirmed by activity staining and used for N-terminal sequence analysis.

Slide-A-Lyzer Dialysis Cassette (Pierce, Rockford, IL, USA) at 4 °C. Subsequently, this protein solution was concentrated using microcon centrifugal filter devices (Millipore, Billerica, MA, USA) and then stored at 4 °C. For ion exchange chromatography, a UPC-900, P-920 and Fac-920 (AKTAFPLC, Amersham Pharmacia Biotech, Sweden), and a Hi Trap_Q_HP_1mL column (Amersham Pharmacia, Uppsala, Sweden) were used. The proteins were consecutively eluted with 0 mol·L^{-1} KCl (in 25 mmol·L^{-1} potassium phosphate buffer) to 1 mol·L^{-1} KCl (in 25 mmol·L^{-1} potassium phosphate buffer). The purified protein was separated by SDS-polyacryamide gel electrophoresis. Activity staining was performed to chase the amylase protein band as described by Lee et al. [10]. To analyze the N-terminal sequence of amylase from the MG1 (amy59), the target protein was transferred to a PVDF membrane according to the method of Sambrook et al. [21]. The eluted protein was sequenced using a Procise 491 HT protein sequencer (Applied Biosystems, USA).

3. RESULTS AND DISCUSSION

3.1. Determination of the cleavage site

For the precise determination of the cleavage site of the ESS gene, amylase was purified from MG1 (amy59), separated using SDS-PAGE and transferred as described in Materials and Methods (Fig. 1). The purified amylase had the N-terminal amino acid sequence: Ser^{45} - Thr^{46} - Asp^{47} - Arg^{48} - Asp^{49} - Ser^{50}. This coincides with the predicted cleavage site of this protein. In general, signal peptides are 14-25 amino acids long and consist of three identifiable domains, i.e., the amino (N-), hydrophobic (-H-), and carboxy-terminal (-C) regions (Fig. 2). The N-region is rich in positively-charged amino acids, and is followed by a hydrophobic region that tends to organize itself into a α-helical conformation when brought into contact with the membrane lipid phase. The C-terminal region is hydrophilic and contains the signal peptide cleavage site that is recognized by the relevant signal peptidase. Hydropathy plot analysis (TMHMM program held at http://www.cbs.dtu.dk/services/) revealed 23 amino acid residues (from 21 to 43; VVV-----AAQ) of the putative trans-membrane sequence with a high score. This site corresponds to a general Ala-X-Ala cleavage site. In addition, the N-region frequently contains the positively-charged amino acid residues lysine and arginine [22]. Very similar features of secretion signals from *Bifidobacterium* have been revealed by a comparative genomics approach [11].

The ESS of amylase from INT-57 corresponded well with the general characteristics of reported signal peptides. Thus, the signal peptide identified in the present study may be used for secreting recombinant proteins in bifidobacteria.

3.2. Construction of a secretion vector and cloning of the phytase gene

Initially, the ESS containing the promoter and signal peptide regions of the *amyB* gene was amplified as described above. The partial sequence of the ESS gene is shown in Figure 3. The amplified product (664 bp) was digested using *Xba*I and *Bam*HI and ligated

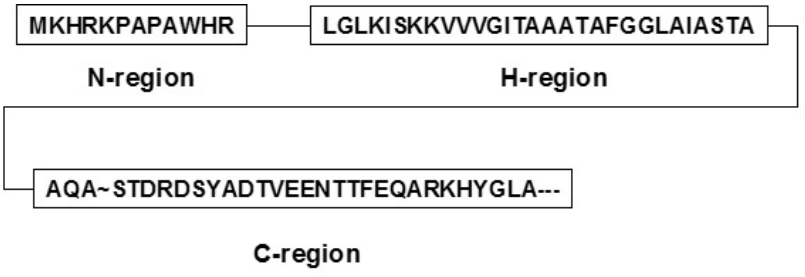

Figure 2. Three domains of the ESS gene. N-region, positively-charged domain; H-region, hydrophobic domain; C-region, hydrophilic domain with cleavage site.

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236 TCTAGAGAAATACCGCAATGCACGGCAAATTCAATTAAACGCAACTCTTCATAGGCCACTTAAAC

301 AAACCACCAC CCCATCAAAT TGGATAGAGC ATATACAAGC CCACATCCAG CAGCACCCCC

361 GTACCCCTAC TTGCGCAAAT GAGAAAGATT TGCAGGGTTG ACGCATCGAC AACAACTGGA

421 TTGCATTTTC AGGGCATCGC CGAATATACT CCCACCACAC AACAAGTTAG GGTGGTACAA

481 AACACCATCA ATTAAGTACC ACCTTTGCAA ACATTTTCAC AAATGAAAGA GTTGTTTCAG

541 CAACGATTTT CATTGTTTTT TCCAAGGCTT TTCGCACTTT AGCACCCTAG AAAAGGTATA

601 AAATAAACAG CATACGTTCG CAATAGTGCA AACGCTATCA AAGAAGATGA ACCCCCGTTA

661 AAGGGATTGA AGAAAAGGAA TAAAGGAGCC ATG AAA CAT CGG AAA CCC GCA CCG GCC TGG
                              RBS    M   K   H   R   K   P   A   P   A   W          10

721 CAT AGG CTG GGG CTG AAG ATT AGC AAG AAA GTG GTG GTC GGC ATC ACC GCC GCG GCG ACC
    H   R   L   G   L   K   I   S   K   K   V   V   V   G   I   T   A   A   A   T    30

781 GCC TTC GGC GGA CTG GCA ATC GCC AGC ACC GCA GCA CAG GCC AGC ACC GAT CGC GAC AGC
    A   F   G   G   L   A   I   A   S   T   A   A   Q   A   S   T   D   R   D   S    50

841 TAC GCC GAC ACC GTT GAA AAC ACC ACG TTC GAA CAG GCG CGC AAG CAT TAC GGA CTG GCG
    Y   A   D   T   V   E   N   T   T   F   E   Q   A   R   K   H   Y   G   L   A    60

901 GGA TCC ATG CAG AGT GAG CCG GAG CTG AAG
    G   S   M   Q[23]  S   E   P   E   L   K        70
```

Figure 3. The partial sequence of the ESS gene containing the promoter region and signal sequence of the *amyB* gene. The putative promoter regions 1 (563–612 bp) and 2 (471–520 bp) and the ribosome binding site (RBS 684 bp) were detected by promoter prediction program (data not shown). The full sequence of the *amyB* gene is available from GenBank under the accession number AY240946. The nucleotide sequence from 907 bp and the amino acid sequence are the cloned phytase gene. Restriction enzyme sites for *Xba*I and *Bam*HI are underlined.

into pBES2 digested with the same enzymes to construct pBESAF2. Subsequently, the gene structure and termination sequences of *appA* (from 250 bp to 1534 bp of *appA*; GenBank accession number M58708) were also PCR-amplified (1297 bp) and digested with *Bam*HI and *Eco*RI. These were ligated in-frame with the ESS gene and the construct was termed pBESAF2-appA (Fig. 4). Cloning was performed in *E. coli* DH5α and the integrity of the plasmid construct was confirmed by restriction pattern analysis. pBESAF2-appA prepared from *E. coli* DH5α was used to transform *B. longum* MG1 by electrotransformation, resulting in MG1(*appA*). PCR amplification using AppA-F and AppA-R for the plasmid prepared from MG1 (*appA*) produced an amplified DNA band of approximately 1.3 kb, which corresponds to the size of *appA*.

3.3. Phytase activity of MG1 (*appA*)

Phytase activity of MG1 (*appA*) was measured as described in Materials and Methods at each time point and the results are shown in Figure 5. More than 95% of phytase activity in MG1 (*appA*) was detected in the culture supernatant. This pattern of phytase activity was the same as that of amylase of *B. adolescentis* INT57 (data not shown). It shows that

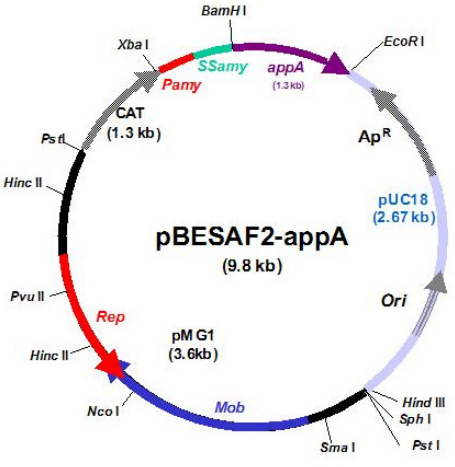

Figure 4. Genetic map of the expression vector pBESAF2-appA. Pamy, promoter of *amyB*; SSamy, 72 N-terminal amino acids of *amyB*, the first 44 amino acids represent the secretion signal peptide sequence and the remainder encompass the mature N-terminus of amylase; *appA*, phytase gene from *E. coli* MC4100; ApR and CAT, ampicillin and chloramphenicol resistance genes, respectively; Ori, origin of replication of *E. coli*; Rep and Mob, replication initiation and plasmid mobilization determinant of *Bifidobacterium*, respectively; pMG1, plasmid originated from *B. longum* MG1.

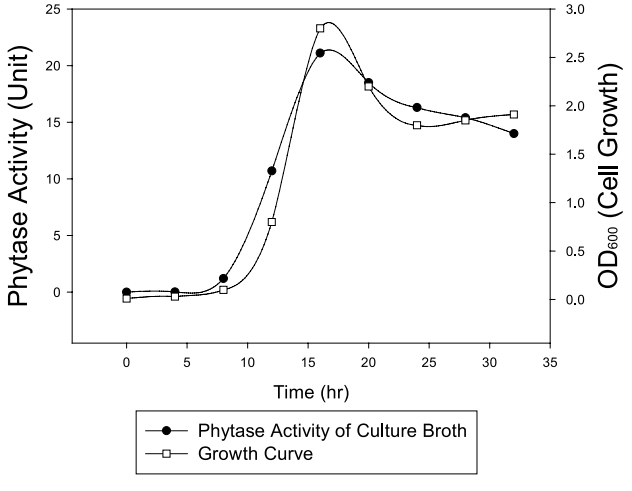

Figure 5. The growth curve and phytase activity of MG1 (*appA*) at indicated time points. -●-, phytase activity of culture broth; -□-, growth curve of MG1 (*appA*).

the activity follows the growth pattern and that therefore the expression of the fusion gene appears to be constitutive.

In this report, we characterized ESS of the *amyB* gene from *B. adolescentis* INT57 and constructed a secretion vector, pBESAF2, using this information. The intracellular phytase gene from *E.coli* was successfully expressed and secreted by *B. longum* into the culture broth by using this system. These results show that *B. longum* is able to produce, secrete, and fold into an active conformation a heterologous enzyme. We expect that pBESAF2 will be a good vehicle for molecular modification and improvement of the genus *Bifidobacterium.*

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