Note

Construction of *Escherichia coli--Bifidobacterium longum* Shuttle Vector Transforming *B. longum* 105-A and 108-A

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A shuttle vector, pBLES100, was constructed by cloning a *Bifidobacterium longum* plasmid and a gene encoding spectinomycin adenyltransferase AAD(9) from *Enterococcus faecalis* into the *Escherichia coli* vector pBR322. Stable transformants with this plasmid were obtained with an efficiency of $2.2 \times 10^5$ transformants/μg DNA or $6.9 \times 10^5$ transformants/cell/μg DNA under the optimal conditions of 10.0 kV/cm, 200 Ω, and 25 μF, using *B. longum* 105-A harvested at late log phase of growth.

Key words: Bifidobacterium longum; Escherichia coli; shuttle vector pBLES100; transformation by electroporation; spectinomycin adenyltransferase AAD(9)

Bifidobacteria are Gram-positive anaerobic bacteria, the genome of which is GC-rich. These microorganisms constitute a major part of the normal microflora in the large intestine of humans and some other animals. These intestinal organisms have been believed to have health-promoting properties for their host, involving increase of the immune response, inhibition of carcinogenesis, protection of the host against virus infection, and possible production of antibacterial substances. Some *Bifidobacterium* species are widely used for the preparation of fermented milk products in many countries. But despite the increasing attention in foods, medicine, and industry, little is known about their genetic properties mainly due to the lack of efficient and reproducible systems for genetic transfer and good selection markers.

Electroporation is an effective technique for introducing DNA into many types of eukaryotic and procaryotic cells. This technique has recently been used for introducing DNAs into several *Bifidobacterium* species, although the efficiencies varied from strain to strain, ranging between $2 \times 10^2$ and $7 \times 10^3$ μg DNA. Here, we report another example of reproducible and stable transformation of *B. longum* by electroporation with a constructed shuttle vector, pBLES100, having two unique restriction sites suitable for gene cloning in the molecule.

A 1.1-kb HindIII--EcoRI fragment containing a spectinomycin resistance (Sp<sup>R</sup>) gene [spectinomycin adenyltransferase AAD(9)] from pDL269<sup>10</sup> was ligated with pBR103<sup>11</sup> to obtain an 8.0-kb plasmid composed of pTB6<sup>11</sup> derived from *B. longum* and pBR322, after digestion with both HindIII and EcoRI. The resulting 9.1-kb plasmid, pBLES100 (Fig. 1), was isolated from a colony resistant to spectinomycin (Sp) after introduction of the ligated DNA into *E. coli* HMS174<sup>12</sup>.

*B. longum* strains JCM1217 (ATCC15707) obtained from the Japan Collection of Microorganisms, and 105-A, 108-A, and M101-2, obtained from Mitsuoka were anaerobically cultured at 37°C to middle log phase in liquid Brigg's broth<sup>13,14</sup> with glucose replaced by 2.0% lactose, and harvested at 4°C. Following processes were handled in the atmosphere. Cells were washed three times with ice-cold glycerol (10% v/v) and resuspended in about 1/100 of the original culture volume of ice-cold glycerol (10% v/v) as described by Missich et al.<sup>9</sup> Fifty-microliter samples [2.0 × 10<sup>6</sup>–2.0 × 10<sup>7</sup> colony-forming units (CFU)] was mixed with 4 μl of CsCl-purified pBLES100 and placed on ice for 5 min before electroporating with a Gene Pulser apparatus (Bio-Rad Labs., U.S.A.; inter-electrode distance 0.2 cm). Electroporation was done at 2.0 kV and 25 μF capacitor setting with the pulse controller at 200 Ω parallel resistance, yielding a pulse duration of 4.1–4.5 ms. One milliliter of Briggs broth was added immediately after electric pulsing, and the cuvette was incubated for 3 h at 37°C without shaking. Cells were spread on Briggs agar (1.5%) plates containing 75 μg Sp per milliliter. The plates were incubated anaerobically at 37°C for 3 to 4 days using a GasPak Anaerobic System (BBL, U.S.A.).

Sp<sup>R</sup> transformants could be obtained from *B. longum* strains 105-A and 108-A with transformation efficiency 1.6 × 10<sup>7</sup> μg DNA and 2.6 × 10<sup>7</sup> μg DNA, respectively, but no transformant was obtained from a strain M101-2 and the type strain JCM1217, showing that Sp<sup>R</sup> transformation with pBLES100 depended on *B. longum* strains.

All the Sp<sup>R</sup> transformants of *B. longum* 105-A tested contained circular plasmid DNA of ~9.1 kb in size from parent pBLES100. Moreover, the restriction fragments of the plasmid with PstI or EcoRV migrated to the same positions as those for parent pBLES100 by agarose gel electrophoresis (data not shown). The plasmids isolated from the Sp<sup>R</sup> transformants...

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**Fig. 1.** Molecular Structure of Shuttle Vector pBLES100. Double line arrow represents a 1.1-kb HindIII--EcoRI fragment containing Sp<sup>R</sup> gene (spectinomycin adenyltransferase AAD(9)) from pDL269 and the transcriptional direction of the gene. Stippled line represents pTB6 DNA (3.6 kb). Single lines represent pBR322 DNA. pBLES100 carries a tetracycline-resistance gene inactivated by deletion of its promoter by EcoRI--HindIII digestion. Only relevant restriction sites are shown and the figure is not drawn to scale.

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**Abbreviations:** Sp, spectinomycin; Sp<sup>R</sup>, spectinomycin resistant or spectinomycin resistance; CFU, colony-forming units.
were therefore concluded to be pBLES100. These plasmids could be successfully re-introduced into *B. longum* 105-A and *E. coli* HMS174 with almost equivalent transformation efficiencies to those with the authentic plasmid pBLES100 (data not shown), suggesting that pBLES100 prepared from *E. coli* HMS174 was not restricted in *B. longum* 105-A, although restriction of the plasmid in *B. longum* 105-A remains to be studied.

It was concluded from these findings that pBLES100 which has two unique restriction sites, EcoRI and HindIII, suitable for gene cloning, is a shuttle vector between *B. longum* and *E. coli*, and a spectinomycin adenyltransferase AAD9 (from *Enterococcus faecalis*) can be expressed also in *B. longum* 105-A and 108-A as reported for *B. longum* B2577 by Missich et al.8)

Figure 2 shows the effect of variation of the applied voltage on the transformation efficiency of *B. longum* 105-A. Transformation occurred over a wide range of voltage. Almost 20 transformants was obtained at the low voltage of 2.5 kV/cm, and the number of transformants increased with the increase of voltage. *B. longum* 105-A was transformed with almost maximized efficiency near at 12.5 kV/cm where the cell survival was approximately 50% of the original CFU.

Transformation of *B. longum* 105-A with pBLES100 was observed at all growth phases tested at 37°C with different efficiencies. Low efficiency of transformation was observed with cells in early log phase, representing $1.0 \times 10^2 \mu g$ DNA or $3.1 \times 10^{-5}$ cell/µg DNA. The maximal efficiency was observed with cells in the middle to late log phase, representing $2.2 \times 10^{1}$ µg DNA or $6.9 \times 10^{-5}$ cell/µg DNA. The transformation of cells decreased after the late log phase, representing $6.0 \times 10^{2}$ µg DNA or $2.5 \times 10^{-5}$ cell/µg DNA.

We finally examined the segregational and structural stabilities of pBLES100 in the *B. longum* cells since stable maintenance of the recombinant plasmid is very important to study molecular genetics of bifidobacteria, involving gene expression, recombination, DNA replication, and also gene engineering. A transformant of *B. longum* 105-A with pBLES100 was picked and cultured in the presence of Sp. Cells were then diluted and incubated at 37°C in Brigg's broth in the absence of Sp. As shown in Fig. 3, more than 85% of cells incubated for 24h showed the Sp^ phenotype. Plasmids extracted from these Sp^ cells were identical to parental pBLES100 in sizes by agarose gel electrophoresis of both closed circular form and restricted fragments (data not shown). Figure 3 also shows that the growth rate of Sp^ transformant is almost identical to non-transformed parental *B. longum* 105-A. These results offered evidence that pBLES100 is maintained fairly stably in *B. longum* 105-A without rearrangement of the molecule and also that the plasmid is not hazardous to host cell physiology.

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References