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Cloned Cytosine Deaminase Gene Expression of *Bifidobacterium longum* and Application to Enzyme/Pro-drug Therapy of Hypoxic Solid Tumors

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***Bifidobacterium longum* is a nonpathogenic anaerobic bacterium among normal bacterial flora. Recently, it was reported that *B. longum* accumulated in hypoxic solid tumors. The gene of interest was expressed in transfected *B. longum* by the shuttle vector pBLES100 in solid tumors. In this report, we constructed pBLES100-S-eCD, which included the cytosine deaminase gene. We confirmed by western blotting that transfected *B. longum* produced cytosine deaminase. In addition, transfected *B. longum* produced cytosine deaminase that converted 5-fluorocytosine into 5-fluorouracil. *B. longum* could be useful for enzyme/pro-drug therapy of hypoxic solid tumors.**

Key words: *Bifidobacterium longum*; cytosine deaminase; enzyme/pro-drug therapy; p-BLES100- S-eCD

The fundamental obstacle to producing gene therapies for cancer is that no vector exists that can carry a particular gene to a corresponding cancer-specific site. In human solid tumors, the oxygen partial pressure is lower than in normal tissue.[1,2] Certain species of anaerobic bacteria, including *Clostridium* and *Bifidobacterium*, can selectively germinate and grow in the hypoxic regions of solid tumors after intravenous injection.[3–6] *Bifidobacterium longum* is one of nonpathogenic anaerobic bacterium among normal bacterial flora and it is found in the lower small intestine and large intestine.

Enzyme/pro-drug therapies for cancer convert nonpathogenic precursors into material with antitumor properties generated by an enzyme in the tumor. If the optional gene could be expressed in *B. longum*, it would be possible to use this species for enzyme/pro-drug therapy. Recently, an *Escherichia coli-B. longum* shuttle vector, pBLES100, was constructed.[7] Yazawa *et al.* reported that *B. longum* transfected by this shuttle vector expressed the antibiotic resistance gene via a plasmid into solid tumors.[8,9] We have also observed that the HU gene, which encodes a histone-like protein, was expressed by *B. longum* at high rates. [10] We constructed a plasmid (pBLES100-S-eCD), which included the HU gene promoter and the gene encoding the cytosine deaminase of *E. coli* in the pBLES100 vector; the cytosine deaminase enzyme converts 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU). To find whether or not transfected *B. longum* produced active cytosine deaminase (CD), we measured the 5-FU concentration *in vitro*.

Materials and Methods

Bacteria. B. longum 105-A, obtained from Mitsuoka, was anaerobically cultured at 37°C to middle log phase in a slightly modified Briggs broth[11,12] using 2% lactose instead of glucose.

Plasmid construction. The plasmid was constructed as follows (Fig. 1). (1) The gene encoding the cytosine deaminase of *E. coli* (eCD) was amplified from pAdex1CACD (RIKEN; (Institute of Physical and Chemical Research), Gene Bank, RDB No. 1591) including the CD gene of *E. coli*. PCR was done using the following primers: sense primer 5′-GGTTCGAATAACGCTTTA-3′ and antisense primer 5′-CGGTTAACTCAACGTTTGTAATC-3′. The sense primer includes an *Nsp*V site and the antisense primer includes an *Hpa*I site. PCR was done with 125 ng of pAdex1CACD in a 40-μl reaction, 2 units of *Pfu Turbo* DNA polymerase, and reaction kits

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Abbreviations: CD, cytosine deaminase; eCD, cytosine deaminase of *Escherichia coli*; cfu, colony-forming units

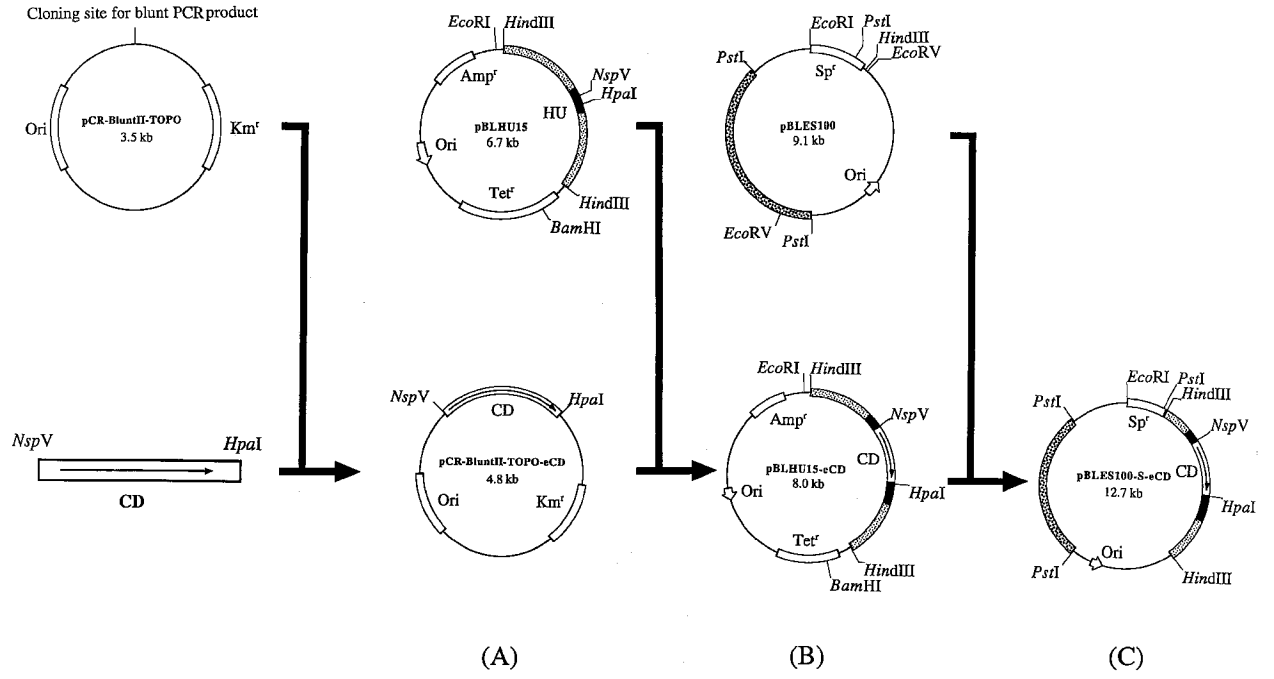

Fig. 1. Construction of pBLES100-S-eCD.

(A) The amplified CD gene was cloned in pCR-BluntII-TOPO, giving pCR-BluntII-TOPO-eCD. (B) pCR-BluntII-TOPO-eCD was digested with *Hpa*I/*Nsp*V. The fragment containing the CD gene was fused with pBLHU15, giving pBLHU15-eCD. (C) pBLHU15-eCD was digested with *Hin*dIII, and the *Hin*dIII-*Hin*dIII fragment was fused with pBLES100, producing pBLES100-S-eCD. (Ori, origin; Km^r, kanamycin resistant; Amp^r, ampicillin resistant; Tet^r, tetracycline resistant; Sp^r, spectinomycin resistant).

(Strategene Cloning Systems, La Jolla, CA), according to the manufacturer's instructions. The PCR profile was as follows: 94°C for 1 min followed by 30 cycles (each cycle: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min). The PCR product was cloned in pCR-BluntII-TOPO (Invitrogen, Carlsbad, CA), thus creating pCR-BluntII-TOPO-eCD. (2) The pCR-BluntII-TOPO-eCD was digested with *Nsp*V/*Hpa*I, and was ligated to pBLHU15[13] and then digested with *Nsp*V/*Hpa*I. Ligation was done with 948 ng of digested pCR-BluntII-TOPO-eCD and 546 ng of digested pBLHU15 in a 20-μl reaction mixture, 2 units of T4 DNA Ligase, and reaction kits (Promega, Madison, WI). (3) The ligation product was used to transform competent *E. coli* JM109 (Takara, Tokyo), according to the manufacturer's instructions. And plasmid minipreps were made by the modified alkaline lysis procedure with RPM-AFS (BIO 101, Vista, CA). This plasmid was digested with *Hin*dIII, and the fragment including the CD gene was inserted. (4) The shuttle vector pBLES100 was digested with *Hin*dIII, then digested with 1.2 units of alkaline phosphatase (*E. coli* C75 (Takara)), to prevent self-ligation. The product of these reactions was used as the vector. (5) Seventy-two nanograms of vector and 199.5 ng of insert were ligated in a 20-μl reaction with 2 units of T4 DNA Ligase and reaction kits (Promega). The ligation product was pBLES100-S-eCD, which contained both the HU promoter and the CD encoding gene.

Polymerase chain reaction. PCR was done using 10 ng of pBLES100-S-eCD or pBLES100 in a 25-μl reaction, 0.5 units of Ampli *Taq* DNA polymerase, and reaction kits (Perkin Elmer/Cetus, Wellesley, MA), according to the manufacturer's instructions. The PCR profile was as follows: 94°C for 5 min followed by 30 cycles (each cycle: 94°C for 30 s, 55°C for 30 s, 72°C for 30 s) and a final extension at 72°C for 1 min. The following primers were used for the amplification of the CD gene specific sequence: sense primer 5′-GGTTCGAATAACGCTTTA-3′ and antisense primer 5′-CGGTTAACTCAACGTTTGTAATC-3′.

Transformation of pBLES100-S-eCD. The pBLES100-S-eCD was transferred directly into *B. longum* 105-A by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, CA; inter-electrode distance, 0.2 cm). Electroporation was done at 2.0 kV and a 25-μF capacitor setting with the pulse controller set at 200 Ω parallel resistance, yielding a pulse duration of 4.1–4.5 ms. Stable transformants were obtained with an efficiency of 1.6×10^4 transformants/μg DNA under optimum conditions. Transfected *B. longum* was grown under anaerobic conditions at 37°C in Briggs broth containing 75 μg/ml spectinomycin.

Protein extract and western blotting. The *B. longum* culture solution was centrifuged ($3{,}000 \times g$ for

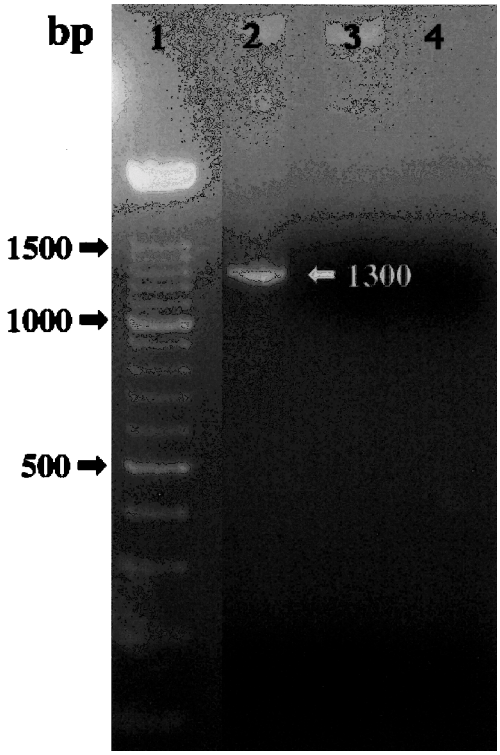

Fig. 2. Results of PCR of pBLES100-S-eCD.
Amplification of the 1300-bp DNA fragment of the CD gene (lane 1, 100-bp DNA ladder; lane 2, pBLES100-S-eCD; lane 3, pBLES100; lane 4, negative control).

30 min at 4°C). Harvested *B. longum* (1.7×10^9) was solubilized in lysis buffer (10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (pH 8.0), 2% glycerol, 40 mM NaCl, 1 mM imidazole, 0.4 mM β-mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride). This mixture was crushed by ultrasonic waves (1 min at 0°C). One hundred microliters was taken from this mixture, and an equivalent of 8 M urea was added. The mixture was then thoroughly stirred. In addition, a 200-μl sample buffer (100 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 1.7 M β-mercaptoethanol) was added to this mixture. After incubation for 10 min at 95°C, samples were separated by SDS-PAGE (Amersham Pharmacia Biotech, Buckinghamshire) and transferred onto a Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membrane was incubated in blocking buffer (3% skim milk, and 3% bovine serum albumin in phosphate-buffered saline (−)). Blots were incubated overnight at 4°C with the primary antibodies (rabbit monoclonal anti-cytosine deaminase (Sawady Technology, Tokyo)). Blots were then washed with washing buffer containing 0.1% Tween 20 (Bio-Rad Laboratories), and were incubated with horseradish peroxidase-conjugated (Amersham Pharmacia) anti-rabbit immunoglobulin G (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as the secondary antibody. Blots were analyzed with the

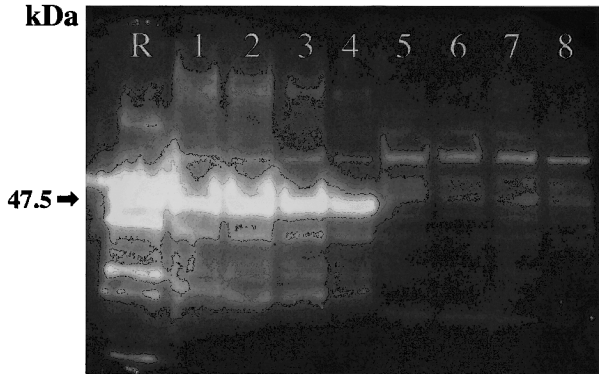

Fig. 3. Western Blotting of Cytosine Deaminase in Transfected *B. longum*.
Both wild-type and transfected *B. longum* cells were examined by western blotting. The enzyme was expressed only in the transfected *B. longum*.
R, recombinant cytosine deaminase; lanes 1-4, transfected *B. longum*; lanes 5-8, wild-type *B. longum*.

ECL western blotting detection system (Amersham Pharmacia).

Measurement of the 5-FU concentration. The 5-FU concentration of the supernatant of the sample was measured by HPLC (Tosoh, Tokyo) (pump: CCPD, autosampler: AS-8000, detector: UV8010, column heater: U-620 Type 30, deggaser: SD8012, data processor: Labchart 180, column: Tsk-gel ODS-80Ts).

Statistical analysis. The statistical significance of differences was evaluated by Student's *t*-test with Statview 5.0 software (SAS Institute Inc., Cary, NC).

Results

Polymerase Chain Reaction

The 1300-bp DNA fragment of the CD gene was amplified using pBLES100-S-eCD as the template (Fig. 2). These results confirmed that the CD gene was cloned in the plasmid pBLES100-S-eCD.

Western blotting

To confirm whether or not the transfected *B. longum* produced CD, proteins extracted from wild-type *B. longum* and transfected *B. longum* were analyzed by western blotting. The molecular mass of recombinant CD was 47.5 kDa, and the transfected *B. longum* had the same molecular mass in all lanes. In contrast, wild-type *B. longum* did not have a band of 47.5 kDa (Fig. 3). Moreover, the finding suggests that the CD gene was expressed and that CD was produced.

Conversion of 5-FC into 5-FU time-dependently

Twenty-five milligrams of 5-FC was added to both culture solutions of wild-type and transfected *B. lon-*

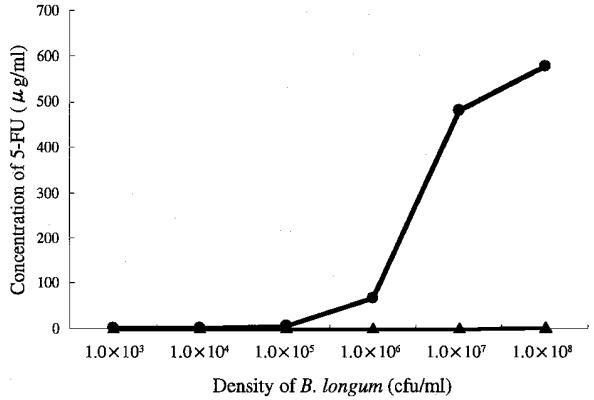

Fig. 4. Conversion of 5-FC into 5-FU in Wild-type and Transfected *B. longum*.

The number of *B. longum* cells was adjusted to 1.0×10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 cfu/ml. 5-FC was added to the culture medium. With transfected *B. longum* (●), the 5-FU concentration was higher with more cells, after a certain point. With wild-type *B. longum* (▲), 5-FU was not detected, whatever the cell density.

gum. The final concentration of wild-type and transfected *B. longum* was 1.4×10^8 cfu/ml each, and the 5-FC concentration was 6.25 mg/ml. The 5-FU concentration of the culture solution was measured by HPLC time-dependently. In transfected *B. longum*, the 5-FU concentration rose with the passage of time. However, in wild-type *B. longum*, 5-FU was hardly detected, even after time had passed. After the 18-hours mark, the 5-FU concentration of transfected *B. longum* was about 2,500 μg/ml. A significantly higher concentration of 5-FU was detected in the transfected *B. longum* than in the wild-type *B. longum*. ($p<0.001$)

Conversion of 5-FC into 5-FU and the density of bacilli

Twenty-five milligrams of 5-FC was added to both wild-type and transfected *B. longum* culture solutions. The total amount of culture solution was 6 ml, and the final 5-FC concentration was 6.25 mg/ml. The number of *B. longum* was adjusted at each concentration: 1.0×10^3, 1.0×10^4, 1.0×10^5, 1.0×10^6, 1.0×10^7, and 1.0×10^8 cfu/ml. After 5 h, the concentration of 5-FU in each culture solution was measured by HPLC. The observed 5-FU concentrations rose with increases in the number of the bacilli. In wild-type *B. longum*, 5-FU was hardly detected, even when the number of bacilli increased (Fig. 4). The concentration of 5-FU increased in proportion to the number of *B. longum*.

Discussion

In this study, we confirmed that *B. longum* transfected by pBLES100-S-eCD was able to produce CD. The CD was able to convert 5-FC into 5-FU *in vitro*, and therefore had enzymatic activity. In addition, the 5-FU concentration increased in proportion to the number of bacilli and the amount of time that had passed.

The treatment of solid tumors by systemic administration of 5-FU has some disadvantages. First, it is necessary to administer large doses of 5-FU to the whole body to distribute a sufficiently dense concentration of 5-FU to the tumor. Second, various side effects accompany massive doses of 5-FU. Five-fluorocytosine is a pro-drug with lower toxicity than that of 5-FU, and therefore it is possible to administer larger doses of 5-FC. If CD, which converts 5-FC into 5-FU, exists only in the tumor, it might be possible to obtain high-density administration of 5-FU without simultaneously distributing 5-FU among normal tissues. We refer to this manner of administration as enzyme/pro-drug therapy.

Some bacterial vectors for cancer gene therapy have been reported. In such cases, bacteria proliferate between tumor cells, and thus the therapeutic gene is not introduced into the tumor cell, but rather the therapeutic protein is produced in the tumor. This mechanism differentiates enzyme/pro-drug therapy from what is called gene therapy, which uses virus and liposome vectors. Candidate bacterial vectors for gene introduction include species of *Bifidobacterium*, *Clostridium*, and *Salmonella*.[3–6,14] Engineered *Clostridium acetobutylicum* has been reported as particularly useful for enzyme/pro-drug therapy.[15] However, *Clostridium* and *Salmonella* are pathogenic, and exothermic reactions related to oral administration have been reported.[16,17] In contrast, *Bifidobacterium* is part of the normal bacterial flora in the intestine, and more importantly, it is non-pathogenic. In addition, *Bifidobacterium* has been reported to suppress carcinogenesis[18] and it also can defend the host against viral infection.[19] Yazawa *et al.* reported that *B. longum* accumulated after systemic administration, and that the gene on the plasmid was expressed in the tumor.[8,9] Therefore, it appears that *B. longum*, transfected by pBLES100-S-eCD, proliferates in a tumor-specific manner. Furthermore, if a sufficient number of *B. longum* cells are present in a tumor, then 5-FC is converted into 5-FU during tumor division after systemic administration of 5-FC, and antitumor effects are expected from such treatments.

These findings suggest that *B. longum* is an excellent gene delivery system and candidate for enzyme/pro-drug therapy. *B. longum* promises to be useful for the treatment of hypoxic solid tumors.

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