Production Example 1: Construction of a Secretory GFP-expressing Plasmid (pSPxA-GFP)

We constructed a plasmid that expresses secretory GFP by a promoter of a signal peptide candidate. A summary is shown in Fig. 1. Details are provided below.

[0105]

Insert Preparation

Among the 25 secretory protein candidates, for 16 whose gene are located on the head of the operon (Table 1, Nos. 1 to 16), putative signal peptide portions comprising a promoter and 60 to 90 nucleotides downstream thereof were amplified by PCR method as described below.

Forward primers were designed 300bps upstream of the translation start site and reverse primers were designed 60 to 90 bps downstream of the DNAs encoding the signal peptides. 30ng of the genomic DNA of *Bifidobacterium longum* 105A was used as template for PCR using 2 x Phusion Flash PCR Master mix (FINNZYMES).

[0106]

The PCR program was as follows: 98°C for 10 seconds, then 30 cycles of 98°C for 1 second plus 55°C for 5 seconds plus 72°C for 9 seconds, and 72°C for 1 minute. PCR primers for each signal peptide are shown in Table 2.1. 15 nucleotides on 5' side of each primer have a homologous sequence to those of the vectors shown below.

[0107]

Table 2.1 Primers for amplification of signal peptides (SPxA)

No.	Primer Name	Sequence (5' -> 3')	PCR product name
1	SP1_F1_primer	cttttctacggatccTCTCGTGTACGCGAATACG (SEQ ID NO: 52)	SP1A
	SP1_R1_primer	ctcctcgcccttggaTTCCACGCGCTCCTTGG (SEQ ID NO: 53)	
2	SP1_F2_primer	cttttctacggatccCGCGCTGCAATGGCGTCGG (SEQ ID NO: 54)	SP2A
	SP1_R2_primer	ctcctcgcccttggaCAAAAACAGCACGCGGGTG (SEQ ID NO: 55)	

3	SP1_F3_primer	cttttctacggatccGGCGTCTGGCAGCGCACAG (SEQ ID NO: 56)	SP3A
	SP1_R3_primer	ctcctcgcccttggaGGCGATGGTCAGCTTGC (SEQ ID NO: 57)	
4	SP1_F4_primer	cttttctacggatccATCAGAGGAGCCGGTGC (SEQ ID NO: 58)	SP4A
	SP1_R4_primer	ctcctcgcccttggaGCCGAACAGACGCGGGGG (SEQ ID NO: 59)	
5	SP1_F5_primer	cttttctacggatccCTCGCGGGCTTGGCGGTC (SEQ ID NO: 60)	SP5A
	SP1_R5_primer	ctcctcgcccttggaTTGGTCGATGATGGCCTTG (SEQ ID NO: 61)	
6	SP1_F6_primer	cttttctacggatccGTTCGGGTCCGGGTGCGG (SEQ ID NO: 62)	SP6A
	SP1_R6_primer	ctcctcgcccttggaATCGACAATAGGACTTTTCC (SEQ ID NO: 63)	
7	SP1_F7_primer	cttttctacggatccAGGCGGTCCATGGTGGATG (SEQ ID NO: 64)	SP7A
	SP1_R7_primer	ctcctcgcccttggaGGTGGAGGTGGATTCGG (SEQ ID NO: 65)	
8	SP1_F8_primer	cttttctacggatccAACCATTCGGACGCGCAG (SEQ ID NO: 66)	SP8A
	SP1_R8_primer	ctcctcgcccttggaCATCGTTGCCTCGCCCG (SEQ ID NO: 67)	
9	SP1_F9_primer	cttttctacggatccCCAGGGCCCGAAGGAAGAG (SEQ ID NO: 68)	SP9A
	SP1_R9_primer	ctcctcgcccttggaGACGATCTGATGCGCCAGC (SEQ ID NO: 69)	
10	SP1_F10_primer	cttttctacggatccCAGCCCATCGCTATGGAG (SEQ ID NO: 70)	SP10A
	SP1_R10_primer	ctcctcgcccttggaTCGCTGCTTGAGTTTGCCG (SEQ ID NO: 71)	

11	SP1_F11_primer	cttttctacggatccTCTGTAGCGGGAGGTTGCG (SEQ ID NO: 72)	SP11A
	SP1_R11_primer	ctcctcgcccttggaCAGCGTGGGCTCCCAAGCC (SEQ ID NO: 73)	
12	SP1_F12_primer	cttttctacggatccGCGTTACTTCCATGTTCGC (SEQ ID NO: 74)	SP12A
	SP1_R12_primer	ctcctcgcccttggaGGAACGGGTCCACAGGGTG (SEQ ID NO: 75)	
13	SP1_F13_primer	cttttctacggatccCCTTCTCAACGCCAGCGGC (SEQ ID NO: 76)	SP13A
	SP1_R13_primer	ctcctcgcccttggaAGACTCGCTAGCACAGCAC (SEQ ID NO: 77)	
14	SP1_F14_primer	cttttctacggatccGACATAGCGCGGTTTCATACC (SEQ ID NO: 78)	SP14A
	SP1_R14_primer	ctcctcgcccttggaTTGGGCCACTATTGTCTTC (SEQ ID NO: 79)	
15	SP1_F15_primer	cttttctacggatccACCGGCACCTGCGCCGGCG (SEQ ID NO: 80)	SP15A
	SP1_R15_primer	ctcctcgcccttggaCTTGCCTGAGGCATCTTG (SEQ ID NO: 81)	
16	SP1_F16_primer	CttttctacggatccATCGCAACACCTCCATATTGTTCC (SEQ ID NO: 82)	SP16A
	SP1_R16_primer	ctcctcgcccttggaGGCCAACGGAGTCGTCTCG (SEQ ID NO: 83)	

[0108]

Analyses of a part of PCR product using 2% agarose gel (1 x TBE buffer, with ethidium bromide) confirmed a single band of putative size.

When a single band was not confirmed, annealing temperature was changed from 55°C to 60°C and performed PCR once more.

PCR products were purified using PCR product purification kit (QIAquick PCR purification kit, QIAGEN) and purified PCR products were quantified by absorption photometer.

A signal peptide fragment comprising its own promoter region is named as a signal peptide xA (SPxA) (x=1 to 16).

[0109]

Vector Preparation

Vectors for cloning SPxA were prepared as follows. A summary of the preparation is shown in Fig. 1. Plasmid pAmyB-GFPuv vector (Fig. 1, top panel, right figure; SEQ ID No.: 1) was completely digested with BamHI and Eco147I (both from Fermentas). Reacting condition was in accordance with the instruction for use of the enzymes. Digested plasmid was fractioned by electrophoresis on 0.8% agarose gel for purification (1 x TBE buffer, with ethidium bromide), a large fragment of approximately 4.2 kbps was cut out, and DNA was extracted from agarose and purified using DNA extraction kit from a gel (QIAquick Gel Extraction Kit, QIAGEN). Purified DNA fragment (vector) was electrophoresed on 0.8% agarose gel (1 x TBE buffer, with ethidium bromide) with a DNA concentration marker to estimate its concentration.

For GFPuv coding sequence in pAmyB-GFPuv vector, codons have been optimized (GenScript) for *Bifidobacterium*.

[0110]

Recombination Reaction

The vector and insert prepared above were mixed in 1: 3 to 10 molar ratio, and linked by recombination reaction (CloneEZ Kit, GenScript). Reacting conditions were in accordance with the product instruction.

[0111]

Transformation of *E. coli*

E. coli TOP10 chemically Competent Cell (Life Technologies Japan) was transformed using 2μL of the recombination reaction solution above, smeared onto a LB (containing 75μg/mL spectinomycin) plate and cultured overnight at 37°C. Transforming conditions were in accordance with the product instruction.

The transformed *E. coli* colonies were cultured overnight in a LB (containing 75μg/mL spectinomycin) liquid medium at 37°C, and plasmid was extracted from the

culture (QIAprep Spin Miniprep Kit, QIAGEN). The insert sequence in this plasmid was determined, and the plasmid was named as pSPxA-GFP (x=1 to 16).

[0112]

Transformation of *Bifidobacterium*

3 to 5 μL of the plasmid DNA extracted from transformed *E. coli* above was used for transforming *Bifidobacterium longum* 105A by electroporation system (Gene Pulser II, Bio-Rad Laboratories). Immediately after an electric shock, a mixture of 800μL of IMR liquid medium and 50μL of vitamin C additive solution was added to the cuvette, which was then collected in a sterilized 2mL microtube. Similar manipulation was performed for each tube, before loosening the lid of these 2mL tubes and placing in a dessicator. The dessicator was deaerated by a vacuum pump and filled with carbon dioxide. This manipulation was repeated three times to replace the air in the dessicator with carbon dioxide, before placing the dessicator in an incubator set to 37°C and incubating for 3 hours.

[0113]

After the incubation, each bacterial suspension was mixed thoroughly, and 100μL thereof was measured and smeared to two IMR agar media (containing 75μg/mL SPCM). These plates were placed in a sealed vessel with deoxygenating/carbon dioxide-generating agent (Anaero Pac®-Anaero, MITSUBISHI GAS CHEMICAL, INC.) and cultured for two days in an incubator set to 37°C.

[0114]

Production Example 2: Construction of a Secretory GFP-expressing Plasmid (pSPxB-GFP)

A plasmid that expresses secretory GFP by histone-like promoter (HU promoter) of *Bifidobacterium*. A summary is shown in Fig. 2. Details are given below.

[0115]

Insert Preparation

Among the 25 secretory protein candidates above, for 22 candidates (Nos. 1-16, 19, 21-25) excluding 3 (Nos. 17, 18, 20) that were assumed to be deficient protein coding

sequences, DNA fragments containing the putative signal peptide coding parts and 60 to 90 nucleotides downstream thereof were amplified by PCR.

Forward primers were designed at the translation start site and reverse primers were designed at 60 to 90 nucleotides downstream of the DNA encoding the signal peptides. PCR primers for each signal peptide are shown in Table 2.2. 15 nucleotides at 5' side of each primer have a homologous sequence to the vector shown below.

[0116]+[0117]

Table 2.2 Primers for amplification of signal peptides (SPx)

	Primer Name	Sequence (5' -> 3')	PCR product
1	SP1_F2_primer	caagaaggatgctttATGGCGGAAACTACCGTTAAGC (SEQ ID NO: 84)	SP1
	SP1_R1_primer	ctcctcgcccttggaTTCCACGCGCTCCTTGG (SEQ ID NO: 53)	
2	SP2_F2_primer	caagaaggatgctttGTGGGTATGACTGAGAACG (SEQ ID NO: 85)	SP2
	SP1_R2_primer	ctcctcgcccttggaCAAAAACAGCACGCGGGTG (SEQ ID NO: 55)	
3	SP3_F2_primer	caagaaggatgctttATGTTCAATAAGCGACAC (SEQ ID NO: 86)	SP3
	SP1_R3_primer	ctcctcgcccttggaGGCGATGGTCAGCTTGC (SEQ ID NO: 57)	
4	SP4_F2_primer	caagaaggatgctttATGACCACTCACAACAGC (SEQ ID NO: 87)	SP4
	SP1_R4_primer	ctcctcgcccttggaGCCGAACAGACGCGGGGG (SEQ ID NO: 59)	
5	SP5_F2_primer	caagaaggatgctttATGACCGCGATTGACGAG (SEQ ID NO: 88)	SP5
	SP1_R5_primer	ctcctcgcccttggaTTGGTCGATGATGGCCTTG (SEQ ID NO: 61)	

6	SP6_F2_primer	caagaaggatgctttATGAAGATTGCGGTTGCAGG (SEQ ID NO: 89)	SP6
	SP1_R6_primer	ctcctcgcccttggaATCGACAATAGGACTTTTCC (SEQ ID NO: 63)	
7	SP7_F2_primer	caagaaggatgctttATGTTTGCGTGCGTAGCC (SEQ ID NO: 90)	SP7
	SP1_R7_primer	ctcctcgcccttggaGGTGGAGGTGGATTCGG (SEQ ID NO: 65)	
8	SP8_F2_primer	caagaaggatgctttATGGTTGGTGACGACACC (SEQ ID NO: 91)	SP8
	SP1_R8_primer	ctcctcgcccttggaCATCGTTGCCTCGCCCG (SEQ ID NO: 67)	
9	SP9_F2_primer	caagaaggatgctttATGGGCACCATGATGCG (SEQ ID NO: 92)	SP9
	SP1_R9_primer	ctcctcgcccttggaGACGATCTGATGCGCCAGC (SEQ ID NO: 69)	
10	SP10_F2_primer	caagaaggatgctttATGATGACTGGTGCACAGG (SEQ ID NO: 93)	SP10
	SP1_R10_primer	ctcctcgcccttggaTCGCTGCTTGAGTTTGCCG (SEQ ID NO: 71)	
11	SP11_F2_primer	caagaaggatgctttATGAAGTTCACCGTTGC (SEQ ID NO: 94)	SP11
	SP1_R11_primer	ctcctcgcccttggaCAGCGTGGGCTCCCAAGCC (SEQ ID NO: 73)	
12	SP12_F2_primer	caagaaggatgctttATGGTGTCTTTCAATAAACTGACC (SEQ ID NO: 95)	SP12
	SP1_R12_primer	ctcctcgcccttggaGGAACGGGTCCACAGGGTG (SEQ ID NO: 75)	
13	SP13_F2_primer	caagaaggatgctttATGGTCGCCGTCCTCAGG (SEQ ID NO: 96)	SP13
	SP1_R13_primer	ctcctcgcccttggaAGACTCGCTAGCACAGCAC (SEQ ID NO: 77)	
14	SP14_F2_primer	caagaaggatgctttTTGCCGGGACCTATATGTCC (SEQ ID NO: 97)	SP14

	SP1_R14_primer	ctcctcgcccttggaTTGGGCCACTATTGTCTTC (SEQ ID NO: 79)	
15	SP15_F2_primer	caagaaggatgctttATGAAACGTAGCGATTATATGTTGG (SEQ ID NO: 98)	SP15
	SP1_R15_primer	ctcctcgcccttggaCTTGCCTGAGGCATCTTG (SEQ ID NO: 81)	
16	SP16_F2_primer	caagaaggatgctttATGAGCAATAGTGCATCATCG (SEQ ID NO: 99)	SP16
	SP1_R16_primer	ctcctcgcccttggaGGCCAACGGAGTCGTCTCG (SEQ ID NO: 83)	
19	SP19_F2_primer	caagaaggatgctttTTGGCAAGATGGGTCACTC (SEQ ID NO: 100)	SP19
	SP19_R2_primer	ctcctcgcccttggaGCCCATGACCGGCATGAAC (SEQ ID NO: 101)	
21	SP21_F2_primer	caagaaggatgctttATGGCATTGACTGATGAACAGG (SEQ ID NO: 102)	SP21
	SP21_R2_primer	ctcctcgcccttggaACGTGCAGTGGTATGGATG (SEQ ID NO: 103)	
22	SP22_F2_primer	caagaaggatgctttTTGGTGTCTATGAGAAGC (SEQ ID NO: 104)	SP22
	SP22_R2_primer	ctcctcgcccttggaGATGCGCTCACGCTTGG (SEQ ID NO: 105)	
23	SP23_F2A_primer	gaaggatgctttATGAACAAGCGATGGAAC (SEQ ID NO: 106)	SP23
	SP23_R2_primer	ctcctcgcccttggaGATCGTCTTGAGAATCTTCAGAC (SEQ ID NO: 107)	
24	SP24_F2_primer	caagaaggatgctttATGGTCGGCATGCGCGAC (SEQ ID NO: 108)	SP24
	SP24_R2_primer	ctcctcgcccttggaGTTGGTGCGGTTCCGGTAG (SEQ ID NO: 109)	
25	SP25_F2_primer	caagaaggatgctttGTGATGTTATCCACACC (SEQ ID NO: 110)	SP25
	SP25_R2_primer	ctcctcgcccttggaCTGCTCATGATCGGCCCAG (SEQ ID NO: 111)	

[0118]

PCR was performed in a similar way to Production Example 1 above, and the prepared PCR products were named as SPx (x=1-16, 19, 21-25).

[0119]

Vector Preparation

Vectors for cloning SPx were prepared as follows. A summary of the preparation is shown in Fig. 2. Plasmid pScHuGFPuv vector (Fig. 2, top panel, right figure; SEQ ID No.: 2) was fully digested with HindIII (Fermentas). Reacting conditions were in accordance to the instruction of the enzyme. Digested plasmid was fractioned by electrophoresis on 0.8% agarose gel for purification (1 x TBE buffer, with ethidium bromide), and a straight chain DNA fragment of approximately 4.6 kbps was cut out, and DNA was extracted from agarose and purified using DNA extraction kit from a gel (QIAquick Gel Extraction Kit, QIAGEN). Purified DNA fragment (vector) was electrophoresed on 0.8% agarose gel (1 x TBE buffer, with ethidium bromide) with a DNA concentration marker to estimate its concentration.

For GFPuv coding sequence in pScHuGFPuv vector, codons have been optimized (GenScript) for *Bifidobacterium*.

[0120]

Recombination Reaction

The vector and insert prepared above were mixed in 1: 3 to 10 molar ratio, and linked by recombination reaction (CloneEZ Kit, GenScript). Reaction conditions were in accordance with the product instruction.

[0121]

Transformation of *E. coli*

E. coli TOP10 chemically Competent Cell (Life Technologies Japan) was transformed using 2μL of the recombination reaction solution above, smeared onto a LB (containing 75μg/mL spectinomycin) plate and cultured overnight at 37°C. Transforming conditions were in accordance with the product instruction.

The transformed *E. coli* colonies were cultured overnight in a LB (containing 75μg/mL spectinomycin) liquid medium at 37°C, and plasmid was extracted from the

culture (QIAprep Spin Miniprep Kit, QIAGEN). The insert sequence in this plasmid was determined, and the plasmid was named as pSPxB-GFP (x=1-16, 19, 21-25).

[0122]

Transformation of *Bifidobacterium*

Bifidobacterium was transformed in a similar way as Production Example 1 above.

[0123]

Production Example 3: Construction of a Secretory GFP-expressing Plasmid (pSec2-GFP)

A secretory peptide Sec2 has been reported in *Bifidobacterium breve* UCC2003 (Laura E. MacConaill et al., Applied and Environmental Microbiology, 2003 Vol. 69: pp6994-7001). From the genomic sequence of *B. longum* 105A, a sequence with high homology to Sec2 was searched and its secretory signal was linked to a coding sequence of GFP. A plasmid which expresses this by a HU promoter was constructed using the plasmid pScHuGFPuv vector of Production Example 2. A summary is shown in Fig. 3. Details are given below.

[0124]

Insert Preparation

Sec2-F1 primer and Sec2-R2 primer were designed at the translation start site of Sec2 gene and at 123bps downstream of the signal peptide coding sequence of *B. longum* 105A, respectively. Primer sequences are shown in Table 2.3. 15 nucleotides at 5' side of each primer have a homologous sequence to the vector shown below.

[0125]

Table 2.3 Primers for amplification of signal peptides (Sec2)

	Primer Name	Sequence (5' -> 3')	PCR product
Sec2	Sec2-F1 primer	caagaaggatgctttTTGGAACATATGAAGATGTTCC (SEQ ID NO: 112)	Sec2
	Sec2-R2 primer	ctcctcgcccttggaGTCGAGTTTCATTGTATCG (SEQ ID NO: 113)	

[0126]

PCR was performed in a similar way to Production Example 1 above, and the prepared PCR product was named as Sec2.

[0127]

Vector Preparation

Preparation was in a similar way as Production Example 2 above, using a plasmid pScHuGFPuv vector (Fig. 3, top panel, right figure; SEQ ID No.: 2)

[0128]

Recombination Reaction

The vector and insert prepared above were mixed in 1:10 molar ratio, linked by a recombination reaction (CloneEZ Kit, GenScript). Reacting conditions were in accordance with the product instruction.

[0129]

Transformation of *E. coli*

E. coli TOP10 chemically Competent Cell (Life Technologies Japan) was transformed using 2μL of the recombination reaction solution above. Transforming conditions were in accordance with the product instruction.

The transformed *E. coli* colonies were cultured overnight in a LB (containing 75μg/mL spectinomycin) liquid medium at 37°C, and plasmid was extracted from the culture (QIAprep Spin Miniprep Kit, QIAGEN). The insert sequence in this plasmid was determined, and the plasmid was named as pSec2-GFP (SEQ ID No.: 3).

[0130]

Transformation of *Bifidobacterium*

Bifidobacterium was transformed in a similar way as Production Example 1 above.

[0131]

Working Example 1: GFP Protein Expression of Recombinant Bifidobacteria

The recombinant bifidobacteria obtained from Production Examples 1 to 3 (*Bifidobacterium longum* 105A/pSPxA-GFP (x=1-16), *Bifidobacterium longum* 105A/pSPxB-GFP (x=1-16, 19, 21-25) and *Bifidobacterium longum* 105A/ pSec2-GFP)

in glycerin stock solution were inoculated at 1% in APS-2S-2.5SE (75μg/mL spectinomycin) liquid medium and cultured in anaerobic condition at 37°C for 24 hours (activating culture solution).

[0132]

Subsequently, the activating culture solution was inoculated at 0.5% in a medium (for each 20mL of APS-2S-2.5SE (75μg/mL spectinomycin) liquid medium, 4mL of 1M sodium phosphate buffer (pH6.8) was added). This was cultured in anaerobic condition at 37°C for 18 hours.

This culture solution was used to prepare culture supernatant and intracellular proteins as follows.

[0133]

The culture solution was centrifuged and then culture supernatant was collected. Proteins in this culture supernatant were precipitated by trichloroacetic acid (TCA), washed with acetone, dissolved in an electrophoresis buffer, and the proteins in the culture supernatant were concentrated. Besides, intracellular proteins were extracted as follows. 1mL of the culture solution was mixed with 4mL of PBS, centrifuged at 12,000rpm for 5 minutes at 4°C, and the supernatant was removed. The precipitation was suspended in 5mL PBS and centrifuged to remove the supernatant, which was repeated twice. After washing, the cells were made to the total volume of 1mL with PBS, homogenized with a sonicator. After centrifugation, the supernatant was collected to provide an intracellular extract.

[0134]

A similar operation was performed for wild type *Bifidobacterium longum* 105A for a negative control. For a positive control for GFP protein, recombinant GFPuv (Clontech) was used.

The culture supernatant concentrate (corresponding to 1mL culture solution) and intracellular protein extract (corresponding to 7.5μL culture solution) above were electrophoresed on 12.5% tris-glycine gel (ATTO Corporation, e-PAGEL®). This was transferred to a PVDF membrane (Invitrogen, iBlot® Transfer Stacks) using iBlot® Transfer Device (Invitrogen). After blotting, the membrane was blocked, then reacted with a rabbit GFP antibody (Clontech, A.v. peptide Antibody Living Colors) as primary

antibody and anti-Rabbit IgG HRP Conjugate (Santa Cruz Biotechnology) as secondary antibody, and developed with ECL Advance Western blotting Detection Kit (GE Healthcare). This was analyzed by an imaging analyzer (Fluor S Max, Bio-Rad).

[0135]

As a result, 13 bacteria (*B. longum* 105A/pSP1B-GFP, *B. longum* 105A/pSP2B-GFP, *B. longum* 105A/pSP3B-GFP, *B. longum* 105A/pSP4B-GFP, *B. longum* 105A/pSP7B-GFP, *B. longum* 105A/pSP9B-GFP, *B. longum* 105A/pSP10B-GFP, *B. longum* 105A/pSP12B-GFP, *B. longum* 105A/pSP16B-GFP, *B. longum* 105A/pSP23B-GFP, *B. longum* 105A/pSP7A-GFP, *B. longum* 105A/pSP12A-GFP and *B. longum* 105A/pSec2-GFP) showed secreting tendency. Similar test was performed twice, and prominent secretory effect was confirmed particular in 5 (*B. longum* 105A/pSP3B-GFP, *B. longum* 105A/pSP7B-GFP, *B. longum* 105A/ pSP23B-GFP, *B. longum* 105A/pSP7A-GFP and *B. longum* 105A/pSec2-GFP) (Fig. 4).

[0136]

Working Example 4: Production of a Secretory TNF alpha-expressing *Bifidobacterium* (pSPxA-TNF alpha and pSPxB-TNF alpha)

Construction of Plasmid Vector pTNF1

The codons of coding sequence of human TNFα (Accession No. X01394) were optimized for *Bifidobacterium* and inserted into pUC57vector (outsource synthesis to GenScript). This plasmid was used as template for PCR (PrimeSTAR® HS Premix, TAKARA BIO, Inc.) targeting to TNFα coding region using TNF-F1 primer and TNF-R1 primer (Table 3). PCR product was purified (QIAquick PCR purification Kit, QIAGEN) and electrophoresed on 0.8% agarose gel, and a DNA fragment of approximately 0.7kbps was cut out. DNA was extracted from this gel (QIAquick Gel Extraction Kit, QIAGEN) to provide the insert.

[0137]

Table 3 Primers for constructing plasmid vector pTNF1

Primers	Sequence (5' -> 3')
TNF-F1 primer	gaaggatgctttATGTCCACCGAATCCATGATCCG (SEQ ID NO: 114)
TNF R1 primer	acgagcagaaggTCACAGGGCGATGATGCCGAAG (SEQ ID NO: 115)

[0138]

Besides, the vector was prepared as follows. 10μL each of the restriction enzymes FastDigest Bsp119 I, FastDigest Pst I, FastDigest Nde I and FastDigest Acl I (Fermentas) were added to 10μg of plasmid pCDshuttle (Patent literature 9; WO2009/128272A1) and incubated at 37°C for 4.5 hours to fully digest the plasmid. This was electrophoresed on0.8% agarose gel, and a DNA fragment of approximately 3.9 kbps was cut out. DNA was extracted from this gel (QIAquick Gel Extraction Kit, QIAGEN) to provide the vector.

[0139]

20 ng of the vector and 36ng of the insert above were linked by recombination of terminal sequences using CloneEZ Kit (GenScript). Details were in accordance with the product instruction of CloneEZ Kit. 2μL of this DNA was used for transforming *E. coli* TOP10 chemically Competent Cell (Life Technologies Japan). Transforming conditions were in accordance with the product instruction.

Transformed *E. coli* colonies were cultured overnight in LB (containing 75μg/mL spectinomycin) liquid medium at 37°C, and plasmid was extracted from this culture (QIAprep Spin Miniprep Kit, QIAGEN). This plasmid was named as pTNF1 (Fig. 5, top panel, right figure; SEQ ID No.: 4).

[0140]

The construction summaries of plasmids pSPxA-TNF alpha and pSPxB-TNF alpha in which the GFP portion of plasmids pSPxA-GFP and pSPxB-GFP has been replaced by TNF alpha were shown in Figs. 5 and 6, respectively.

Plasmid pTNF1 was used as template for PCR (PrimeSTAR® HS Premix, TAKARA BIO, Inc.) using TNFvec F1 primer and TNFvec R1 primer (Table 4), and PCR product of approximately 3.8 kbps was obtained to provide the vector.

[0141]

Table 4 Vector primers for constructing pSPxA-TNF alpha and pSPxB-TNF alpha

Primers	Sequence (5' -> 3')
TNFvec_F1_primer	GTGCGCTCCTCCTCCCGTAC (SEQ ID NO: 116)
TNFvec_R1_primer	GCCGTAGTTAGGCCACCACTTCAAG (SEQ ID NO: 117)

[0142]

Besides, the plasmid pSPxA-GFP (x=7 or 12) or pSPxB-GFP (x=1-4, 7, 9, 10, 12, 16 or 23) which showed secreting tendency in Working Example 1 was used as template for PCR amplification (PrimeSTAR® HS Premix, TAKARA BIO, Inc.) of the insert using primers of Table 5, to provide the insert.

[0143]

Table 5 Insert primers for constructing pSPxA-TNF alpha, pSPxB-TNF alpha and pSec2-TNF alpha

PCR product for	Primers	Sequence (5' -> 3')	Template Plasmid
pSP7A-TNF	pUC_ori_F2 primer	tggcctaactacggctacac (SEQ ID NO: 118)	pSP7A-GFP
	SP7-TNF_R1 primer	ggaggaggagcgcacGGTGGAGGTGGATTCGGCGAAC (SEQ ID NO: 119)	
pSP12A-TNF	pUC_ori_F2 primer	tggcctaactacggctacac (SEQ ID NO: 118)	pSP12A-GFP
	SP12-TNF_R1 primer	ggaggaggagcgcacGGAACGGGTCCACAGGGTGAT (SEQ ID NO: 120)	

pSP1B-TNF	pUC_ori_F2 primer	tggcctaactacggctacac (SEQ ID NO: 118)	pSP1B-GFP
	SP1B-TNF_R1 primer	ggaggaggagcgcacTTCCACGCGCTCCTTGGCGATG (SEQ ID NO: 121)	
pSP2B-TNF	pUC_ori_F2 primer	tggcctaactacggctacac (SEQ ID NO: 118)	pSP2B-GFP
	SP2B-TNF_R1 primer	ggaggaggagcgcacCAAAAACAGCACGCGGGTG (SEQ ID NO: 122)	
pSP3B-TNF	pUC_ori_F2 primer	tggcctaactacggctacac (SEQ ID NO: 118)	pSP3B-GFP
	SP3B-TNF_R1 primer	ggaggaggagcgcacGGCGATGGTCAGCTTGC (SEQ ID NO: 123)	
pSP4B-TNF	pUC_ori_F2 primer	tggcctaactacggctacac (SEQ ID NO: 118)	pSP4B-GFP
	SP4B-TNF_R1 primer	ggaggaggagcgcacGCCGAACAGACGCGGGGGAA (SEQ ID NO: 124)	
pSP7B-TNF	pUC_ori_F2 primer	tggcctaactacggctacac (SEQ ID NO: 118)	pSP7B-GFP
	SP7-TNF_R1 primer	ggaggaggagcgcacGGTGGAGGTGGATTCGGCGAAC (SEQ ID NO: 119)	
pSP9B-TNF	pUC_ori_F2 primer	tggcctaactacggctacac (SEQ ID NO: 118)	pSP9B-GFP
	SP9B-TNF_R1 primer	ggaggaggagcgcacGACGATCTGATGCGCCAGCGCATC (SEQ ID NO: 125)	
pSP10B-TNF	pUC_ori_F2 primer	tggcctaactacggctacac (SEQ ID NO: 118)	pSP10B-GFP
	SP10B-TNF_R1 primer	ggaggaggagcgcacTCGCTGCTTGAGTTTGCCGGAAATC (SEQ ID NO: 126)	
pSP12B-TNF	pUC_ori_F2 primer	tggcctaactacggctacac (SEQ ID NO: 118)	pSP12B-GFP
	SP12-TNF_R1 primer	ggaggaggagcgcacGGAACGGGTCCACAGGGTGAT (SEQ ID NO: 120)	
pSP16B-TNF	pUC_ori_F2 primer	tggcctaactacggctacac (SEQ ID NO: 118)	pSP16B-GFP
	SP16B-TNF_R1 primer	ggaggaggagcgcacGGCCAACGGAGTCGTCTC (SEQ ID NO: 127)	

pSP23B-TNF	pUC_ori_F2 primer	tggcctaactacggctacac (SEQ ID NO: 118)	pSP23B-GFP
	SP23B-TNF_R1 primer	ggaggaggagcgcacGATCGTCTTGAGAATCTTCAGACG (SEQ ID NO: 128)	
pSec2-TNF	Sec2_out1_primer	tacGGATCCgtcttcctgctg (SEQ ID NO: 129)	pSec2-GFP
	Sec2a_R1_primer	GTACGGGAGGAGGAGCGCACGTCGAGTTTCATTGTATCG (SEQ ID NO: 130)	
pSec2-TNF	Sec2a_F1_primer	CGATACAATGAAACTCGACGTGCGCTCCTCCTCCCGTAC (SEQ ID NO: 131)	pTNF1
	TNF_out1_primer	aggACTAGTccggaataatacgg (SEQ ID NO: 132)	

[0144]

100ng of the vector and 40ng of the insert above were linked by In-Fusion™ Advantage PCR Cloning Kit (TAKARA BIO, Inc.). 2μL of this DNA was used for transforming *E. coli* TOP10 chemically Competent Cell (Life Technologies Japan). Transforming conditions were in accordance with the product instruction.

[0145]

Transformed *E. coli* colonies were cultured overnight in LB (containing 75μg/mL spectinomycin) liquid medium at 37°C, and plasmids were extracted from this culture (QIAprep Spin Miniprep Kit, QIAGEN). These plasmids were fully sequenced and their plasmid names were assigned as pSP7A-TNF alpha, pSP12A-TNF alpha, pSP1B-TNF alpha, pSP2B-TNF alpha, pSP3B-TNF alpha, pSP4B-TNF alpha, pSP7B-TNF alpha, pSP9B-TNF alpha, pSP10B-TNF alpha, pSP12B-TNF alpha, pSP16B-TNF alpha, pSP23B-TNF alpha.

[0146]

Transformation of Bifidobacteria with pSPxA-TNF alpha and pSPxB-TNF alpha

Plasmids pSPxA-TNF alpha and pSPxB-TNF alpha were used for transforming *B. longum* 105A in a similar way as Production Example 1.

[0147]

Reference Example 2: Construction of Plasmid pTNF3

We constructed a shuttle vector (*Bifidobacterium* - *E. coli*) in which the mature human TNFα coding sequence is located downstream of Hu promoter derived from *Bifidobacterium*. A summary is shown in Fig. 12. Details are as follows.

[0148]

Insert Preparation

We constructed a plasmid human TNFalpha_in_pUC57 containing an artificial DNA having human TNFα (Accession No:X01394; from 153th to 854th nucleotides of an immature TNFα coding sequence) of which codons are optimized for *Bifidobacterium*, and Hu promoter derived from *Bifidobacterium* located upstream thereof and Hu terminator derived from *Bifidobacterium* located downstream thereof (custom-synthesized by GenScript).

1ng of the plasmid human TNFalpha_in_pUC57 was used as template for PCR amplification of the mature TNFα portion of the TNFα coding sequence by PrimeSTAR® HS Premix (TAKARA BIO, Inc.). TNF F3 and TNF R1 primers were used, wherein the 15 nucleotides of the 5' side of each primer had a homologous sequence to the vector terminal (Table 6). The PCR program consisted of 30 cycles of 10 seconds at 98°C, 5 seconds at 60°C and 30 seconds at 72°C, followed by 30 seconds at 72°C.

A part of PCR product was electrophoresed with DNA concentration marker on 2% agarose gel (1 x TBE buffer, containing ethidium bromide), confirming a single band of approximately 0.5kbp and estimating its concentration.

[0149]

Vector Preparation

1ng of the plasmid pCDshuttle was used as template for PCR amplification of the vector skeletal by PrimeSTAR® HS Premix (TAKARA BIO, Inc.). Primers pCDshuttle F1 and pCDshuttle R1 were used, wherein the 15 nucleotides on the 5' side of each primer had a homologous sequence to the insert terminal (Table 6). The PCR program consisted of 30 cycles of 10 seconds at 98°C, 5 seconds at 55°C and 4 minutes at 72°C, followed by 30 seconds at 72°C.

A part of PCR product was electrophoresed with DNA concentration marker on 0.8% agarose gel (1 x TBE buffer, containing ethidium bromide), confirming a single band of approximately 3.9kbps.

[0150]

Table 6: Primers for pTNF3 construction

Primers	Sequence (5' -> 3')	PCR product
TNF F3_primer	GAAGGATGCTTTATGGTGCGCTCCTCCCG (SEQ ID NO: 141)	insert
TNF_R1_primer	ACGAGCAGAAGGTCACAGGGCGATGATGCCC AAG (SEQ ID NO: 142)	insert
pCDshuttle_F1_primer	TGACCTTCTGCTCGTAGCG (SEQ ID NO: 143)	vector
pCDshuttle_R1_primer	CATAAAGCATCCTTCTTGGGTCAG (SEQ ID NO: 144)	vector

[0151]

Cloning

100ng of the vector and 50ng of the insert above were ligated by recombination of terminal sequences using In-Fusion Advantage PCR Cloning Kit (TAKARA BIO, Inc.). At this time, Cloning Enhancer (TAKARA BIO, Inc.) was also added into the reacting solution, concurrently degrading the template plasmid contained in the vector and the insert. Details were in accordance with the product instruction of In-Fusion Advantage PCR Cloning Kit.

2μL of the In-Fusion reaction solution above was used for transforming *E. coli* TOP10 chemically Competent Cell (Invitrogen). Transforming conditions were in accordance with the product instruction. Transformed *E. coli* colonies were cultured overnight at 37°C in LB (containing 75μg/mL spectinomycin) liquid medium, and the plasmid was extracted from this culture (QIAprep Spin Miniprep Kit, QIAGEN). This plasmid was full-sequenced and named pTNF3 (SEQ ID No: 51).

[0152]

Transformation of *Bifidobacterium*

The plasmid pTNF3 was used for transforming *B. longum* 105A using a similar method as Production Example 1.

[0153]

Production Example 5: Production of a Secretory TNF alpha-expressing *Bifidobacterium* (pSec2-TNF alpha)

Summary of the construction of pSec2-TNF alpha, a plasmid in which the GFP portion of the plasmid pSec2-GFP was replace by TNF alpha, is shown in Fig. 7.

[0154]

Vector Preparation

Plasmid pCDshuttle was fully digested with BamHI, BcuI and PstI (all from Fermentas). Reacting conditions were in accordance with the instruction of the enzymes. Digested plasmid was fractioned by electrophoresis on 1% agarose gel for purification (1 x TBE buffer, with ethidium bromide), and a large fragment of approximately 3.4kbps was cut out, and DNA was extracted from the agarose gel by DNA extraction kit (QIAquick Gel Extraction Kit, QIAGEN). Purified DNA fragment (vector) was electrophoresed on 0.8% agarose gel (1 x TBE buffer, with ethidium bromide) with DNA concentration marker to estimate its concentration.

[0155]

Insert Preparation

Plasmid pSec2-GFP was used as template for PCR amplification of Sec2 signal peptide coding sequence including HU promoter with Sec2_out1 primer and Sec2a_R1 primer (Table 5) (PCR product 1). Besides, plasmid pTNF1 was used for PCR amplification of TNF alpha coding sequence including HU terminator with Sec2a_F1 primer and TNF_out1 primer (Table 5) (PCR product 2). PCR products 1 and 2 were purified with PCR product purification kit (QIAquick PCR purification kit, QIAGEN), and the amount of PCR products was estimated by absorption measurement. PCR product 1 and PCR product 2 were mixed in equimolar amount. This PCR product mixture solution 1ng plus 2 x PCR Solution PrimeSTAR HS (TAKARA BIO, Inc.) was made to 49μL with 0.1 x TE buffer. This solution was set in a thermal cycler, and two PCR fragments were linked by the reaction of 5 cycles, each cycle consisting of 98°C for 10 seconds and 72°C for 36 seconds. Then, the linked PCR product was amplified by adding Sec2_out1 primer and TNF_out1 primer, reacting 25 cycles, each cycle

consisting of 98°C for 10 seconds, 55°C for 5 seconds and 72°C for 70 seconds, before elongation at 72°C for 30 seconds.

[0156]

This was fractioned by electrophoresis on 1% agarose gel for purification (1 x TBE buffer, with ethidium bromide), and a fragment of approximately 1.2kbp was cut out, and DNA was extracted and purified from the agarose gel using DNA extraction kit (QIAquick Gel Extraction Kit, QIAGEN). This purified DNA fragment was fully digested with BamHI and BcuI. Reacting conditions were in accordance with the instruction of the enzymes. Digested plasmid was fractioned by electrophoresis on 1% agarose gel for purification (1 x TBE buffer, with ethidium bromide), and a DNA fragment of approximately 1.2kbp was cut out, and DNA was extracted and purified from agarose gel using DNA extraction kit (QIAquick Gel Extraction Kit, QIAGEN). Purified DNA fragment (insert) was electrophoresed on 0.8% agarose gel (1 x TBE buffer, with ethidium bromide) with DNA concentration marker to estimate its concentration.

[0157]

Ligation

The vector and the insert above were mixed in 1:3 molar ratio for ligation (Rapid DNA Ligation Kit, Fermentas). Details were in accordance with the product instruction.

[0158]

Transformation of *E. coli*

2μL of the ligation reaction solution above was used for transforming *E. coli* TOP10 chemically Competent Cell (Life Technologies Japan). Transforming conditions were in accordance with the product instruction.

Transformed *E. coli* colonies were cultured overnight in LB (containing 75μg/mL spectinomycin) liquid medium at 37°C, and plasmids were extracted from this culture (QIAprep Spin Miniprep Kit, QIAGEN). The insert part of this plasmid was fully sequenced to confirm that there was no PCR error, and the plasmid was named as pSec2-TNF alpha.

[0159]

Transformation of *Bifidobacterium* with pSec2-TNF alpha

Plasmid pSec2-TNF alpha was used for transforming *B. longum* 105A in a similar way as Production Example 1.

[0160]

Working Example 2: TNF alpha Protein Expression by Recombinant *Bifidobacterium*

The recombinant bifidobacteria obtained from Production Example 4 and Production Example 5 (*Bifidobacterium longum* 105A/ pSPxA-TNF alpha (x=7, 12)、*Bifidobacterium longum* 105A/pSPxB-TNF alpha(x=1-4, 7, 9, 10, 12, 16 or 23) and *Bifidobacterium longum* 105A/pSec2-TNF alpha) in glycerin stock solution were inoculated at 1% in APS-2S-2.5SE (75μg/mL spectinomycin) liquid medium and cultured in anaerobic condition at 37°C for 24 hours (activation culture solution).

[0161]

Subsequently, the activating culture solution was inoculated at 0.5% in a medium (for each 20mL of APS-2S-2.5SE (75μg/mL spectinomycin) liquid medium, 4mL of 1M sodium phosphate buffer (pH6.8) was added). This was cultured in anaerobic condition at 37°C for 18 hours.

After centrifuging the culture solution, culture supernatant was collected. Proteins in this culture supernatant was precipitated by trichloroacetic acid (TCA), washed with acetone, dissolved in a buffer for electrophoresis, and proteins in the culture supernatant were concentrated.

[0162]

Besides, intracellular proteins were extracted as follows. 1mL of the culture solution was mixed with 4mL of PBS, centrifuged at 12,000rpm for 5 minutes at 4°C, and the supernatant was removed. The precipitation was suspended in 5mL PBS and centrifuged to remove the supernatant, which was repeated twice. After washing, the cells were made to the total volume of 1mL with PBS, homogenized with a sonicator. After centrifugation, the supernatant was collected to provide an intracellular extract, which was then subjected to westernblot analysis.

[0163]

A similar operation was performed for wild type *Bifidobacterium longum* 105A for a negative control. For a positive control for TNF alpha, human recombinant TNF alpha (PEPRO TECH, INC.) was used.

[0164]

The culture supernatant (corresponding to 7.5μL culture solution), culture supernatant concentrate (corresponding to 1mL culture solution) and intracellular protein extract (corresponding to 7.5μL culture solution) above were electrophoresed on 16% Tris-Glycine gel (Invitrogen). Note that, for following samples, the amount applied was adjusted as follows. The supernatant of SP3B-TNF alpha corresponding to 0.15μL culture solution, the intracellular protein extract of SP16B-TNF alpha corresponding to 0.15μL, the intracellular protein extract of SP23B-TNF alpha corresponding to 0.75μL, the culture supernatant concentrate of the same corresponding to 20μL and 100μL were subjected for electrophoresis. These were transferred to PVDF membranes (Invitrogen, iBlot® Transfer Stacks) using iBlot® Transfer Device (Invitrogen). After blotting, the membranes were blocked, then reacted with anti-human TNF-alpha (goat) (R&D Systems) as primary antibody and anti-Goat IgG HRP Conjugate (Santa Cruz Biotechnology) as secondary antibody, and developed with ECL Advance Western blotting Detection Kit (GE Healthcare). These were analyzed by an imaging analyzer (Fluor S Max, Bio-Rad). The results of the analyses are shown in Fig. 8.

[0165]

As a result, secretion was confirmed in 9 bacteria (*B. longum* 105A/pSP1B- TNF alpha, *B. longum* 105A/pSP3B- TNF alpha, *B. longum* 105A/pSP4B- TNF alpha, *B. longum* 105A/pSP7B- TNF alpha, *B. longum* 105A/pSP12B- TNF alpha, *B. longum* 105A/pSP16B- TNF alpha, *B. longum* 105A/pSP23B- TNF alpha, *B. longum* 105A/pSP7A- TNF alpha and *B. longum* 105A/pSec2-TNF alpha), with particularly prominent expression in the culture supernatant of 2 bacteria (*B. longum* 105A/SP3B-TNF alpha and *B. longum* 105A/SP23B-TNF alpha).

[0166]

Reference Example 3: Confirmation of Secretion by a non-TNFα-secretory bacterium *B. longum* 105A/pTNF 3

The glycerin stocks of *B. longum* 105A/pTNF3 obtained in Reference Example 2 and wild-type *B. longum* 105A were inoculated at 1% to APS-2S-2.5SE (75μg/mL spectinomycin) liquid medium, cultured in anaerobic condition at 37°C for 24 hours (activating culture solution). The activating culture solution was inoculated at 0.5% to a medium (75μg/mL spectinomycin) (for each 20mL of APS-2S-2.5SE liquid medium added 4mL of 1M sodium phosphate buffer (pH6.8)), which was cultured in anaerobic condition at 37°C for 18 hours. Note that wild-type was cultured in a medium which was not supplemented with spectinomycin. This culture solution was centrifuged to collect a culture supernatant. Meanwhile, an intracellular extract was prepared as follows. 1mL of the culture solution was washed with PBS buffer, then the cells were suspended in PBS buffer to make 1mL and homogenized with a sonicator. This was centrifuged, and the supernatant was collected to give an intracellular extract.

[0167]

A sample obtained from wild-type was used as a negative control. As a positive control, human-derived recombinant TNF alpha (PEPRO TECH, INC.) was used. The culture supernatant (corresponding to 7.5μL of the culture solution) and intracellular extract (corresponding to 0.075μL of the culture solution) above were electrophoresed on 15% polyacrylamide gel (ATTO Corporation). This was transferred to a PVDF membrane (Invitrogen, iBlotTransfer Stacks) using iBlot Transfer Device (Invitrogen). After blotting, the membrane was blocked and reacted using anti-human TNF-alpha(goat) (R&D Systems) as a primary antibody and anti-Goat IgG HRP Conjugate (Santa Cruz Biotechnology) as a secondary antibody, and developed by ECL Advance Western blotting Detection Kit (GE Healthcare). It was analyzed with an imaging analyzer (Fluor S Max, Bio-Rad). The results of the analyses are shown in Fig. 9.

[0168]

As a result, when the intracellular extracts of *B. longum* 105A/pTNF3 and wild-type *B. longum* 105A were compared, a band indicating TNFα expression was confirmed in *B. longum* 105A/pTNF3 but not in wild-type *B. longum* 105A. Thus, it was shown that the cells transformed with the plasmid pTNF3 normally express TNFα. However, comparing both culture supernatants confirmed no TNFα in either culture supernatant, indicating that TNFα is not extracellulary secreted from *B. longum* 105A/pTNF3.

[0169]

Production Example 6: Construction of pBifi-SP3B-TNF

Plasmid pBifi-SP3B-TNF was constructed from plasmid pSP3B-TNF alpha (*E. coli - Bifidobacterium* shuttle vector) by removing the origin of replication in *E. coli*. Details of the construction are shown in Fig. 10.

[0170]

Preparation of pUCori-removed Fragment

2.4μg of plasmid extracted from the recombinant *E. coli.* TOP10/pSP3B-TNF alpha (shuttle vector) was digested by BamHI and BglII at 37°C. This was fractioned by electrophoresis using 0.8% agarose gel for purification, and a DNA fragment of approximately 3.8kbps was cut out. DNA was extracted and purified from the cut-out gel (QIAquick Gel Extraction Kit, QIAGEN), and DNA concentration was measured by measuring the absorbance.

[0171]

Self-ligation of pUCori-removed fragment

The pUCori-removed fragment above was self-ligated in 6 tubes. For each tube, 50ng of pUCori-removed fragment was used for self-ligation in 50μL reaction system at 25°C for 5 minutes (RAPID DNA LIGATION KIT, Fermentas), then Ligase was deactivated by heating at 65°C for 5 minutes. 6 ligation reaction solutions were assembled to one tube, and subjected to protein degradation by Proteinase K and subsequent protein removal by phenol/chloroform extraction and ethanol precipitation thereafter. DNA was dissolved in 10μL 0.1 x TE.

[0172]

Transformation of *Bifidobacterium*

Bifidobacterium longum 105A competent cell was transformed (electroporation, Gene Pulser II, Bio-Rad Laboratories, Inc.) using 150ng (5μL) of the purified product after the ligation above. Immediately after an electric shock, a mixture of 800μL of IMR liquid medium and 50μL of vitamin C additive solution was added to the cuvette, which was then collected in a sterilized 2mL microtube. The lid of tube was loosen, and the tube was placed in a dessicator, which was then deaerated by a vacuum pump and filled with carbon dioxide This manipulation was repeated three times to replace the air in the dessicator with carbon dioxide, before placing the dessicator in an incubator set to 37°C and incubating for 3 hours.

[0173]

After the incubation, the bacterial suspension was mixed thoroughly and smeared to two IMR agar media (containing 75μg/mL SPCM). These plates were placed in a sealed vessel with deoxygenating/carbon dioxide-generating agent (Anaero Pac®-Anaero, MITSUBISHI GAS CHEMICAL, INC.) and cultured for two days in an incubator set to 37°C.

[0174]

Confirmation of the Transformant and Production of a Glycerin Stock of Recombinant *Bifidobacterium*

The colonies of candidate recombinant formed on the IMR agar media (containing 75μg/mL SPCM) above were streaked on BL-bS agar media (BL agar media containing spectinomycin, excluding horse defibrinated blood), placed in a sealed vessel with deoxygenating/carbon dioxide-generating agent (Anaero Pac®-Anaero, MITSUBISHI GAS CHEMICAL, INC.) and cultured for one day at 37°C. The streak-cultured bifidobacteria was cultured in anaerobic condition in APS-2S-2.5SE (75μg/mL spectinomycin) liquid medium at 37°C for one day, and plasmid DNA was extracted from this (QIAprep Spin Miniprep Kit, QIAGEN). The extracted DNA was used as template for PCR amplification with Check primer F1 (on AAD9 cassette) and Check primer R2 (on HU promoter), and PCR product size was confirmed by agarose-gel electrophoresis. Primer sequences are shown in Table 6. Locations of

PCR primers are shown in Fig. 9. PCR product size was approximately 0.5kbps, confirming the exclusion of pUC ori fragment. This result confirmed that this recombinant *Bifidobacterium* possesses pBifi-SP3B-TNF alpha, a plasmid in which pUC ori has been removed from pSP3B-TNF alpha.

[0175]

Table 7 Primers for confirmation of shuttle and non-shuttle vectors

Primers	Sequence (5' -> 3')
Check primer F1	TGACTTAGAGGAATTACTACCTG (SEQ ID NO: 133)
Check primer R2	AAAGTGGCGGAAAGCGCCAC (SEQ ID NO: 134)

[0176]

The streak culture on BL-bS agar medium was inoculated in APS-2S-2.5SE (75μg/mL spectinomycin) liquid medium and cultured at 37°C for 24 hours. To this culture solution glycerin solution was added to make a final concentration of 20%, to give a glycerin stock.

[0177]

Nucleotide Sequencing of Plasmid pBifi-SP3B-TNF

The glycerin stock of *Bifidobacterium longum* 105A/pBifi-SP3B-TNF was cultured in anaerobic condition in APS-2S-2.5SE (75μg/mL spectinomycin) liquid medium. Bacterial cells were collected from the culture solution by centrifugation, suspended in 30mM GTA buffer, then treated with *N*-acetyl muramidase. It was further treated with Proteinase K (QIAGEN) before purification by plasmid DNA purification kit (QIAprep Spin Miniprep Kit, QIAGEN). This plasmid DNA was used for determination of full nucleotide sequence, confirming the exclusion of pUCori (SEQ ID No.: 5).

[0178]

Working Example 3: Confirmation of TNF alpha protein secretion from recombinant *Bifidobacterium*

The glycerin stock of *Bifidobacterium longum* 105A/pBifi-SP3B-TNF alpha obtained in Production Example 6 was inoculated at 1% in APS-2S-2.5SE (75μg/mL spectinomycin) liquid medium and cultured in anaerobic condition at 37°C for 24 hours (activating culture solution). The activating culture solution was inoculated at 0.5% in a medium (for each 20mL of APS-2S-2.5SE(75μg/mL spectinomycin) liquid medium, 4mL of 1M sodium phosphate buffer (pH6.8) was added), which was cultured in anaerobic condition at 37°C for18 hours. This culture solution was centrifuged and the culture supernatant was collected. Besides, the intracellular extract was prepared as follows. 1mL of the culture solution was washed with PBS, suspended in PBS to make 1mL, then homogenized by a sonicator. This was centrifuged and the supernatant was collected to give the intracellular extract. Similar manipulation was performed for a shuttle vector *Bifidobacterium longum* 105A/ pSP3B-TNF alpha and wild type *Bifidobacterium longum* 105A (wild type). Note that the wild type was cultured in a medium excluding spectinomycin. A sample obtained from the wild type was used as a negative control. For a positive control, human-derived recombinant TNF alpha (PEPRO TECH, INC.) was used.

[0179]

The culture supernatant (corresponding to 0.75μL culture solution) and intracellular protein extract (corresponding to 1.5μL culture solution) above were electrophoresed on 15% polyacrylamide gel (ATTO Corporation). This was transferred to a PVDF membrane (Invitrogen, iBlot® Transfer Stacks) using iBlot® Transfer Device (Invitrogen). After blotting, the membrane was blocked, then reacted with anti-human TNF-alpha (goat) (R&D Systems) as primary antibody and anti-Goat IgG HRP Conjugate (Santa Cruz Biotechnology) as secondary antibody, and developed with ECL Advance Western blotting Detection Kit (GE Healthcare). This was analyzed by an imaging analyzer (Fluor S Max, Bio-Rad). The result of the analysis is shown in Fig. 11.

[0180]

Working Example 4: Transformation of *E. coli* with pBifi-SP3B-TNF alpha and pSP3B-TNF

Plasmids obtained from Production Example 6 (pBifi-SP3B-TNF alpha and pSP3B-TNF alpha) were used for transforming *E. coli.* TOP10 strain.

Transformation was performed in accordance with the product instruction of *E. coli.* TOP10competent cell (Life Technologies Japan), and 100μL each was smeared onto a LB (containing 75μg/mL spectinomycin) agar medium in duplicate, cultured overnight at 37°C. Colonies were formed only when the shuttle vector pSP3B-TNF alpha was introduced (266cfu and 226cfu), while *E. coli* in which pBifi-SP3B-TNF alpha was transferred formed no colony on the selection medium.

[0181]

Reference Example 4: Construction of Plasmid pBEshuttle

We constructed pBEshuttle as a mock vector having a protein expression unit containing no insert, as follows. A summary is shown in Fig. 13.

[0182]

PCR fragment Preparation

5ng of the plasmid pCDshuttle was used as template for amplifying two PCR fragments A and B using PrimeSTAR® HS Premix (TAKARA BIO, Inc.). MCS F1 primer and TNFvec R1 primer were used for the amplification of PCR fragment A, and pUC ori F2 primer and MCS R1 primer was used for the amplification of PCR fragment B (Table 8).

The 15 nucleotides on 5' side of the primer for the amplification of PCR fragment A was designed to have a homologous sequence to the terminal of PCR fragment B, while the 15 nucleotides on 5' side of the primer for the amplification of PCR fragment B was designed to have a homologous sequence to the terminal of PCR fragment A.

The PCR program consisted of 30 cycles of 10 seconds at 98°C, 5 seconds at 55°C and X seconds (PCR fragment A: X=3 minutes 20 seconds, PCR fragment B: X=35 seconds) at 72°C, followed by 30 seconds at 72°C.

A part of PCR product was electrophoresed on an agarose gel (1 x TBE buffer, containing ethidium bromide; 0.8% agarose gel for PCR product A, 2% agarose gel for PCR product B) with DNA concentration marker, confirming a single band (PCR product A: approximately 3.3kbps, PCR product B: approximately 0.6kbps) and estimating its concentration.

[0183]

Table 8 Primers for pBEshuttle Construction

Primers	Sequence (5' -> 3')	PCR product
MCS_F1 primer	AAGCTTATCCTGCAGTGACCTTCTGCTCGTAGCGA (SEQ ID NO: 135)	A
TNFvec_R1_primer	GCCGTAGTTAGGCCACCACTTCAAG (SEQ ID NO: 117)	A
pUC_ori_F2_primer	TGGCCTAACTACGGCTACAC (SEQ ID NO: 118)	B
MCS_R1 primer	CTGCAGGATAAGCTTCATAAAGCATCCTTCTTG (SEQ ID NO: 136)	B

[0184]

Cloning

100ng of the PCR product A and 35ng of the PCR product B above were ligated by recombination of terminal sequences using In-Fusion Advantage PCR Cloning Kit (TAKARA BIO, Inc.). At this time, Cloning Enhancer (TAKARA BIO, Inc.) was also added into the reacting solution, concurrently degrading the template plasmid contained in the vector and the insert. Details were in accordance with the product instruction of In-Fusion Advantage PCR Cloning Kit.

2μL of the In-Fusion reaction solution above was used for transforming *E. coli* TOP10 chemically Competent Cell (Invitrogen). Transforming conditions were in

accordance with the product instruction. Transformed *E. coli* colonies were cultured overnight at 37°C in LB (containing 75μg/mL spectinomycin) liquid medium, and the plasmid was extracted from this culture (QIAprep Spin Miniprep Kit, QIAGEN). This plasmid was full-sequenced and named pBEshuttle (SEQ ID No: 50).

[0185]

Transformation of *Bifidobacterium*

The plasmid pBEshuttle was used for transforming *B. longum* 105A using a method as used in Production Example 1.

[0186]

Production Example 7: Production of Recombinant *Bifidobacterium B. breve*/pSP3B-TNF alpha

Bifidobacterium breve JCM1192 was transformed with the plasmid pSP3B-TNF alpha in a method as used in the transformation of *Bifidobacterium* in Production Example 1.

[0187]

Working Example 5: Confirmation of TNFα protein expression by Recombinant *Bifidobacterium*

The glycerin stocks of *B. longum* 105A/pBEshuttle obtained in Reference Example 4, *B. longum* 105A/pSP3B-TNF alpha obtained in Production Example 4, *B. longum* 105A/pBifiSP3B-TNF alpha obtained in Production Example 6 and *B. breve*/pSP3B-TNF alpha obtained in Production Example 7 were inoculated at 1% to APS-2S-2.5SE (75μg/mL spectinomycin) liquid media, cultured at 37°C for 24 hours in anaerobic condition (activating culture). The activating culture solution was inoculated at 0.5% to a medium (75μg/mL spectinomycin) (for each 20mL of APS-2S-2.5SE liquid medium added 4mL of 1M sodium phosphate buffer (pH6.8)), which was cultured at 37°C for 18 hours in anaerobic condition. This culture solution was centrifuged to collect a culture supernatant. TNFα content in the culture supernatant was measured by ELISA of the culture supernatant (Quantikine Human TNF alpha/TNFSF1A Immunoassay, R&D Systems, Inc.). The measurement results are shown in Table 9.

[0188]

Table 9

sample name	culture time (hrs)	OD(600nm)	TNFalpha conc. (μg/mL)
B. longum 105A /pBEshuttle	18	2.539	0
B. longum 105A/pSP3B-TNF alpha	18	1.806	0.69
B. longum 105A /pBifiSP3B-TNF alpha	18	1.509	0.42
B. breve /pSP3B-TNF alpha	12	6.864	1.94

[0189]

TNFα secretion was observed in the culture supernatant in either of *B. longum* 105A/pSP3B-TNF alpha, *B. longum* 105A/pBifiSP3B-TNF alpha and *B. breve*/pSP3B-TNF alpha, but not in *B. longum* 105A/pBEshuttle.

[0190]

Working Example 6: The Physiological Activity of TNFα Protein Secreted by Recombinant *Bifidobacteirum* and the Neutralization of the Physiological Activity with Anti-hTNFα Antibody

Culture of Test Bacterium and Preparation of Culture Supernatant

The glycerin stocks of *B. longum* 105A/pBEshuttle obtained in Reference Example 4, *B. longum* 105A/pSP3B-TNF alpha obtained in Production Example 4 and *B. longum* 105A/pBifiSP3B-TNF alpha obtained in Production Example 6 were inoculated at 1% to APS-2S-2.5SE (75μg/mL spectinomycin) liquid media, cultured at 37°C for 24 hours in anaerobic condition (activating culture). The activating culture solution was inoculated at 0.5% to a medium (75μg/mL spectinomycin) (for each 20mL of APS-2S-2.5SE liquid medium added 4mL of 1M sodium phosphate buffer (pH6.8)), which was cultured at 37°C for 18 hours in anaerobic condition. This culture solution was centrifuged to collect a culture supernatant.

[0191]

TNFα Cytotoxicity Assay

The physiological activity and neutralization of rhTNFα was assessed by examining the cytotoxicity via TNFα receptor, which is a physiological activity of TNFα. As a test cell, a human breast cancer cell line KPL-1 cell was used. KPL-1 cell was cultured in a DMEM medium (a DMEM medium supplemented with 10% (v/v) FBS and

0.1% (v/v) penicillin (50000U/mL)/streptomycin (50mg/mL) solution) at 37°C, in 5% CO_2 condition. This cell was seeded onto 96 well plate at 1 x 10^4 cells per well, cultured at 37°C in 5% CO_2 for 24 hours to give confluent cells. The old medium was removed from these cells by aspiration, and freshly added thereto were 80μL each per well of 10% (v/v) FBS supplemented with actinomycin D to make an actinomycin D final concentration of 5μg/mL and DMEM medium supplemented with 0.1% penicillin (50000U/mL)/streptomycin (50mg/mL) solution. Subsequently added were, as samples for measurement, a medium for *Bifidobacterium* (APS-2S-2.5SE), rhTNF alpha prepared at 100ng/mL as rhTNFα standard, five times dilution of *B. longum* 105A/pBEshuttle culture supernatant, five times dilution of *B. longum* 105A/pSP3B-TNF alpha culture supernatant and five times dilution of *B. longum* 105A/pBifiSP3B-TNF alpha culture supernatant, 10μL each per well. Added thereto in order to measure the neutralizing ability against rhTNFα physiological activity were anti-hTNFα antibody (anti-human TNF alpha, R&D Systems, 0.0125 - 0.1mg/mL), normal goat IgG (normal Goat IgG, R&D Sytems, 0.0125 - 0.1mg/mL), and 10% (v/v) FBS and DMEM medium supplemented with 0.1%(v/v) penicillin (50000U/mL)/ streptomycin (50mg/mL) solution, 10μL each per well. This plate was cultured at 37°C in 5% CO_2 for 48 hours.

[0192]

Measuring cytotoxicity employed Cell Counting Kit-8 (DOJINDO), wherein 10μL per well of this solution was added to each well, before further culturing for 4 hours at 37°C in 5% CO_2 and measuring of the absorbance at wavelength of 450nm and 630nm (630nm was used as reference wavelength). The results of the analyses are shown in Fig. 14, in which the culture supernatant of the recombinant bacteria *B. longum* 105A/pSP3B-TNF alpha and *B. longum* 105A/pBifiSP3B-TNF alpha showed cytotoxicity against KPL-1 cells while being neutralized by anti-TNFα antibody, confirming that the recombinant hTNFα secreted in the culture supernatant had a physiological activity.

[0193]

Working Example 7: Measurement of Antitumor Effect of *B. longum* 105A/pSP3B-TNF alpha and *B. breve*/pSP3B-TNF alpha

The antitumor effect of *B. longum* 105A/pSP3B-TNF alpha prepared in Production Example 4 and B.breve/pSP3B-TNF alpha prepared in Production Example 7 were measured.

[0194]

(1) Culturing of transplant tumor cells

Human breast cancer cell line KPL-1 cells were cultured in a DMEM medium supplemented with 10% (v/v) FBS and 0.1%(v/v) penicillin (50000U/mL)/streptomycin (50mg/mL) solution at 37°C in 5%CO_2 condition.

Upon reaching confluent, the cells were detached by washing with 1 x PBS(-) and adding trypsin-EDTA, and the cells were collected by centrifugation (1000 spins/5 minutes) and appropriately diluted with DMEM medium and subcultured.

Cells after 5 passages were used for transplantation experiments. The number of viable cells which were not stained with trypan blue was counted on Thoma hemocytometer (Thoma deep 0.1 mm ERMA, Tokyo), suspended in Hank's solution and the cell number was adjusted to at 2.5 x 10^6 cells/mL.

[0195]

(2) Production of a cancer-bearing nude mouse and measurement of tumor volume

0.2mL of the prepared KPL-1 cell suspension was subcutaneously transplanted to a nude mouse on the dosal side of the right anterior limb (5 x 10^5 cells/mouse).

Tumor volume after transplantation was assessed by measuring tumor diameter (long axis, short axis and thickness) using calipers and calculated by following equation:

Tumor volume (mm^3) = long axis (mm) x short axis (mm) x thickness (mm)/2

[0196]

(3) Grouping and group constitution

From KPL-1 cancer-bearing nude mice, 24 mice whose tumor volumes were around approximately 80 to 135 mm^3 were selected and divided into 3 groups (8 animals for each group) such that the average tumor volume would be similar. This day was set to Day 0.

The constitution of the test groups is as shown in Table 10. That is, Group I: a group with no treatment, Group II: a group receiving *B. longum* 105A/pSP3B-TNF alpha, Group III: a group receiving *B. breve*/pSP3B-TNF alpha.

[0197]

Table 10: Group constitution

Group	Given substance	Dosage	Number of dosage (times/day)	Administration date (Day)	
Group I	—	—	—	—	8
	—	—	—	—	
Group II	B. longum 105A/pSP3B-TNF alpha	0.2 mL/body/time	2	1, 4, 8, 11	8
	Maltose	200 mg/body/day	2	1～21	
Group III	B. breve/pSP3B-TNF alpha	0.2 mL/body/time	2	1, 4, 8, 11	8
	Maltose	200 mg/body/day	2	1～21	

[0198]

(4) Culturing of bacteria and preparation of bacterial suspension for administration

Culturing of bacteria

The glycerin stocks of the bifidobacteria *B. longum* 105A/pSP3B-TNF alpha prepared in Production Example 4 and *B. breve*/pSP3B-TNF alpha prepared in Production Example 7 were inoculated at 1% to APS-2S-2.5SE (75μg/mL spectinomycin) liquid media, cultured at 37°C for 23.5 hours in anaerobic condition (activating culture solution). Next, the activating culture solution was inoculated at

1% to 20mL of APS-2S-2.5SE (75μg/mL spectinomycin) liquid medium, cultured at 37°C for 18 hours in anaerobic condition (main culture solution).

[0199]

Preparation of cultured viable cells for administration (*B. longum* 105A/pSP3B-TNF alpha)

10mL of themain culture solution obtained as above was measured by a measuring pipette and added to a conical tube containing 40mL of well-cooled PBS buffer, gently mixed by inversion, and then centrifuged in a centrifuge cooled at 4°C, at 8000 rpm for 10 minutes. After centrifugation, the supernatant was removed and 40mL of fresh PBS buffer was added and gently mixed by a vortex. This manipulation was repeated four times to wash the cells. The washed cells were suspended in 5mL PBS buffer to give a cultured viable cells for administration.

[0200]

Preparation of cultured viable cells for administration (*B. breve*/pSP3B-TNF alpha)

10mL of the main culture solution obtained as above was measured by a measuring pipette and added to a conical tube containing 40mL of well-cooled PBS buffer, gently mixed by inversion, and then centrifuged in a centrifuge cooled at 4°C, at 8000 rpm for 10 minutes. After centrifugation, the supernatant was removed and 40mL of fresh PBS buffer was added and gently mixed by a vortex. This manipulation was repeated four times to wash the cells. The washed cells were suspended in 10mL PBS buffer to give cultured viable cells for administration.

[0201]

(5) Administration of the bacterium and maltose

Administering the bacterium

For Group II and Group III, 0.2mL per mouse of each cultured viable cells (Group II :*B. longum* 105A/pSP3B-TNF alpha, Group III :*B. breve*/pSP3B-TNF alpha) was administered intravenously twice a day (AM/PM), at a pace of twice a week (Day 1, 4, 8, 11), for two weeks. The cultured viable cells were administered in the administered total volume of 1.6mL, i.e., the total cell number of 3.1×10^9 cfu/mouse for *B. longum* 105A/pSP3B-TNF alpha, and 4.8×10^9cfu/mouse for *B. breve*/pSP3B-TNF alpha. The number of administered viable cells was measured as follows.

[0202]

Measuring viable cell number

The cultured viable cells were diluted 10^6 times with an anaerobic dilutant, 100μL of which was smeared to three BLFS plates each and cultured in anaerobic condition in a sealed vessel (Anaero Pac Rectangular jar, MITSUBISHI GAS CHEMICAL, INC.) with a deoxygenating/carbon dioxide-generating agent in an incubator at 37°C for three days. For each plate in which colonies of 30 to 300 were detected, the number of the cells administered was calculated by the formula below. Number of the cells administered (cfu) = number of colonies (a) x dilution ratio at the time of being smeared to the plate (b) x conversion coefficient for 1mL of cultured viable cells (c) x dosage (mL)

(a): (P1+P2+P3)/3 [average number of colonies of 3 plates (P1,P2,P3)]

(b): x 10^6 [10^6 times dilution]

(c): x 10 [smeared 100μL per plate]

[0203]

Administering maltose

For Group II and III, 1mL of 10% maltose solution was administered intraperitoneally as carbohydrate source twice a day (200mg/body/day). Administration period was for 21 days from the day of administering the cultured viable cells (Day 1 - 21).

[0204]

(6) Confirming tumor-growth suppressing effect

For all mice, tumor diameter was measured before the initiation of the treatment (at grouping) and for 22 days after the initiation of the treatment, at frequency of once in 3 to 4 days, to confirm the effect against tumor growth.

The average tumor volume ± SD for each group of mice was calculated, and antitumor effect was assessed using relative tumor volume ratio to the control group (Group I) [T/C (%)] as an index. Also, statistical analyses (comparison between two groups: t-test) between Group I and Group II and between Group I and Group III were performed.

The tumor volume for each group (average ± SD) is shown in Table 11 below.

Chronological variation of tumor volume at the time was also shown in Fig. 15.

[0205]

Table 11: Average tumor volume of each group

Group / Given cell	Number of animals	Tumor volume (mm3) after grouping (Day0)								T/C (%)#1 at Day22	Two tailed t-test (p-value) Group I vs
		Measurement date (Day)	0	3	7	10	14	18	22		
一) No treatment	8	Average	107.2	168.6	284.4	426.5	658.3	1347.7	2128.8	—	—
		S.D.	19.6	33.0	52.1	139.0	248.3	647.2	1040.1		
二) B. lonum 105A /pSP3B-TNF alpha	8	Average	105.9	146.7	196.6	274.4	420.0	690.2	1028.8	48.3	0.021
		S.D.	18.1	23.6	33.8	64.7	127.5	225.9	348.6		
三) B. breve /pSP3B-TNF alpha	8	Average	105.5	142.4	151.7	181.9	201.6	337.3	579.4	27.2	0.004
		S.D.	18.6	48.8	56.7	64.5	61.5	146.6	292.2		

#1:T/C(%)= Average tumor volume of Group II or Group III/ Average tumor volume of Group I x 100

[0206]

In either group receiving *B. longum* 105A/pSP3B-TNF alpha or *B. breve*/pSP3B-TNF alpha, a significant decrease in tumor volume was observed compared with untreated group.

[0207]

Working Example 8: Measurement of antitumor effect of *B. longum* 105A/pSP3B-TNF alpha

We measured the antitumor effect of *B. longum* 105A/pSP3B-TNF alpha prepared in Production Example 4 in concomitant use with adriamycin.

(1) Culturing of the transplant tumor cells

Human breast cancer cell line KPL-1 cell was cultured in DMEM medium supplemented with 10% (v/v) FBS and 0.1% (v/v) penicillin (50000U/mL)/ streptomycin (50mg/mL) under the condition at 37°C in 5%CO_2.

Upon reaching confluent, the cells were detached by washing with 1 x PBS(-) and adding trypsin-EDTA, and the cells were collected by centrifugation (1000 spins/5 minutes) and appropriately diluted with DMEM medium and subcultured.

Cells after 5 passages were used for transplantation experiments. The number of viable cells which were not stained with trypan blue was counted on Thoma hemocytometer (Thoma deep 0.1 mm ERMA, Tokyo), suspended in Hank's solution and the cell number was adjusted to at 2.5 x 10^6 cells/mL.

[0208]

(2) Production of a cancer-bearing nude mouse and measurement of the tumor volume

0.2mL of the prepared KPL-1 cell suspension was subcutaneously transplanted to a nude mouse on the dorsal side of the right anterior limb (5 x 10^5 cells/mouse). Tumor volume after transplantation was assessed by measuring tumor diameter (long axis, short axis and thickness) using calipers and calculated by following equation:

Tumor volume (mm^3) = long axis (mm) x short axis (mm) x thickness (mm)/2

[0209]

(3) Grouping and group constitution

From KPL-1 cancer-bearing nude mice, 18 mice whose tumor volumes were around approximately 80 to 120 mm^3 were selected and divided into 3 groups (6 animals for each group) such that the average tumor volume would be similar. This day was set to be as Day 0.

The constitution of the test groups are as shown in Table 12. That is, Group I: untreated group, Group II: the group receiving adriamycin alone, Group III: the group receiving the combination of bacterium (*B. longum* 105A/pSP3B-TNF alpha) + adriamycin.

[0210]

Table 12: Group constitution

Group	Given substance	Dosage	Number of dosage (times/day)	Administration date (Day)	Number of animals
Group I	Bacterium	—	—	—	6
	Maltose	—	—	—	
	Adriamycin	—	—	—	
Group II	Bacterium	—	—	—	6
	Maltose	—	—	—	
	Adriamycin	5 mg/kg	1	0 *	
Group III	Bacterium	0.2 mL/body/time	2	1, 5, 8, 12	6
	Maltose	200 mg/body/day	2	1 to 20	
	Adriamycin	5 mg/kg	1	0 *	

[0211]

(4) Culturing of the bacterium (*B. longum* 105A/pSP3B-TNF alpha)

The glycerin stock of the *Bifidobacterium B. longum* 105A/pSP3B-TNF alpha prepared in Production Example 4was inoculated at 1% to APS-2S-2.5SE (75μg/mL spectinomycin) liquid medium, cultured at 37°C for 23.5 hours in anaerobic condition (activating culture solution). Next, the activating culture solution was inoculated at 1% to 20mL of APS-2S-2.5SE (75μg/mL spectinomycin) liquid medium, cultured at 37°C for 18 hours in anaerobic condition (main culture solution).

[0212]

Preparation of cultured viable cells for administration

5mL of the main culture solution above was measured by a measuring pipette and added to a conical tube containing 20mL of well-cooled PBS buffer, gently mixed by inversion, and then centrifuged in a centrifuge cooled at 4°C, at 8000 rpm for 10 minutes. After centrifugation, the supernatant was removed and 20mL of fresh PBS

buffer was added and gently mixed by a vortex. This manipulation was repeated four times to wash the cells. The washed cells were suspended in 2.5mL PBS buffer to give a cultured viable cells for administration.

[0213]

(5) Administration of the bacterium, maltose and adriamycin

Administering the bacterium

For Group III, 0.2mL per mouse of cultured viable cells (test drug) was administered intravenously twice a day (AM/PM), twice a week (Day 1, 5, 8, 12). The cultured viable cells were administered in the total administered volume of 1.6mL, i.e., the total cell number of 3.0 x 10^9cfu/mouse. The number of administered viable cells was measured as follows.

[0214]

Measuring viable cell number

The cultured viable cells were diluted 10^6 times with an anaerobic dilutant, 100μL of which was smeared to three BLFS plates each and cultured in anaerobic condition in a sealed vessel (Anaero Pac Rectangular jar, MITSUBISHI GAS CHEMICAL, INC.) with a deoxygenating/carbon dioxide-generating agent in an incubator at 37°C for three days. For each plate in which colonies of 30 to 300 were detected, the number of the cells administered was calculated by the formula below. Number of the cells administered (cfu) = number of colonies (a) x dilution ratio at the time of being smeared to the plate (b) x conversion coefficient for 1mL of cultured viable cells (c) x dosage (mL)

(a): (P1+P2+P3)/3 [average number of colonies of 3 plates (P1,P2,P3)]

(b): x 10^6 [10^6 times dilution]

(c): x 10 [smeared 100μL per plate]

[0215]

Administering maltose

For Group III, 1mL of 10% maltose solution was administered intraperitoneally as carbohydrate source twice a day (200mg/body/day). Administration period was for 20 days from the day of administering the cultured viable cells (Day 1 - 20).

Administering adriamycin

For Group II and Group III, 0.1mL adriamycin solution (1.0mg/mL) was administered intravenously to mice only on a day before the first administration of bacterium (Day0).

[0216]

(6) Confirming tumor-growth suppressing effect

For all mice, tumor diameter was measured before the initiation of the treatment (at grouping) and for 21 days after the initiation of the treatment, at frequency of once in 3 to 4 days, to confirm the effect against tumor growth.

The average tumor volume ± SD for each group of mice was calculated, and antitumor effect was assessed using relative tumor volume ratio to the control group (Group I) [T/C (%)] as an index. Also, in order to assess the antitumor ability of the present bacterium secreting TNFα, a statistical analysis (comparison between two groups: t-test) between Group II and Group III was performed.

The tumor volume for each group (average ± SD) is shown in Table 13 below.

Chronological variation of tumor volume at the time was also shown in Fig. 16.

[0217]

Table 13: Average tumor volume of each group

Group / Given cell	Number of animals	Tumor volume (mm3) after grouping (Day0)								T/C (%)#2 at Day21	Two tail t-test (p-value)
		Measurement date (Day	0	4	7	11	15	19	21		
一) No treatment	8	Average	100.3	156.5	238.5	347.4	613.5	1002.2	1337.0	–	–
		S.D.	13.8	44.4	77.5	157.2	274.9	561.6	726.0		–
二) Receiving adriamycin#1	8	Average	97.7	133.6	145.4	257.9	415.2	625.2	852.6	63.8	0.168
		S.D.	14.3	21.5	25.3	42.5	92.4	137.5	199.0		–
三) Receiving bacterium and adriamycin#1	8	Average	97.8	96.9	100.7	120.4	148.5	220.4	265.0	19.8	0.015
		S.D.	12.6	30.0	29.1	26.8	57.8	57.7	104.6		0.0003

#1: Adriamycin 5.0mg/kg
#2:T/C(%)=Average tumor volume of Group II or Group III/ Average tumor volume of Group I x 100

[0218]

In the group received a concomitant use of *B. longum* 105A/pSP3B-TNF alpha and adriamycin, tumor volume was significantly reduced, not only when compared with untreated group but also when compared with the group receiving adriamycin alone. This means, namely, the concomitant use of adriamycin and *B. longum* 105A/pSP3B-TNF alpha may increase their effects.

[0219]

Production Example 8: Production of a Non-Secretory Human IL-18-Expressing *Bifidobacterium*

Construction of Plasmid phIL18mut-His

We constructed a shuttle vector (*Bifidobacterium* -*E. coli*) having only the human IL-18 located downstream of Hu promoter derived from *Bifidobacterium* but

having no secretory signal. A summary is shown in Fig. 17. Details are as follows.

[0220]

Insert Preparation

We used a plasmid human IL18_opt_in_pUC57 having an artificial DNA of human IL-18 (Accession No: NM_001562, 329th to 799th nucleotide sequence in mature protein coding region) of which codons were optimized for *Bifidobacterium*, and Hu promoter located upstream thereof and Hu terminator located downstream thereof (custom-synthesized by GenScript). Upon synthesizing the artificial DNA, amino acid substitutions were introduced to the mature human IL-18 at 2 sites, i.e., at 7th amino acid (from E to A) and at 54th amino acid (from K to A), to decrease the neutralization with a IL-18-binding protein, and a histidine tag was added to the C-terminal (the amino acid sequence of the mature human IL-18: SEQ ID No: 47).

[0221]

Added to 2μg of the plasmid human IL18_opt_in_pUC57 25unit of BamHI and 15unit of BcuI (both enzymes from Fermentas), which was incubated at 37ºC for 3 hours to allow a complete digestion. After the digestion, the plasmid was electrophoresed on 1% agarose gel for purification (1 x TBE buffer, containing ethidium bromide) to separate DNA fragments. A small fragment of approximately 1kbp was cut out, and DNA was extracted and purified from the agarose gel by a DNA extraction kit (QIAquick Gel Extraction Kit, QIAGEN). Purified DNA fragment (insert) was electrophoresed on 0.8% agarose gel (1 x TBE buffer, containing ethidium bromide) with DNA concentration marker to estimate its concentration.

[0222]

Vector Preparation

The plasmid pCDshuttle was completely digested with BamHI, BcuI, PstI and Bsp119I (all from Fermentas; PstI and Bsp119I has their recognition sites on CD). Reacting conditions were in accordance with the instruction for use of the enzymes. After the digestion, the plasmid was electrophoresed on 1% agarose gel for purification (1 x TBE buffer, containing ethidium bromide) for separation, a large fragment of approximately 3.4kbps was cut out, and DNA was extracted and purified from the agarose gel by a DNA extraction kit (QIAquick Gel Extraction Kit, QIAGEN).

Purified DNA fragment (vector) was electrophoresed on 0.8% agarose gel (1 x TBE buffer, containing ethidium bromide) with DNA concentration marker to estimate its concentration.

[0223]

Cloning

The vector and the insert above were mixed in 1:3 (molar ratio) and ligated (Rapid DNA Ligation Kit, Fermentas). Details were in accordance with the product instruction.

2μL of the ligation reaction solution above was used for transforming *E. coli* TOP10 chemically Competent Cell (Invitorogen). Transforming conditions were in accordance with the product instruction. Transformed *E. coli* colonies were cultured overnight in LB (containing 75μg/mL spectinomycin) liquid medium at 37ºC, from which the plasmid was extracted (QIAprep Spin Miniprep Kit, QIAGEN). The plasmid was named as phIL18mut-His (SEQ ID No: 48).

[0224]

Construction of pSP3B-hIL18mut

We constructed a shuttle vector (*Bifidobacterium* -*E. coli*) having human IL18mut fused to a signal peptide downstream of Hu promoter derived from *Bifidobacterium*. A summary is shown in Fig. 18. Details are as follows.

Insert Preparation

5ng of the plasmid phIL18mut-His was used as template for PCR amplification of hIL18mut coding region by PrimeSTAR® HS Premix (TAKARA BIO, Inc.). IL18 F2 and IL18 R2 primers were used, in which the 15 nucleotides on the 5' side of each primer had a homologous sequence to the vector terminal (Table 14). Primers were designed such that the PCR product would not contain the histidine tag from C-terminal of IL-18. PCR program consisted of 30 cycles of 10 seconds at 98°C, 5 seconds at 55°C, 30 seconds at 72°C, followed by 30 seconds at 72°C.

A part of the PCR product was electrophoresed on 2% agarose gel (1 x TBE buffer, containing ethidium bromide) with DNA concentration marker, confirming a single band of approximately 0.5kbp and estimating its concentration.

[0225]

Vector Preparation

5ng of the plasmid pSP3B-TNFalpha was used as template for PCR amplification of a signal peptide SP3 and vector skeletal by PrimeSTAR® HS Premix (TAKARA BIO, Inc.). The primers vector F3 and vector R2 was used (Table 14), in which the 15 nucleotides on the 5' side of each primer had a homologous sequence to the insert terminal. PCR program consisted of 30 cycles of 10 seconds at 98°C, 5 seconds at 55°C, 4 minutes at 72°C, followed by 30 seconds at 72°C.

A part of the PCR product was electrophoresed on 0.8% agarose gel (1 x TBE buffer, containing ethidium bromide) with DNA concentration marker, confirming a single band of approximately 4kbps and estimating its concentration.

[0226]

Table 14 Primers for constructing pSP3B-hIL18mut

Primers	Sequence (5' -> 3')	PCR product
IL18 F2 primer	TACTTCGGCAAGCTGGC (SEQ ID NO: 137)	insert
IL18 R2 primer	GAGCAGAAGGTCATCAATCCTCGTTCTGGACGGTG (SEQ ID NO: 138)	insert
vector_F3 primer	GATGACCTTCTGCTCGTAGCG (SEQ ID NO: 139)	vector
vector_R2 primer	CAGCTTGCCGAAGTAGGCGATGGTCAGCTTGCC (SEQ ID NO: 140)	vector

[0227]

Cloning

100ng of the vector and 40ng of the insert above were ligated by the recombination of terminal sequences using In-Fusion Advantage PCR Cloning Kit (TAKARA BIO, Inc.). At this time, Cloning Enhancer (TAKARA BIO, Inc.) was also added into the reaction solution for concurrently degrading the template plasmid contained within the insert and the vector. Details were in accordance with the product instruction of In-Fusion Advantage PCR Cloning Kit.

[0228]

2μL of the In-Fusion reaction solution above was used for transforming *E. coli*

TOP10 chemically Competent Cell (Invitorogen). Transforming conditions were in accordance with the product instruction. Transformed *E. coli* colonies were cultured overnight in LB (containing 75μg/mL spectinomycin) liquid medium at 37°C, from which the plasmid was extracted (QIAprep Spin Miniprep Kit, QIAGEN). This plasmid was fully sequenced and named as pSP3B-hIL18mut (SEQ ID No: 49).

[0229]

Transformation of *Bifidobacterium*

The plasmid pSP3B-hIL18mut was used for transforming *B. longum* 105A and *B. breve* JCM1192 in a similar method as Production Example 1.

[0230]

Working Example 9: Human IL-18 Protein Expression by Recombinant *Bifidobacterium*

Sample Preparation

The glycerin stocks of the recombinant bifidobacteria *Bifidobacterium longum* 105A/ pSP3B-hIL18mut obtained from Production Example 9 and *Bifidobacterium longum* 105A/ pBEshuttle obtained from Reference Example 4 were inoculated at 1% to APS-2S-2.5SE (75μg/mL spectinomycin) liquid media, cultured at 37°C for 24 hours in anaerobic condition (activating culture solution). Subsequently, the activating culture solution was inoculated at 0.5% to a medium (for each 20mL of APS-2S-2.5SE (75μg/mL spectinomycin) liquid medium 4mL of 1M sodium phosphate buffer (pH6.8) was added), which was cultured at 37°C for 18 hours in anaerobic condition (main culture solution).

[0231]

Bifidobacterium breve JCM1192/ pSP3B-hIL18mut was cultured in a similar method as above, except that themain culture was cultured for 14 hours.

1.3mL of the main culture solution was measured to a tube with a capacity of 1.5mL, centrifuged (14,000rpm for 5 minutes at 4°C), and the supernatant was collected to give a sample for IL-18 measurement.

[0232]

IL-18 Measurement

The protein content of the human IL-18 in each supernatant was measured

using Human IL-18 ELISA kit (MBL). As a result, 986pg/mL of IL-18 was detected in *Bifidobacterium longum* 105A/ pSP3B-hIL18 and 1632pg/mL in *Bifidobacterium breve* JCM1192/ pSP3B-hIL18mut, although none was detected in the mock.

[Industrial Applicability]

[0233]

Introducing the secretory signal peptide of the present invention into an expression cassette enables an efficient secretion of an expressed protein from a transformed bacterium without impairing its physiological activity. Accordingly, the vector of the present invention and the anaerobic microorganism transformed with said vector are capable of more efficiently providing a therapeutic agent to a disease site in an anaerobic disease tissue compared with those of conventional use, thereby being capable of providing a high therapeutic effect.

CLAIMS

[Claim 1] A transforming plasmid for producing a transformed anaerobic bacterium, the plasmid comprising an expression cassette containing a secretory signal that functions in the anaerobic bacterium, wherein the secretory signal comprises a nucleotide sequence encoding a secretory signal peptide encoded by SEQ ID NO: 6, 7, 8, 9, 12, 14, 15, 17, 21 or 25 ~~and the plasmid being a non-shuttle plasmid~~.

[Claim 2] The transforming plasmid according to Claim 1, wherein the anaerobic bacterium is *Bifidobacterium*.

~~[Claim 3] The transforming plasmid according to Claim 1 or 2, wherein the secretory signal peptide is derived from *Bifidobacterium*.~~

~~[Claim 4] The transforming plasmid according to Claim 3, wherein the secretory signal peptide is derived from *Bifidobacterium longum*.~~

~~[Claim 5] The transforming plasmid according to any one of Claims 1 to 4, wherein the secretory signal is a DNA according to any one of the nucleotide sequences of SEQ ID No.: 6 to 28, or said sequence in which one or several nucleotide thereof are deleted, substituted or added.~~

~~[Claim 6] The transforming plasmid according to Claim 5, wherein the secretory signal is a nucleotide sequence of SEQ ID No.: 6, 7, 8, 9, 12, 14, 15, 17, 21, 25 or 28, or said sequence in which one or several nucleotide thereof are deleted, substituted or added.~~

[Claim ~~7~~ 3] The transforming plasmid according to Claim ~~6~~ 1, wherein the secretory signal ~~is a DNA according to the nucleotide sequence of either~~ comprises a nucleotide sequence encoding a secretory signal peptide encoded by SEQ ID ~~No~~ NO.: 8 or 25 ~~or a single nucleotide polymorphism thereof~~.

~~[Claim 8] The transforming plasmid according to any one of Claims 1 to 7, wherein a promoter contained in the expression cassette is a DNA according to any one of nucleotide sequences of promoter regions of SEQ ID Nos.: 29 to 44 or the nucleotide sequence of SEQ ID No.: 45, or said sequence in which one or several nucleotide thereof are deleted, substituted or added.~~

[Claim ~~9~~ 4] The transforming plasmid according to Claim ~~8~~ 1, wherein ~~the~~ a promoter contained in the expression cassette is ~~a nucleotide sequence of a promoter region of SEQ ID No.: 35 or~~ the nucleotide sequence of SEQ ID ~~No~~ NO.: 45~~, or said sequence in which one or several nucleotide thereof are deleted, substituted or added~~.

[Claim ~~10~~ 5] The transforming plasmid according to ~~any one of Claims 1 to 9~~ Claim 1, wherein a terminator contained in the expression cassette is a DNA according to the nucleotide sequence of SEQ ID ~~No~~ NO.: 46~~, or said sequence in which one or several nucleotide thereof are deleted, substituted or added~~.

~~[Claim 11] The transforming plasmid according to any one of Claims 1 to 10, wherein a target gene contained in the expression cassette is a gene encoding a fluorescent protein.~~

[Claim ~~12~~ 6] The transforming plasmid according to ~~any one of Claims 1 to 10~~ Claim 1, wherein a target gene contained in the expression cassette is a gene encoding a protein having an antitumor activity.

[Claim ~~13~~ 7] The transforming plasmid according to Claim ~~12~~ 6, wherein the protein having an antitumor activity is one selected from the group consisting of ~~cytokines such as interferon (IFN)-α, IFN-β, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF),~~ interleukin (IL)~~-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IL~~-18, ~~and~~ tumor necrosis factor (TNF)-α~~, lymphotoxin (LT)-β, TNF-related apoptosis inducing ligand (TRAIL), granulocyte colony-stimulating factor (G-CSF),~~

~~macrophage colony-stimulating factor (M-CSF), macrophage migration-inhibitory factor (MIF), leukemia-inhibitory factor (LIF), T cell activator co-stimulators B7 (CD80) and B7-2 (CD86), Kit ligand and oncostatin M, and anti-angiogenic agents such as endostatin, angiostatin, kringle-1, kringle-2, kringle-3, kringle-4 and kringle-5~~.

[Claim ~~14~~ 8] The transforming plasmid according to Claim ~~13~~ 7, wherein the protein having an antitumor activity is ~~either~~ tumor necrosis factor (TNF)-α ~~or TNF-related apoptosis inducing ligand (TRAIL)~~.

~~[Claim 15] The transforming plasmid according to any one of Claims 1 to 10, wherein the target gene is a gene encoding a protein having an activity to convert a precursor of an antitumor substance to the antitumor substance.~~

~~[Claim 16] The transforming plasmid according to Claim 15, wherein the protein having an activity to convert a precursor of an antitumor substance to the antitumor substance is one selected from the group consisting of cytosine deaminase, nitroreductase and β-glucuronidase.~~

~~[Claim 17] The transforming plasmid according to any one of Claims 1 to 10, wherein the target gene is a gene encoding a protein having a therapeutic activity for an ischemic disease.~~

~~[Claim 18] The transforming plasmid according to Claim 17, wherein the protein having a therapeutic activity for an ischemic disease is one selected from the group consisting of proteins having a proangiogenic activity such as fibroblast growth factor 2 (FGF2), endothelial cell growth factor (ECGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF).~~

~~[Claim 19] The transforming plasmid according to any one of Claims 1 to 10, wherein the target gene is a nucleic acid having a therapeutic activity for an anaerobic disease.~~

~~[Claim 20] The transforming plasmid according to Claim 19, wherein the nucleic acid having a therapeutic activity for an anaerobic disease is an siRNA associated with at least one tumor cell growth factor selected from the group consisting of fibroblast growth factor 2(FGF2), endothelial cell growth factor (ECGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF).~~

[Claim ~~21~~ 9] The transforming plasmid according to ~~any one of Claims 1 to 20~~ Claim 1, comprising a DNA sequence according to the nucleotide sequence of SEQ ID ~~No~~ NO.: ~~5, or said sequence in which one or several nucleotide thereof are deleted, substituted or added~~ (pBifi-SP3B-TNF alpha).

[Claim ~~22~~ 10] A gene transfer carrier consisting of an anaerobic bacterium transformed with the transforming plasmid according to ~~any one of Claims 1 to 21~~ Claim 1.

[Claim ~~23~~ 11] The gene transfer carrier according to Claim ~~22~~ 10, wherein the anaerobic bacterium is an avirulent enterobacterium.

[Claim ~~24~~ 12] The gene transfer carrier according to Claim ~~22 or 23~~ 10, wherein the anaerobic bacterium is *Bifidobacterium*.

[Claim ~~25~~ 13] The gene transfer carrier according to Claim ~~24~~ 12, wherein the *Bifidobacterium* is a species selected from the group consisting of *Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *Bifidobacterium animalis*, *Bifidobacterium asteroides*, *Bifidobacterium bifidum*, *Bifidobacterium boum*, *Bifidobacterium breve*, *Bifidobacterium catenulatum*, *Bifidobacterium choerinum*, *Bifidobacterium coryneforme*, *Bifidobacterium cuniculi*, *Bifidobacterium denticolens*, *Bifidobacterium dentium*, *Bifidobacterium gallicum*, *Bifidobacterium gallinarum*, *Bifidobacteria globosum*, *Bifidobacteria indicum*, *Bifidobacterium infantis*, *Bifidobacteria inopinatum*, *Bifidobacterium lactis*, *Bifidobacterium lactentis*, *Bifidobacterium liberorum*,

Bifidobacterium longum, *Bifidobacterium magnum*, *Bifidobacterium merycicum*, *Bifidobacterium minimum*, *Bifidobacterium mongoliense*, *Bifidobacterium parvulorum*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium pseudolongum*, *Bifidobacterium psychroaerophilum*, *Bifidobacterium pullorum*, *Bifidobacterium ruminale*, *Bifidobacterium ruminantium*, *Bifidobacterium saeculare*, *Bifidobacterium scardovii*, *Bifidobacterium subtile*, *Bifidobacterium suis*, *Bifidobacterium thermacidophilum* and *Bifidobacterium thermophilum*.

[Claim ~~26~~ 14] The gene transfer carrier according to Claim ~~25~~ 13, wherein the *Bifidobacterium* is *Bifidobacterium longum*.

[Claim ~~27~~ 15] The gene transfer carrier according to ~~any one of Claims 22 to 26~~ Claim 10, being capable of growing in a tumor tissue in an anaerobic environment and being capable of expressing and secreting at least one protein ~~or nucleic acid~~ that is useful for ~~diagnosis or~~ treatment of an anaerobic disease.

~~[Claim 28] The gene transfer carrier according to Claim 27, wherein the protein that is useful for diagnosis of an anaerobic disease is a fluorescent protein.~~

~~[Claim 29] The gene transfer carrier according to Claim 27, wherein the protein that is useful for treatment of an anaerobic disease is a protein having an antitumor activity.~~

[Claim ~~30~~ 16] The gene transfer carrier according to Claim ~~28~~ 15, wherein the protein ~~having~~ has an antitumor activity and is ~~one~~ selected from the group consisting of ~~cytokines such as interferon (IFN)-α, IFN-ß, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF),~~ interleukin (IL)~~-1α, IL-1ß, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IL~~-18, and tumor necrosis factor (TNF)-α, ~~lymphotoxin (LT)-ß, TNF-related apoptosis inducing ligand (TRAIL), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), macrophage migration-inhibitory factor (MIF), leukemia-inhibitory factor (LIF), T cell

~~activator co-stimulators B7 (CD80) and B7-2 (CD86), Kit ligand and oncostatin M, and anti-angiogenic agents such as endostatin, angiostatin, kringle-1, kringle-2, kringle-3, kringle-4 and kringle-5~~.

~~[Claim 31] The gene transfer carrier according to Claim 27, wherein the protein that is useful for treatment of an anaerobic disease is a protein having an activity to convert a precursor of an antitumor substance to the antitumor substance.~~

~~[Claim 32] The gene transfer carrier according to Claim 31, wherein the protein having an activity to convert a precursor of an antitumor substance to the antitumor substance is selected from the group consisting of cytosine deaminase, nitroreductase and ß-glueronidase.~~

~~[Claim 33] The gene transfer carrier according to Claim 27, wherein the nucleic acid that is useful for treatment of an anaerobic disease is an siRNA associated with an anaerobic disease factor.~~

~~[Claim 34] The gene transfer carrier according to Claim 33, wherein the siRNA associated with an anaerobic disease factor is an siRNA associated with at least one tumor cell growth factor selected from the group consisting of fibroblast growth factor 2(FGF2), endothelial cell growth factor (ECGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF).~~

[Claim ~~35~~ 17] A pharmaceutical composition comprising the gene transfer carrier according to ~~any one of Claims 22 to 34~~ Claim 10.

[Claim 18] The gene transfer carrier according to Claim 16, wherein the protein is tumor necrosis factor (TNF)-α.

[Claim 19] A pharmaceutical composition comprising the gene transfer carrier according to Claim 12.

[Claim 20] A pharmaceutical composition comprising the gene transfer carrier according to Claim 13.

[Claim 21] A pharmaceutical composition comprising the gene transfer carrier according to Claim 14.

[Claim 22] The transforming plasmid according to Claim 7, wherein the protein having an antitumor activity is interleukin (IL)-18.

[Claim 23] The gene transfer carrier according to Claim 16, wherein the protein is interleukin (IL)-18.