DESCRIPTION

TITLE OF THE INVENTION

HETEROGENEOUS POLYPEPTIDE EXPRESSION CASSETTE

Technical Field

[0001]

The present invention relates to an expression cassette comprising a promoter DNA, a DNA encoding a secretory signal peptide, a DNA encoding a linker peptide, a DNA encoding a heterogeneous polypeptide and a terminator DNA sequentially in this order and expressed in a bacterium of the genus *Bifidobacterium*. The present invention also relates to a vector comprising the expression cassette, a bacterium of the genus *Bifidobacterium* transformed with the vector and a pharmaceutical composition comprising the bacterium.

Background Art

[0002]

Recently, for treating diseases such as cancer, therapies using a gene transport carrier have been increasingly developed. For example, an anaerobic enteric bacterium, if it is systemically administered, is accumulated in a hypoxic solid tumor. Using this property, a transformed microorganism which can express a gene encoding a protein having an anti-tumor activity or a protein having an activity to convert a precursor of an anti-tumor agent into the anti-tumor agent at a target

site of disease (see, for example, Patent Document 1) has been proposed.

[0003]

Also, an expression cassette, which comprises a promoter, a DNA encoding a signal sequence, a DNA encoding a polypeptide or a cloning site for inserting the DNA (see, for example, Patent Document 2); and a recombinant obligatory anaerobic gram-positive bacterium, which comprises a nucleic acid encoding a fusion protein comprising a signal peptide, a single-chain antibody and one or more heterogeneous polypeptides and which can express the nucleic acid therein (see, for example, Patent Document 3) have been proposed.

[0004]

However, signal peptides proposed in the above documents are found in membrane proteins and secretory proteins. In the context, a signal peptide having better secretion efficiency is desirably identified.

Prior Art Documents

Patent Documents

[0005]

Patent Document 1: International Publication No. WO2006/109619

Patent Document 2: International Publication No. WO2010/126073

Patent Document 3: International Publication No. WO2014/010758

Summary of Invention

Object to be Solved by the invention

[0006]

An object of the present invention is to provide, in producing a heterogeneous polypeptide by a bacterium of the genus *Bifidobacterium*, a signal peptide which enables to secrete the heterogeneous polypeptide with high efficiency outside the bacterial cell; a heterogeneous polypeptide expression cassette, which enables to secrete the heterogeneous polypeptide outside the bacterial cell with high efficiency by using such a signal peptide; a heterogeneous polypeptide expression vector having the expression cassette integrated therein; and a bacterium of the genus *Bifidobacterium*, which is transformed with the heterogeneous polypeptide expression vector and can secrete the heterogeneous polypeptide.

Means to Solve the object

[0007]

The present inventors have already developed an anti-cancer therapy using a bacterium of the genus *Bifidobacterium*, which is transformed with a vector prepared by integrating a gene for cytosine deaminase, i.e., an enzyme for converting 5-fluoro-cytosine into 5-fluorouracil having an antitumor activity, and which specifically accumulates and grows in solid cancer. This time, the present inventors found that various diseases can be treated by locally expressing and secreting an antibody peptide specifically recognizing a target cell and a heterogeneous polypeptide in a bacterium of the

genus *Bifidobacterium*. Based on the finding, they tried to identify a secretory signal peptide which enables expression and secretion of a heterogeneous polypeptide more efficiently than a secretory signal peptide known in the art and which is applicable to a bacterium of the genus *Bifidobacterium*. As a result, they found a plurality of secretory signal peptides including a secretory signal peptide designated as SP7. In addition, they found that *Bifidobacterium longum* transformed with a vector, in which an expression cassette comprising a promoter DNA functioning in a bacterium of the genus *Bifidobacterium*; a DNA encoding the secretory signal peptide as mentioned above; a DNA encoding a linker peptide; a DNA encoding a single-chain antibody(scFv antibody) having an antitumor activity; and a terminator DNA functioning in the bacterium of the genus *Bifidobacterium*, sequentially in this order, is integrated, secretes the scFv antibody outside the bacterial cell with high efficiency. They further found that the scFv antibody secreted outside the bacterial cell varies in the secretion amount outside the bacterial cell, binding ability thereof to an antigen and competitive binding inhibitory activity, depending upon the type of secretory signal peptide sequence as mentioned above and the number of amino acids of a linker peptide sequence located downstream thereof. Based on these findings, the present invention was accomplished.

[0008]

The present invention is more specifically as follows.

[1] An expression cassette expressed in a bacterium of the genus *Bifidobacterium* and sequentially comprising the following DNAs (1) to (4).

(1) a promoter DNA functioning in the bacterium of the genus *Bifidobacterium*;

(2) a DNA encoding a secretory signal peptide consisting of an amino acid sequence represented by the following a) or b);

a) an amino acid sequence represented by any one of SEQ ID Nos: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16; and

b) an amino acid sequence obtained by deletion, replacement or addition of one or several amino acids in an amino acid sequence represented by any one of SEQ ID Nos: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16, wherein a peptide consisting of the amino acid sequence functions as a secretory signal peptide in the bacterium of the genus *Bifidobacterium*;

(3) a DNA encoding a heterogeneous polypeptide; and

(4) a terminator DNA functioning in the bacterium of the genus *Bifidobacterium*.

[2] The expression cassette according to [1], wherein a DNA encoding a linker peptide is linked downstream of a DNA encoding a secretory signal peptide.

[3] The expression cassette according to [2], wherein the linker peptide consists of each amino acid sequence obtained by deletion of 0 to 29 amino acid residues from the C terminal of the amino acid sequence represented by any one of SEQ ID Nos: 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 and 35.

[4] The expression cassette according to [2], wherein the linker peptide consists of each amino acid sequence obtained by deletion of 10 to 29 amino acid residues from the C terminal of the amino acid sequence represented by any one of SEQ ID Nos: 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 and 35.

[5] The expression cassette according to any one of [1] to [4], wherein the heterogeneous polypeptide is a single-chain antibody.

[6] The expression cassette according to [5], wherein the single-chain antibody is an anti-PD-1 antibody.

[7] The expression cassette according to [5], wherein the single-chain antibody is an anti-CTLA-4 antibody.

[8] A vector comprising the expression cassette according to any one of [1] to [7].

[9] A bacterium of the genus *Bifidobacterium* transformed with the vector according to [8].

[10] The bacterium of the genus *Bifidobacterium* according to [9], which is *Bifidobacterium longum*.

[11] A pharmaceutical composition comprising the bacterium of the genus *Bifidobacterium* according to [9] or [10].

[12] A secretory signal peptide consisting of the amino acid sequence represented by any one of SEQ ID Nos: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16.

[13] A DNA encoding the secretory signal peptide according to [12].

[14] A secretory signal peptide-linker conjugate consisting of each amino acid sequence obtained by

deletion of 0 to 29 amino acid residues from the C terminal of the amino acid sequence represented by any one of SEQ ID Nos: 105, 106, 107, 108, 109, 110, 111, 112, 113, 114 and 115.

[15] The secretory signal peptide-linker conjugate according to [14], consisting of each amino acid sequence obtained by deletion of 10 to 29 amino acid residues from the C terminal of the amino acid sequence represented by any one of SEQ ID Nos: 105, 106, 107, 108, 109, 110, 111, 112, 113, 114 and 115.

[16] A DNA encoding the secretory signal peptide-linker conjugate according to [14] or [15].

As the other aspects except those mentioned above, a therapeutic method comprising administering an effective amount of bacterium of the genus *Bifidobacterium* according to [9] or [10]; use of the bacterium of the genus *Bifidobacterium* according to [9] or [10] for preparing a therapeutic agent; and the bacterium of the genus *Bifidobacterium* according to [9] or [10] for use in treating a disease can be mentioned.

Effect of Invention

[0009]

According to the present invention, it is possible to provide a bacterium of the genus *Bifidobacterium* capable of secreting a heterogeneous polypeptide outside the bacterial cell with high efficiency by using an expression cassette excellent in secretion of the heterogeneous polypeptide. If the heterogeneous polypeptide is an antibody having an anti-cancer effect,

such as PD-1 and CTLA-4, the bacterium of the genus *Bifidobacterium* is extremely useful as an anti-cancer agent.

Brief Description of Drawings

[0010]

[Figure 1] Figure 1 is a schematic view of expression cassettes of the present invention; (a) shows an expression cassette using an anti-PD-1 antibody as a scFv antibody; and (b) shows an expression cassette using an anti-CTLA-4 antibody as a scFv antibody.

[Figure 2] Figure 2 schematically shows how to prepare an anti-PD-1 or anti-CTLA-4 scFv antibody secretory plasmid: pHuSP3L22-scFv-PD-1-1, pHuSP7L20-scFv-PD-1-1, pHuSP23L27-scFv-PD-1-1, pHuSP7L20-scFv-PD-1-2, pHuSP7L20-scFv-PD-1-3, pHuSP7L20-scFv-CTLA-4-1 or pHuSP7L20-scFv-CTLA-4-2.

[Figure 3] Figure 3 schematically shows how to prepare an anti-PD-1-1 scFv antibody secretory plasmid using various signal peptide-linker conjugates.

[Figure 4] Figure 4 shows the results of Western analysis of an anti-PD-1-1 scFv antibody secreted in the culture supernatant of a bacterium of the genus *Bifidobacterium* transformed with a vector obtained by inserting an expression cassette comprising various signal peptide-linker peptide conjugates and an anti-PD-1-1 scFv antibody.

[Figure 5] Figure 5 shows the results of Western analysis of an anti-PD-1-1 scFv antibody secreted in the culture supernatant of a bacterium of the genus

Bifidobacterium transformed with a vector obtained by inserting an expression cassette comprising various signal sequences and an anti-PD-1-1 scFv antibody.

[Figure 6] Figure 6 shows the results of Western analysis of antibodies each secreted in the culture supernatant of a bacterium of the genus *Bifidobacterium*.

[Figure 7] Figure 7 is a graph showing the results of the presence or absence of binding of an anti-PD-1-2 scFv antibody, an anti-CTLA-4-1 scFv antibody and an anti-CTLA-4-2 scFv antibody secreted from a bacterium of the genus *Bifidobacterium* to human PD-1 immobilized on a plate.

[Figure 8] Figure 8 is a graph showing the results of the presence or absence of binding of an anti-PD-1-2 scFv antibody, an anti-CTLA-4-1 scFv antibody and an anti-CTLA-4-2 scFv antibody secreted from a bacterium of the genus *Bifidobacterium* to human CTLA-4 immobilized on a plate.

[Figure 9] Figure 9 is a graph showing the results of the presence or absence of binding of an anti-PD-1-2 scFv antibody, an anti-PD-1-3 scFv antibody and an anti-CTLA-4-2 scFv antibody secreted from a bacterium of the genus *Bifidobacterium* to mouse PD-1 immobilized on a plate.

[Figure 10] Figure 10 is a graph showing the results of the presence or absence of binding of an anti-PD-1-2 scFv antibody, an anti-PD-1-3 scFv antibody and an anti-CTLA-4-2 scFv antibody secreted from a bacterium of the genus *Bifidobacterium* to human CTLA-4 immobilized on a plate.

[Figure 11] Figure 11 is a graph obtained by flow cytometry and showing the binding of an anti-PD-1-2 scFv

antibody secreted from a bacterium of the genus *Bifidobacterium* to a Jurkat cell expressing human PD-1.

[Figure 12] Figure 12 is a graph obtained by flow cytometry and showing the binding of an anti-PD-1-2 scFv antibody secreted from a bacterium of the genus *Bifidobacterium* to a HEK293T cell expressing human PD-1.

[Figure 13] Figure 13 is a graph showing an inhibition rate of human CD80 against the binding of an anti-CTLA-4-1 scFv antibody and an anti-CTLA-4-2 scFv antibody to human CTLA-4.

[Figure 14] Figure 14 is a graph showing an inhibition rate of human CD86 against the binding of an anti-CTLA-4-1 scFv antibody and an anti-CTLA-4-2 scFv antibody to human CTLA-4.

[Figure 15] Figure 15 is a graph showing the competitive inhibition activity of an anti-mouse PD-1 scFv antibody against the binding reaction between mouse PD-1 and mouse PD-L1, where the vertical axis shows inhibitory activity and the horizontal axis shows antibody concentration.

[Figure 16] Figure 16 shows graphs obtained by flow cytometry. Of the graphs showing expression induction of mPD-1 into a CD4-positive T cell by stimulation with CD3/CD28, graph A shows the case where an anti-mouse PD-1 antibody was reacted with a non-stimulated CD4-positive cell and detected by FITC, and graph B shows the case where an anti-mouse PD-1 antibody was reacted with a CD3/CD28-stimulated CD-positive cell and detected by FITC. Of the graphs showing binding of the anti-mouse PD-1 scFv antibody to CD3 and CD28-stimulated CD4 positive cell

(obtained by flow cytometry), graph C shows the case where an anti-mouse PD-1 scFv antibody was reacted with a non-stimulated CD4-positive cell and then an Alexa 488-anti-His-tag antibody was reacted with His thereof and detection was made; and graph D shows the case where an anti-mouse PD-1 scFv antibody was reacted with a CD3/CD28-stimulated CD-positive cell and then an Alexa 488-anti-His-tag antibody was reacted with His thereof and detection was made.

[Figure 17] Figure 17(A) shows immunohistochemical staining of a tumor tissue of an anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium* administered mouse; and Figure 17(B) shows immunohistochemical staining of a tumor tissue of a not administered mouse.

[Figure 18] Figure 18 is a graph showing a change of CT26 tumor volume in a single agent administration group and a non-administration group.

[Figure 19] Figure 19 is a graph showing a change of CT26 tumor volume in a single agent administration group, a combined administration group of two agents and non-administration group.

[Figure 20] Figure 20 is a graph showing a change of CT26 tumor volume in a single agent administration group and a combined administration group of two agents.

[Figure 21] Figure 21 shows the presence or absence of antibody secretion in each of *Bifidobacterium longum* 105-A/pHuSP69Ly-hPD-1scFv03 strains.

[Figure 22] Figure 22 is a graph showing the binding amount of *Bifidobacterium longum* 105-A/pHuSP69L5-hPD-

1scFv03 strain and *Bifidobacterium longum* 105-A/pHuSP69L20-hPD-1scFv03 strain by ELISA.

[Figure 23] Figure 23 shows the binding amount of PD-L1 not competitively inhibited by each of scFv secreted from *Bifidobacterium longum* 105-A/pHuSP69L5-hPD-1scFv03 strain and *Bifidobacterium longum* 105-A/pHuSP69L20-hPD-1scFv03 strain, by ELISA.

[Figure 24] Figures 24(a) and 24(b) show the presence or absence of antibody secretion from each of *Bifidobacterium longum* 105-A/pHuSPxL20-hPD-1scFv03 strains.

[Figure 25] Figures 25(a) to 25(d) show the presence or absence of antibody secretion from each of *Bifidobacterium longum* 105-A/pHuSPxLy-hPD-1scFv03 strains (note that, y = 5, 10, 15, 20).

[Figure 26] Figures 26(a) to 26(c) show the presence or absence of antibody secretion from each of *Bifidobacterium longum* 105-A/pHuSPxLy-hPD-1scFv03 strains.

[Figure 27] Figure 27 shows the presence or absence of antibody secretion from each of *Bifidobacterium longum* 105-A/pHuSPxLy-hPD-1scFv03 strains.

[Figure 28] Figure 28 shows the presence or absence of antibody secretion from each of *Bifidobacterium longum* 105-A/pP30SPxL20-hCTLA-4scFv02 strains.

[Figure 29] Figures 29(a) to 29(c) shows the presence or absence of antibody secretion from each of *Bifidobacterium longum* 105-A/pP30SPxLy-hCTLA-4scFv02 strains.

Mode of Carrying Out the Invention

[0011]

The expression cassette of the present invention is not particularly limited as long as it is an expression cassette sequentially comprising (1) a promoter DNA functioning in a bacterium of the genus *Bifidobacterium*, (2) a DNA encoding a secretory signal peptide, (3) a DNA encoding a linker peptide, (4) a DNA encoding a heterogeneous polypeptide, and (5) a terminator DNA functioning in a bacterium of the genus *Bifidobacterium*, in which the secretory signal peptide consists of a) an amino acid sequence represented by any one of SEQ ID Nos: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16, or b) an amino acid sequence obtained by deletion, replacement or addition of one or several of amino acids in the amino acid sequence represented by any one of SEQ ID Nos: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16; and constituting a peptide functioning as a secretory signal peptide in the bacterium of the genus *Bifidobacterium*; and used for expressing the heterogeneous polypeptide in a bacterium of the genus *Bifidobacterium*. In the DNA fragments as mentioned above, the 3' end of one DNA fragment and the 5' end of another DNA fragment located downstream of the former DNA fragment may not be directly linked as long as the effect of the invention is obtained; however it is preferable that the ends are directly linked.

[0012]

The promoter DNA is not particularly limited as long as it is a promoter DNA functioning in a bacterium of the genus *Bifidobacterium*. Examples thereof include Hu promoter DNA, which is a promoter involved in expression of a gene encoding histone-like DNA binding protein

derived from *Bifidobacterium longum*; a promoter DNA for Gap gene derived from *Bifidobacterium breve* (Biotechnol. Lett. 2008 30: 1983-1988); a promoter DNA for AmyB gene derived from *Bifidobacterium longum* (Biotechnol. Lett. 2006 28: 163-168); 16S rRNA Promoter DNA (Biotechnol. Lett. 2008 30: 165-172), a promoter DNA for GAPDH (pr-BL1363) gene (Appl Environ Microbiol. 2006 72(11): 7401-7405); P_RP_L promoter DNA (Cancer Gene Ther. 2007 14: 151-157); a promoter DNA for p572 (β-glycosidase from *B. animalis* subsp lactis) gene (J Microbiol Biotechnol. 2012 Dec; 22(12): 1714-23), p919 (rplM promoter) DNA (J Microbiol. 2012 Aug; 50(4): 638-43); and p895 (rplR promoter) DNA (JMicrobiol. 2012 Aug; 50(4): 638-43).

[0013]

Examples of the secretory signal peptide include a) a peptide consisting of an amino acid sequence represented by any one of SEQ ID Nos. 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16; namely, SP7, SP45, SP50, SP52, SP55, SP58, SP64, SP66, SP67, SP68, SP69 respectively,

and b) a peptide consisting of an amino acid sequence obtained by deletion, replacement or addition of one or several of amino acids in the amino acid sequence represented by any one of SEQ ID Nos. 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16 and functioning as a secretory signal peptide in a bacterium of the genus *Bifidobacterium* (mutated secretory signal peptide). The "an amino acid sequence obtained by deletion, replacement or addition of one or several of amino acids" refers to an amino acid sequence obtained by deletion, replacement or addition of e.g., 1 to 5, preferably 1 to 3, more preferably 1 to 2

and further preferably a single amino acid. The amino acid sequence of the mutated secretory signal peptide has a sequence identity of preferably 90% or more with any of the amino acid sequence represented by SEQ ID Nos: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16, more preferably 95% or more and further preferably, 98% or more.

[0014]

DNA encoding a secretory signal peptide consisting of the amino acid sequence described in a) above is not particularly limited as long as it is DNA having a nucleotide sequence corresponding to the amino acid sequence described in a). Accordingly, examples of DNA include, although different DNAs due to codon degeneracy are included, DNA consisting of a nucleotide sequence represented by any one of SEQ ID Nos: 116, 117, 118, 119, 120, 121, 122, 123, 124, 125 and 126. These DNAs can be prepared by a method known to those skilled in the art, such as chemical synthesis and genetic engineering procedure.

[0015]

DNA encoding a secretory signal peptide consisting of the amino acid sequence described in b) above is not particularly limited as long as it is DNA having a nucleotide sequence corresponding to the amino acid sequence described in b). Accordingly, different DNAs due to codon degeneracy are included. These DNAs molecules can be also prepared by a method known to those skilled in the art such as chemical synthesis, genetic engineering procedure and mutation induction. For example, mutated DNAs can be obtained by introducing a mutation into these

DNAs, by a method of bringing a mutagenic drug into contact with DNA consisting of a nucleotide sequence represented by any of SEQ ID Nos: 116, 117, 118, 119, 120, 121, 122, 123, 124, 125 and 126; a UV irradiation method; and/or genetic engineering techniques. One of the genetic engineering procedures, i.e., site-specific mutagenesis, is useful since a specific mutation can be introduced into a specific site, and carried out in accordance with the method described in e.g., Molecular Cloning: A laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY., 1989., Current Protocols in Molecular Biology, Supplement 1 to 38, John Wiley & Sons (1987-1997).
[0016]

The secretory signal peptide is preferably used in conjunction with a linker peptide in the form of a signal peptide-linker conjugate, in view of expression/secretion efficiency of a heterogeneous polypeptide. The linker peptide to be used herein is not particularly limited as long as it is a peptide that can be conjugated to the C terminal of a secretory signal peptide as mentioned above and successfully enhance expression/secretion efficiency of a heterogeneous polypeptide. As an example, a peptide consisting of an amino acid sequence having 1 to 30 amino acid residues can be mentioned. More specifically, in each of the following 11 types of amino acid sequences positioned downstream of the individual secretory signal peptides and identified in *Bifidobacterium longum* 105-A strain, a linker peptide consisting of amino acid sequence having any number of amino acids within the range of the 1st to 30th amino acid, such as a linker peptide

consisting of the 1st to 5th, 1st to 10th, 1st to 15th or 1st to 20th, is mentioned.

[0017]

1) Linker peptide sequence represented by SEQ ID No: 25 (downstream of SP7);

2) Linker peptide sequence represented by SEQ ID No: 26 (downstream of SP45);

3) Linker peptide sequence represented by SEQ ID No: 27 (downstream of SP50);

4) Linker peptide sequence represented by SEQ ID No: 28 (downstream of SP52);

5) Linker peptide sequence represented by SEQ ID No: 29 (downstream of SP55);

6) Linker peptide sequence represented by SEQ ID No: 30 (downstream of SP58);

7) Linker peptide sequence represented by SEQ ID No: 31 (downstream of SP64);

8) Linker peptide sequence represented by SEQ ID No: 32 (downstream of SP66);

9) Linker peptide sequence represented by SEQ ID No: 33 (downstream of SP67);

10) Linker peptide sequence represented by SEQ ID No: 34 (downstream of SP68);

11) Linker peptide sequence represented by SEQ ID No: 35 (downstream of SP69).

[0018]

DNA encoding the secretory signal peptide-linker conjugate of the present invention refers to a DNA region from the 5' end of the DNA (2) encoding a secretory signal peptide to the 3' end of the DNA (3) encoding a linker

peptide. Since the 3' end of DNA (2) encoding a secretory signal peptide is connected to the 5' end of DNA (3) encoding a linker peptide, the secretory signal peptide-linker conjugate is hereinafter sometimes referred to as SPxLy (where x represents the number of a secretory signal peptide designated in the specification; and y represents the number of amino acid residues of a linker peptide). As the secretory signal peptide-linker conjugate, the following 11 types can be specifically mentioned.

[0019]

1) Secretory signal peptide-linker conjugate (SP7L30) represented by SEQ ID No: 105;

2) Secretory signal peptide-linker conjugate (SP45L30) represented by SEQ ID No: 106;

3) Secretory signal peptide-linker conjugate (SP50L30) represented by SEQ ID No: 107;

4) Secretory signal peptide-linker conjugate (SP52L30) represented by SEQ ID No: 108;

5) Secretory signal peptide-linker conjugate (SP55L30) represented by SEQ ID No: 109;

6) Secretory signal peptide-linker conjugate (SP58L30) represented by SEQ ID No: 110;

7) Secretory signal peptide-linker conjugate (SP64L30) represented by SEQ ID No: 111;

8) Secretory signal peptide-linker conjugate (SP66L30) represented by SEQ ID No: 112;

9) Secretory signal peptide-linker conjugate (SP67L30) represented by SEQ ID No: 113;

10) Secretory signal peptide-linker conjugate (SP68L30) represented by SEQ ID No: 114;

11) Secretory signal peptide-linker conjugate (SP69L30) represented by SEQ ID No: 115.

[0020]

For example, the 5' end of DNA encoding a linker peptide, which is obtained by deletion of amino acid residues of 0 to 29 (e.g., 10 to 29, 15 to 29, 20 to 29, or 25 to 29) from the C terminal of a linker peptide consisting of the amino acid sequence represented by SEQ ID No: 25, is connected to the 3' end of DNA encoding a secretory signal peptide consisting of the amino acid sequence represented by SEQ ID No: 6. Similarly, the 5' end of DNA encoding a linker peptide, which is obtained by deletion of amino acid residues of 0 to 29 (e.g., 10 to 29, 15 to 29, 20 to 29, or 25 to 29) from the C terminal of a linker peptide consisting of the amino acid sequence represented by any one of SEQ ID Nos: 26 to 35 is correspondingly connected to the 3' end of DNA encoding a secretory signal peptide consisting of the amino acid sequence represented by any of SEQ ID Nos: 7 to 16.

[0021]

Such DNA encoding the secretory signal peptide-linker conjugate can be prepared by a method known to those skilled in the art such as chemical synthesis and genetic engineering procedure based on the nucleotide sequence information represented by any of, for example, SEQ ID No: 116 + SEQ ID No: 127 (SP7L30), SEQ ID No: 117 + SEQ ID No: 128 (SP45L30), SEQ ID No: 118 + SEQ ID No: 129 (SP50L30), SEQ ID No: 119 + SEQ ID No: 130 (SP52L30), SEQ ID No: 120 + SEQ ID No: 131 (SP55L30), SEQ ID No: 121 + SEQ ID No: 132 (SP58L30), SEQ ID No: 122 + SEQ ID No: 133

(SP64L30), SEQ ID No: 123 + SEQ ID No: 134 (SP66L30), SEQ ID No: 124 + SEQ ID No: 135 (SP67L30), SEQ ID No: 125 + SEQ ID No: 136 (SP68L30) and SEQ ID No: 126 + SEQ ID No: 137 (SP69L30).

[0022]

In the expression cassette of the present invention, more specifically, in a linker peptide consisting of each amino acid sequence obtained by deletion of 0 to 29 amino acid residues from the C terminal of the amino acid sequence represented by any of SEQ ID Nos: 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 and 35, reference symbol "y" of "Ly" mentioned above can be represented by any of 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 and 1.

[0023]

Examples of the secretory signal peptide-linker conjugate (SPxLy) of the present invention include SP7Ly (y = any of integers of 1 to 30 (hereinafter, the same is applied), for example, y = 1 to 20, y = 1 to 15, y = 1 to 10, y = 1 to 5); SP45Ly (y = 1 to 30, for example, y = 1 to 20, y = 1 to 15, y = 1 to 10, y = 1 to 5); SP50Ly (y = 1 to 30, for example, y = 1 to 20, y = 1 to 15, y = 1 to 10, y = 1 to 5); SP52Ly (y = 1 to 30, for example, y = 1 to 20, y = 1 to 15, y = 1 to 10, y = 1 to 5); SP55Ly (y = 1 to 30, for example, y = 1 to 20, y = 1 to 15, y = 1 to 10, y = 1 to 5); SP58Ly (y = 1 to 30, for example, y = 1 to 20, y = 1 to 15, y = 1 to 10, y = 1 to 5); SP64Ly (y = 1 to 30, for example, y = 1 to 20, y = 1 to 15, y = 1 to 10, y = 1 to 5); SP66Ly (y = 1 to 30, for example, y = 1 to 20, y = 1 to 15, y = 1 to 10, y = 1 to 5); SP67Ly (y =

1 to 30, for example, y = 1 to 20, y = 1 to 15, y = 1 to 10, y = 1 to 5); SP68Ly (y = 1 to 30, for example, y = 1 to 20, y = 1 to 15, y = 1 to 10, y = 1 to 5); and SP69Ly (y = 1 to 30, for example, y = 1 to 20, y = 1 to 15, y = 1 to 10, y = 1 to 5).

[0024]

In *Bifidobacterium longum* 105-A strain, which is transformed with a vector containing an expression cassette having anti-hPD-1scFv03 integrated downstream of a secretory signal peptide or a secretory signal peptide-linker conjugate according to the present invention, specific examples of the secretory signal peptide-linker conjugates by which successful secretion of anti-hPD-1scFv03 was observed, include SP69L1, SP69L2, SP69L3, SP69L4, SP69L5, SP69L6, SP69L7, SP69L8, SP69L9, SP69L10, SP69L15, SP69L20; SP7L20, SP45L20, SP50L20, SP52L20, SP55L20, SP58L20, SP64L20, SP66L20, SP67L20, SP68L20; SP7L5, SP7L10, SP7L15, SP45L5, SP45L10, SP45L15, SP50L5, SP50L10, SP50L15, SP52L5, SP52L10, SP52L15, SP55L5, SP55L10, SP55L15, SP58L15, SP64L5, SP64L10, SP64L15, SP66L5, SP66L10, SP66L15, SP67L5, SP67L10, SP67L15, SP68L5, SP68L10, SP68L15, SP69L5, SP69L10, SP69L15; SP45L0, SP45L1, SP45L2, SP45L3, SP50L0, SP50L1, SP50L2, SP50L3, SP50L4, SP64L0, SP64L1, SP64L2, SP64L3, SP64L4, SP68L0, SP68L1, SP68L2, SP68L3, SP68L4; and SP67L10 and SP69L7. Among them, SPSP50L0, SP50L1, SP50L2, SP50L3, SP50L4, SP50L5, SP50L10, SP50L15, SP50L20, SP64L0, SP64L1, SP64L2, SP64L3, SP64L4, SP64L5, SP64L10, SP64L15, SP64L20, SP68L1, SP68L2, SP68L3, SP68L4, SP68L5, SP68L10, SP68L15, SP68L20, SP69L7, SP69L8, SP69L9, SP69L10, SP69L15 and SP69L20, which

secreted a large amount of anti-hPD-1scFv03, can be preferably mentioned.

[0025]

In *Bifidobacterium longum* 105-A strains similarly transformed, when anti-hPD-1scFv03 was secreted and found to have binding ability to PD-1, specific examples of the secretory signal peptide-linker conjugate used there include SP7L5, SP7L20, SP68L5, SP68L20, SP69L5, SP69L20, SP45L20, SP50L20, SP52L20, SP55L20, SP58L20, SP64L20, SP66L20 and SP67L20. Of them, SP7L5, SP68L5 and SP69L5 secreting anti-hPD-1scFv03 binding to PD-1 in a larger amount can be preferably mentioned.

[0026]

In *Bifidobacterium longum* 105-A strain similarly transformed, when anti-hPD-1scFv03 was secreted and found to inhibit the binding between hPD-1 and PD-L1, specific examples of the secretory signal peptide-linker conjugate used there include SP69L5, SP69L20, SP50L5, SP64L5, SP67L10, SP68L1, SP68L5, SP69L1 and SP69L7. SP50L5, SP68L1 and SP69L1 are preferable. Among them, SP50L5 inhibits 50% of binding between hPD-1 and PD-L1 at a low concentration (antibody concentration (IC50) is low) is particularly preferable.

[0027]

In *Bifidobacterium longum* 105-A strain transformed with a vector containing an expression cassette in which anti-hCTLA-4scFv02 is integrated downstream of a secretory signal peptide or a secretory signal peptide-linker conjugate according to the present invention, when anti-hCTLA-4scFv02 was secreted, specific examples of the

secretory signal peptide-linker conjugate used there include SP7L20, SP45L20, SP50L20, SP52L20, SP55L20, SP58L20, SP64L20, SP66L20, SP67L20, SP68L20, SP69L20, SP45L0, SP45L1, SP45L2, SP45L3, SP45L4, SP45L5, SP50L0, SP50L1, SP50L2, SP50L3, SP50L4, SP50L5, SP64L0, SP64L1, SP64L2, SP64L3, SP64L4, SP64L5, SP68L1, SP68L2, SP68L3, SP68L4, SP68L5, SP69L0, SP69L1, SP69L2, SP69L3, SP69L4, SP69L5, SP69L6, SP69L7, SP69L8, SP69L9 and SP69L10.

[0028]

The DNA encoding a heterogeneous polypeptide as mentioned above is not particularly limited as long as it can be expressed in the expression cassette of the present invention and is not derived from a bacterium of the genus *Bifidobacterium*. Examples thereof include DNAs encoding a cytokines such as interferon (IFN)-α, β, γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukins (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IL-18, IL-27, tumor necrosis factor (TNF)-α, lymphotoxin (LT)-β, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), macrophage migration inhibitory factor (MIF); and DNAs encoding an angiogenesis-inhibiting substance such as endostatin and angiostatin. Besides, DNA encoding a cytosine deaminase, which is an enzyme of converting a prodrug of 5-fluorouracil, i.e., 5-fluoro-cytosine, to 5-fluorouracil, can be preferably mentioned. In addition, DNA encoding an antibody polypeptide can be used. To such DNA encoding a heterogeneous polypeptide, DNA encoding an affinity tag such as a histidine tag can

be appropriately added for the convenience of isolating the polypeptide.

[0029]

Examples of the antibody include an antibody targeting an interleukin-6 (IL-6) receptor for use in an antirheumatic drug and an antibody targeting α4-integrin for use in a multiple sclerosis therapeutic agent and antibodies targeting CD20, CD33, PD-1, CTLA-4, CD80 or CD86 and having an anti-cancer effect.

[0030]

Of them, the anti-PD-1 antibody can be preferably mentioned as an example, since the anti-PD-1 antibody binds to a PD-1 receptor expressed on an activated lymphocyte (T cell, B cell) to inhibit binding of PD-L1 or PD-L2 expressed by a cancer cell to the PD-1 receptor, with the result that an immune reaction with a tumor cell is augmented. Also, the anti-CTLA-4 antibody can be preferably mentioned as an example, since the anti-CTLA-4 antibody suppresses the function of CTLA-4, known as a molecule suppressing an autoimmune function to augment an anti-tumor immune response and inhibit the binding of CTLA-4 to CD80 and CD86 expressed on an antigen-presenting cell, with the result that negative downregulation of immune response induced by interaction of these molecules is conceivably inhibited.

[0031]

Examples of the DNA encoding the antibody polypeptide include DNA encoding a chimera antibody, a humanized antibody, Fab, Fab', F $(ab')_2$ and a scFv antibody; however, DNA encoding a scFv antibody is

preferable, since it can recognize and bind to a target substance by itself; it is not excessively large in view of molecular weight and it can be expressed even if it is introduced into a bacterium of the genus *Bifidobacterium* cell. DNA encoding a heterogeneous polypeptide including these antibodies can be prepared by a method known in the art such as chemical synthesis and genetic engineering procedure based on its sequence information appropriately obtained from documents publicly known and database such as GenBank.

[0032]

The terminator DNA is not particularly limited as long as it is terminator DNA functioning in a bacterium of the genus *Bifidobacterium*. Specific examples thereof include Hu terminator and T572 terminator derived from *Bifidobacterium longum* (J Microbiol Biotechnol. 2012 Dec; 22(12): 1714-23).

[0033]

A method for preparing an expression cassette of the present invention, preferably an expression cassette comprising DNA encoding a linker peptide sequentially comprises

(1) a promoter DNA functioning in a bacterium of the genus *Bifidobacterium*;

(2) a DNA encoding a secretory signal peptide consisting of an amino acid sequence represented by the following a) or b);

a) an amino acid sequence represented by any of SEQ ID Nos: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16;

b) an amino acid sequence obtained by deletion, replacement or addition of one or several of amino acids in the amino acid sequence represented by any of SEQ ID Nos: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16 and constituting a peptide functioning as a signal peptide in the bacterium of the genus *Bifidobacterium*;

(3) a DNA encoding a linker peptide;

(4) a DNA encoding a heterogeneous polypeptide; and

(5) a terminator DNA functioning in the bacterium of the genus *Bifidobacterium*;

in this order from upstream to downstream.

The expression cassette of the present invention can be prepared in accordance with a method described in a commercially available experimental certificate, for example, Gene Manual (Kodansha Ltd.), Gene Manipulation Experimental Technique edited by Yasutaka Takagi, (Kodansha Ltd.), Molecular Cloning [Cold Spring Harbor Laboratory (1982)], Molecular Cloning, second edition [Cold Spring Harbor Laboratory (1989)], Methods in Enzymology 194 (1991) and Gene Experimental Technique by Yeast, Experimental Medicine, Supplementary Volume, Yodosha (1994).

[0034]

The vector of the present invention is not particularly limited, as long as it is a vector which allows to express a heterogeneous polypeptide when the expression cassette of the present invention is inserted in the vector and a bacterium of the genus *Bifidobacterium* is transformed with the vector. One or more expression cassette of the present invention can be inserted into the

vector. A plasmid replication unit functioning in a bacterium of the genus *Bifidobacterium* is included in the vector of the present invention. Examples thereof include replication units such as pTB6 (Biosci Biotechnol Biochem. 2005 Feb; 69(2): 422-5); pMB1 (Lett Appl Microbiol. 1990 Oct; 11(4): 220-3); pTB4 (Structural Analysis and Application of *Bifidobacterium longum*-derived Plasmid pTB4, General Lecture, Poster Display program-Molecular Biology Society of Japan, 1994); pFI2576 (J Microbiol Biotechnol. 2009 Apr; 19(4): 403-8); pCIBAO (Appl Environ Microbiol. 2007 Dec; 73(24): 7858-66); pBC1 (Plasmid. 2007 Mar; 57(2): 165-74); pDOJH10S (Appl Environ Microbiol. 2006 Jan; 72(1): 527-35); and PKJ50 (Microbiology 1999 Mar; 145(Pt): 585-92). A pTB6 replication unit consisting of a pTB6 derived OriV region and RepB gene can be preferably mentioned. A marker gene such as an antibiotic resistant gene can be added to the above vector.

[0035]

Examples of the marker gene include spectinomycin, chloramphenicol, erythromycin and ampicillin.

[0036]

As a method for introducing the vector of the present invention into a bacterium of the genus *Bifidobacterium*, electroporation can be mentioned.

[0037]

Examples of a bacterium of the genus *Bifidobacterium* according to the present invention include *Bifidobacterium longum*, *Bifidobacterium breve* (*B. breve*), *Bifidobacterium adolescentis* (*B. adolescentis*), *Bifidobacterium bifidum* (*B. bifidum*), *Bifidobacterium pseudolongum* (B. *pseudolongum*),

Bifidobacterium thermophirum (*B. thermophirum*), *Bifidobacterium infantis* (B. *infantis*), *Bifidobacterium animalis* (*B. animalis*), *Bifidobacterium angulatum* (*B. angulatum*), *Bifidobacterium asteroides* (*B. asteroides*), *Bifidobacterium boum* (*B. boum*), *Bifidobacterium catenulatum* (*B. catenulatum*), *Bifidobacterium choerinum* (*B. choerinum*), *Bifidobacterium coryneforme* (*B. coryneforme*), *Bifidobacterium cuniculi* (*B. cuniculi*), *Bifidobacterium denticolens* (*B. denticolens*), *Bifidobacterium dentium* (*B. dentium*), *Bifidobacterium gallicum* (*B. gallicum*), *Bifidobacterium gallinarum* (*B. gallinarum*), *Bifidobacterium globosum* (*B. globosum*), *Bifidobacterium indicum* (*B. indicum*), *Bifidobacterium inopinatum* (*B. inopinatum*), *Bifidobacterium lactis* (*B. lactis*), *Bifidobacterium lactentis* (*B. lactentis*), *Bifidobacterium magnum* (*B. magnum*), *Bifidobacterium merycicum* (*B. merycicum*), *Bifidobacterium minimum* (*B. minimum*), *Bifidobacterium Mongolia Enns* (*B. Mongolia Enns*), *Bifidobacterium parvulorum* (*B. parvulorum*), *Bifidobacterium pseudocatenulatum* (*B. pseudocatenulatum*), *Bifidobacterium psychraerophilum* (*B. psychraerophilum*), *Bifidobacterium pullorum* (*B. pullorum*), *Bifidobacterium ruminale* (*B. ruminale*), *Bifidobacterium ruminantium* (*B. ruminantium*), *Bifidobacterium saeculare* (*B. saeculare*), *Bifidobacterium scardovii* (*B. scardovii*), *Bifidobacterium subtile* (*B. subtile*), *Bifidobacterium suis* (*B. suis*) and *Bifidobacterium thermacidophilum* (*B. thermacidophilum*). Of them, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium adolescentis*, *Bifidobacterium bifidum* and *Bifidobacterium infantis* are preferably used as a host

cell since they are known to be endemic bacteria in the intestines of humans regardless of age, and *Bifidobacterium longum* is more preferably used. These bacteria are all commercially available or can be easily obtained from depositories.

[0038]

The strain of each bacterium is not particularly limited. Examples of the strain of *Bifidobacterium longum* include *Bifidobacterium longum* 105-A strain, *Bifidobacterium longum* aE-194b strain, *Bifidobacterium longum* bs-601 strain, *Bifidobacterium longum* M101-2 strain and *Bifidobacterium longum* ATCC-15707 strain. Of them, *Bifidobacterium longum* 105-A strain is preferable. Examples of the strain of *B. breve* include *B. breve* standard strain (JCM1192), *B. breve* aS-1 strain and *B. breve* I-53-8W strain. Of them, *B. breve* standard strain and *B. breve* aS-1 strain are preferable. Examples of the strain of *B. infantis* include *B. infantis* standard strain (JCM1222) and *B. infantis* I-10-5 strain. Examples of the strain of *B. lactentis* include *B. lactentis* standard strain (JCM1210). Examples of the strain of *Bifidobacterium bifidum* include *Bifidobacterium bifidum* ATCC-11863 strain.

[0039]

The vectors, transformed bacterium of the genus *Bifidobacterium*, etc. of the present invention can be prepared in accordance with a method described in a commercially available experimental certificate, for example, Gene Manual (Kodansha Ltd.), Gene Manipulation Experimental Technique edited by Yasutaka Takagi (Kodansha

Ltd.), Molecular Cloning [Cold Spring Harbor Laboratory (1982)], Molecular Cloning, second edition [Cold Spring Harbor Laboratory (1989)], Methods in Enzymology 194 (1991) and Gene Experimental Technique by Yeast, Experimental Medicine, Supplementary Volume, Yodosha (1994).

[0040]

A transformed bacterium of the genus *Bifidobacterium* as mentioned above does not proliferate in normal tissues but proliferates only in tumor tissues under an anaerobic environment and can express a polypeptide useful for therapy within tumor tissues. Accordingly, such a transformed bacterium of the genus *Bifidobacterium* brings about a tumor growth suppression effect and a tumor volume suppression effect, preferably a complete regression action (effect) against a tumor under an anaerobic environment, and thus can be used as a pharmaceutical composition effective for treating a solid tumor, particularly as an anti-cancer agent. Accordingly, the pharmaceutical composition of the present invention is not particularly limited as long as it contains a bacterium of the genus *Bifidobacterium* of the present invention as mentioned above capable of secreting a heterogeneous polypeptide, preferably an antibody having an anti-cancer effect, as active ingredient, and can contain optional ingredients such as a pharmacologically acceptable carrier, an excipient and a diluent, as long as the optional ingredients do not suppress the function and effect of the polypeptide to be secreted.

[0041]

If the pharmaceutical composition of the present invention is an anti-cancer agent, examples of the applicable cancer include colorectal cancer, head and neck cancer, breast cancer, lung cancer, esophageal cancer, stomach cancer, liver cancer, gallbladder cancer, bile duct cancer, pancreatic cancer, pancreatic islet cell carcinoma, choriocarcinoma, colon cancer, renal cell cancer, adrenal cortical cancer, bladder cancer, testis cancer, prostate cancer, testicular cancer, ovarian cancer, uterine cancer, choriocarcinoma, thyroid cancer, squamous cell cancer, skin cancer, brain tumor, malignant carcinoid tumor, osteosarcoma, soft tissue sarcoma, neuroblastoma, Wilms' tumor, retinoblastoma and melanoma.

[0042]

As the dosage form of the pharmaceutical composition of the present invention, a liquid preparation or solid preparation is mentioned. The liquid preparation can be produced by purifying the culture of the bacterium of the genus *Bifidobacterium* of the present invention, optionally adding appropriate saline, a replacement fluid or pharmaceutical additive(s), and charging ampoules or vials with the resultant suspension. The solid preparation can be produced by adding a suitable protective agent to a liquid preparation and charging ampoules or vials with the resultant solution, and subjecting them to lyophilization or by adding a suitable protective agent to a liquid preparation, subjecting lyophilization and thereafter charging ampoules or vials with the resultant material. As a method for administering the pharmaceutical composition of the present invention, both oral

administration and parenteral administration can be employed; however, parenteral administration is preferable. Examples thereof include intravenous administration and local administration.

[0043]

The dosage amount of a pharmaceutical composition of the present invention is not particularly limited as long as it is sufficient for the bacterium of the genus *Bifidobacterium* of the present invention to grow at a site of disease and express an effective therapeutic amount of active antibody. The dosage can be appropriately selected depending upon the extent of the disease and the weight, age and gender of a patient; and can be appropriately increased or decreased depending upon the degree of improvement. However, the dosage amount is preferably as low as possible within the range where a requisite therapeutic effect can be obtained, from an economic point of view and in order to avoid a side effect as much as possible.

[0044]

For example, in the case of intravenous administration, it is required to reduce a risk such as embolus due to bacterial mass. Because of this, it is preferable that an injectable preparation having a lowest possible concentration is injected in a plurality of divided doses or diluted with an appropriate replacement fluid and continuously injected. For example, in the case of an adult, bacterial cells of the bacterium of the genus *Bifidobacterium* of the present invention, 10^4 to 10^{12} cfu of the bacterial cells per body weight (1 kg), is

administered once or in a plurality of divided doses per day for one to several days, continuously or at appropriate intervals. More specifically, a preparation containing the bacterial cells of bacterium of the genus *Bifidobacterium* of the present invention in an amount of 10^4 to 10^{10} cfu/mL is administered directly in a dose of 1 to 1000 mL per adult or diluted with an appropriate replacement fluid and administered once or in a plurality of divided portions per day continuously for one to several days.

[0045]

For example, in the case of local administration directly to a diseased tissue, bacterial cells are required to be colonized in the whole diseased tissue as much as possible and proliferate there. Because of this, a highly concentrated injection is desirably injected to a plurality of sites in the diseased tissue. For example, in the case of an adult, bacterial cells of the bacterium of the genus *Bifidobacterium* of the present invention, 10^4 to 10^{12} cfu of the bacterial cells per body weight (1 kg) is administered once or in a plurality of divided doses per day, if necessary, for one to several days, continuously or at appropriate intervals. More specifically, a preparation containing the bacterial cells of the bacterium of the genus *Bifidobacterium* of the present invention in an amount of 10^4 to 10^{10} cfu/mL is administered directly in a dose of 0.1 to 100 mL per adult in several divided portions per day, if necessary, continuously for one to a plurality of days.

[0046]

Now the present invention will be more specifically described below by way of Examples; however the technical range of the present invention is not limited by these Examples.

Examples

[0047]

[Example 1]

[Preparation of pHuSP3L22-scFv-PD-1-1, pHuSP7L20-scFv-PD-1-1 and pHuSP23L27-scFv-PD-1-1]

Anti-PD-1-1 scFv antibody secretory plasmids were prepared by using secretory signal peptides known in the art and a novel secretory signal peptide.

[0048]

The details of the primers used in Examples 1 and 5 to 8 are shown in Table 1 and Table 2 below.

[0049]

[Table 1]

PCR primers used in insert and vector amplification

Insert	Forward primer	Reverse primer	PCR product (bp)
PD-1-1	Ins_PD-1-1_F1	Ins_PD-1-1_R1	747
PD-1-2	Ins_PD-1-2_F1	Ins_PD-1-2_R1	759
PD-1-3	Ins_PD-1-3_F1	Ins_PD-1-3_R1	771
CTLA-4-1	Ins_CTLA-4-1_F1	Ins_CTLA-4-1_R1	774
CTLA-4-2	Ins_CTLA-4-1_F1	Ins_CTLA-4-2_R1	756
Vector	Forward primer	Reverse primer	PCR product (bp)
Vec-SP3L20	TGA_Hu_Terminator_F	vec-SP3L20-PD-1-1_R1	4019
Vec-SP7L20-1	TGA_Hu_Terminator_F	vec-SP7L20-PD-1-1_R1	4019
Vec-SP23L20	TGA_Hu_Terminator_F	vec-SP23L20-PD-1-1_R1	4019
Vec-SP7L20-2	TGA_Hu_Terminator_F	vec-SP7L20-PD-1-2_R1	4019
Vec-SP7L20-3	TGA_Hu_Terminator_F	vec-SP7L20-PD-1-3_R1	4019
SP3L22	Ins_PD-1-1_F1 (5'-phosphorylated)	SP3L22_R1 (5'- phosphorylated)	4742
SP23L27	Ins_PD-1-1_F1 (5'-phosphorylated)	SP23L27_R1 (5'-phosphorylated)	4757

[0050]

[Table 2]

Primer sequence in insert and vector amplification

Primer name	Sequence (5 -> 3)
Ins_PD-1-1_F1	CAGGTCCAGCTGGTCCAGAGCGGCAGCGAA (SEQ ID No: 36)
Ins_PD-1-2_F1	CAGGTCCAGCTGCAGGAATCGGGCCCGGGC (SEQ ID No: 37)
Ins_PD-1-3_F1	GAAGTGCGTCTGCTGGAATCGGGCGGCGGC (SEQ ID No: 38)
Ins_CTLA-4-1_F1	CAGGTCCAGCTGGTCGAATCGGGCGGCGGC (SEQ ID No: 39)
Ins_PD-1-1_R1	ACGAGCAGAAGGTCAGTGGTGGTGATGATGGTGCTT (SEQ ID No: 40)
vec-SP3L20-PD-1-1_R1	GACCAGCTGGACCTGGGTCAGCTTGCCCGGCTTGTA (SEQ ID NO: 41)
vec-SP7L20-PD-1-1_R1	GACCAGCTGGACCTGCACCGAACTCGCCTTCGGGAA (SEQ ID NO: 42)
vec-SP23L20-PD-1-1_R1	GACCAGCTGGACCTGACGAATCTTCTTCTCCTGCGC (SEQ ID NO: 43)
Ins_PD-1-2_R1	ACGAGCAGAAGGTCAGTGATGATGATGGTGGTGACG (SEQ ID NO: 44)
vec-SP7L20-PD-1-2_R1	CTGCAGCTGGACCTGCACCGAACTCGCCTTCGGGAA (SEQ ID NO: 45)
Ins_PD-1-3_R1	ACGAGCAGAAGGTCAGTGATGATGGTGGTGATGGCC (SEQ ID NO: 46)
vec-SP7L20-PD-1-3_R1	CAGCAGACGCACTTCCACCGAACTCGCCTTCGGGAA (SEQ ID NO: 47)
Ins_CTLA-4-1_R1	ACGAGCAGAAGGTCAGTGATGATGGTGATGATGCTT (SEQ ID NO: 48)
Ins_CTLA-4-2_R1	ACGAGCAGAAGGTCAGTGATGATGATGATGATGCTT (SEQ ID NO: 49)
SP3L22_R1	GGCGATGGTCAGCTTGCCCGGCTTGTACGT (SEQ ID NO: 50)
SP23L27_R1	GATCGTCTTGAGAATCTTCAGACGAATCTTCTTCTCCTGCGC (SEQ ID NO: 51)
TGA_Hu_Terminator_F	TGACCTTCTGCTCGTAGCGATTAC (SEQ ID NO: 52)

[0051]

[Expression cassette for Anti-PD-1 scFv antibody]

An expression cassette for an anti-PD-1 scFv antibody was, as shown in Figure 1(a), constituted of Hu promoter DNA (derived from *Bifidobacterium longum*), a DNA encoding a secretory signal peptide, a DNA encoding a linker peptide following the secretory signal peptide, a DNA encoding an anti-PD-1 scFv antibody (containing a heavy chain sequence, a linker $((GGGGS)_3)$, a light chain sequence), a His tag sequence and a Hu terminator (derived from *Bifidobacterium longum*). As the anti-PD-1 scFv antibodies (containing nucleotide sequences containing a heavy chain sequence, a linker $((GGGGS)_3)$ and a light chain sequence) in individual expression cassettes, two

anti-human PD-1 scFv antibodies and a single anti-mouse PD-1 scFv antibody were used, with reference to the documents shown in Table 3 below.

[0052]

[Table 3]

Antibody	Sequence	Reference document
scFv-PD-1-1 (Anti-human PD-1 antibody)	SEQ ID No: 1 (738 nucleotides) SEQ ID No: 138 (245 amino acids) Heavy chain sequence(1-354 nucleotides) $(GGGGS)_3$linker (355-399 nucleotides) Light chain sequence (400-717 nucleotides) Histidine tag (718-735 nucleotides)	US Patent 7332582B2
scFv-PD-1-2 (Anti-human PD-1 antibody)	SEQ ID No: 2 (750 nucleotides) SEQ ID No: 139 (249 amino acids) Heavy chain sequence(1-360 nucleotides) $(GGGGS)_3$linker (361-405 nucleotides) Light chain sequence (406-729 nucleotides) Histidine tag (730-747 nucleotides)	Japanese Patent No. 4532409
scFv-PD-1-3 (Anti-mouse PD-1 antibody)	SEQ ID No: 3 (762 nucleotides sequence) SEQ ID No: 140 (253 amino acids) Heavy chain sequence(1-366 nucleotides) $(GGGGS)_3$ linker (367-411 nucleotides) Light chain sequence (412-741 nucleotides) Histidine tag (742-759 nucleotides)	Japanese Patent No. 4249013

[0053]

The genes of the anti-PD-1 scFv antibodies, namely scFv-PD-1-1, scFv-PD-1-2 and scFv-PD-1-3, were separately subcloned in a plasmid for *E. coli*, i.e., pUC57 by GenScript Japan Inc. and provided as artificially

synthesized plasmids, namely, pUC57-scFv-PD-1-1, pUC57-scFv-PD-1-2 and pUC57-scFv-PD-1-3, respectively.

[0054]

As the secretory signal peptide sequence, secretory signal peptide-linker conjugates, namely SP3L22, SP7L20 and SP23L27, were used.

[0055]

As SP3L22 mentioned above, a known sequence represented by SEQ ID No: 8 described in International Publication No. WO2011/093467 was used. As SP23L27, a known sequence represented by SEQ ID No: 25 described in International Publication No. WO2011/093467 was used. As SP7L20, a nucleotide sequence, which was prepared by adding a 66-nucleotide: ATGGCGTTGATGATGAGCGTTAAGACTATTATTTCCACATCAGTGGCGATTATCGCCA CGGGTGCC (starting with ATG) to the upstream of the 5' side of a nucleotide sequence represented by SEQ ID No: 12 described in International Publication No. WO2011/093467 (used as a basic sequence), and represented by SEQ ID No: 81, was used. Such a nucleotide sequence was added because the SP7 was estimated to have a secretory signal peptide sequence at the N terminal of a protein when SP7 was analyzed by a signal sequence estimation program, Signal P 4.1 server (http://www.cbs.dtu.dk/services/SignalP/), which is a program of estimating the presence or absence of a secretory signal. In this case, the linker peptide sequence following SP7 was 20 amino acid residues at the C terminal side of SP7.

[0056]

How to prepare an anti-PD-1 scFv antibody secretory plasmid is schematically shown in Figure 2. On the left side in Figure 2, scFv-gene is PD-1. In preparation of pHuSP7L20-scFv-PD-1-1, x = 7 and n = 1; in the preparation of pHuSP7L20-scFv-PD-1-2, x = 7 and n = 2; and in preparation of pHuSP7L20-scFv-PD-1-3, x = 7 and n = 3. On the right side of Figure 2, in the preparation of pHuSP3L22-scFv-PD-1-1, x = 3 and y = 22; and in the preparation of pHuSP23L27-scFv-PD-1-1, x = 23 and y = 27. A construction method will be more specifically described below.

[0057]

[Preparation of plasmid, pHuSP3L22-scFv-PD-1-1]

In preparing a plasmid, pHuSP3L22-scFv-PD-1-1, first, a plasmid, pHuSP3L20-scFv-PD-1-1 was prepared.

[0058]

[Preparation of plasmid, pHuSP3L20-scFv-PD-1-1]

(Preparation of anti-PD-1-1 scFv antibody insert)

PCR amplification was performed by using a plasmid, pUC57-scFv-PD-1-1 (500 pg) as a template and a primer set consisting of Ins_PD-1-1_F1 primer and Ins_PD-1-1_R1 primer listed in Table 1 and Table 2. The primer sequences were designed such that an insert fragment and a vector fragment were mutually overlapped at the end of 15 bp. The PCR amplification was performed by setting each primer concentration at 0.2 μM and the reaction volume at 30 μL and using PrimeSTAR HS (Premix) kit (manufactured by Takara Bio Inc.). As an amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 72°C for

60 seconds was repeated 30 times. The PCR product obtained by the amplification was subjected to electrophoresis on 2% agarose gel and purified by QIAquick Gel Extraction Kit (manufactured by QIAGEN) to prepare an anti-PD-1-1 scFv antibody insert.

[0059]

(Preparation of vector fragment)

PCR amplification was performed by using a linearized vector fragment (500 pg) represented by SEQ ID No: 20 as a template and a primer set consisting of TGA_Hu_Terminator_F primer and vec-SP3L20-PD-1-1_R1 primer listed in Table 1 and Table 2 above, setting each primer concentration at 0.2 μM and the reaction volume at 30 μL and using PrimeSTAR HS (Premix) kit. As an amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 72°C for 5 minutes was repeated 30 times. The PCR product obtained by the amplification was subjected to electrophoresis on 0.8% agarose gel and purified by QIAquick Gel Extraction Kit (manufactured by QIAGEN) to prepare 5'-Hu terminator-pTB6rep unit-SPCMr-pUCori-Hu promoter-SP3L20-3' vector fragment.

[0060]

The linearized vector fragment is the nucleotide sequence consisting of the 4th nucleotide to the 3845th nucleotide of the nucleotide sequence represented by SEQ ID No: 20 and constituted of:

Hu terminator: the nucleotide sequence consisting of the 4th nucleotide to the 117th nucleotide of the nucleotide sequence represented by SEQ ID No: 20;

Origin of replication pTB6 rep unit of bacterium of the genus *Bifidobacterium*: the nucleotide sequence consisting of the 124th nucleotide to the 1719th nucleotide of the nucleotide sequence represented by SEQ ID No: 20;

Spectinomycin resistant gene, SPCMr: the nucleotide sequence consisting of the 1726th nucleotide to the 2804th nucleotide of the nucleotide sequence represented by SEQ ID No: 20;

Origin of replication of *E. coli*, pUCori: the nucleotide sequence consisting of the 2811st nucleotide to the 3478th nucleotide of the nucleotide sequence represented by SEQ ID No: 20; and

Hu promoter: the nucleotide sequence consisting of the 3485th nucleotide to the 3845th nucleotide of the nucleotide sequence represented by SEQ ID No: 20.

[0061]

(Infusion reaction)

The vector fragment and an anti-PD-1-1 scFv antibody insert prepared above were ligated by use of In-Fusion (registered trademark) HD Cloning Kit (manufactured by Takara Bio Inc.). More specifically, in a microtube, the vector and the insert were added at a molar ratio of 1: 5 and thereafter 2 μL of 5 × In-Fusion HD Enzyme premix was added. The volume of the reaction solution was adjusted to be 10 μL. The reaction solution was kept at 50°C for 15 minutes. The procedure described in the instruction of the kit except the aforementioned procedure was performed to prepare an infusion reaction solution.

[0062]

(Transformation of *E. coli* and sequencing of plasmid DNA)

Using the infusion reaction solution (5 μL), *E. coli* HST08 competent cell (manufactured by Takara Bio Inc.) was transformed in accordance with the instruction thereof. After transformation, the bacterial suspension was spread onto a 75 μg/mL spectinomycin-containing LB agar medium and subjected to static culture at 37°C overnight. *E. coli* colonies formed on the agar medium were cultured in a 75 μg/mL spectinomycin-containing LB liquid medium at 30°C overnight. From the cultured medium, a plasmid was extracted by use of QIAprep Spin Miniprep Kit (manufactured by QIAGEN). An anti-PD-1-1 scFv antibody expression cassette (containing Hu promoter to Hu terminator) of the plasmid extracted was subjected to a sequencing reaction by BigDye (registered trademark) Terminator v3.1 Cycle Sequencing Kit (manufactured by Applied Biosystems) for determining the sequence thereof. The extracted plasmid was designated as pHuSP3L20-scFv-PD-1-1.

[0063]

[Preparation of pHuSP3L22-scFv-PD-1-1]

(Preparation of phosphorylated primer)

Each of Ins_PD-1-1_F1 primer and SP3L22_R1 primer listed in Table 1 and Table 2 was phosphorylated with T4 polynucleotide kinase (manufactured by Takara Bio Inc.) in accordance with the instruction. Ins_PD-1-1_F1 phosphorylated primer and SP3L22_R1 phosphorylated primer each were adjusted so as to have a final concentration of

20 μM with 0.1 × TE buffer (1mM Tris-HCl, 0.1mM EDTA, pH7.5).

[0064]

(Preparation of linearized fragment of pHuSP3L22-scFv-PD-1-1 vector)

PCR amplification was performed by using a plasmid, pHuSP3L20-scFv-PD-1-1 (500 pg) obtained above as a template and a primer set consisting of Ins_PD-1-1_F1 phosphorylated primer and SP3L22_R1 phosphorylated primer, setting each primer concentration at 0.2 μM and the reaction volume at 30 μL and using PrimeSTAR HS (Premix) kit. As an amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 72°C for 5 minutes was repeated 30 times. The PCR product obtained by amplification was subjected to electrophoresis on 0.8% agarose gel, purified by QIAquick Gel Extraction Kit to prepare a linearized fragment pHuSP3L22-scFv-PD-1-1 vector.

[0065]

(Ligation reaction)

The linearized vector fragment obtained above was subjected to a ligation reaction using T4 DNA Ligase (manufactured by Thermo Fisher Scientific Inc.) in accordance with the instruction. The accompanying 10 × buffer (2 μL) for T4 DNA ligase was added and the volume of a reaction solution was set to be 20 μL. This was subjected to a ligation reaction at 22°C for 10 minutes to prepare a ligation reaction solution.

[0066]

(Transformation of *E. coli* and sequencing of plasmid DNA)

Using the ligation reaction solution (10 μL) obtained above, *E. coli* HST08 competent cell was transformed. After transformation, a bacterial suspension was spread onto a 75 μg/mL spectinomycin-containing LB agar medium and subjected to static culture at 37°C overnight. Transformation was carried out in accordance with the conditions described in the instruction for *E. coli* HST08 competent cell as outlined below. *E. coli* colonies formed on the agar medium were cultured in a 75 μg/mL spectinomycin-containing LB liquid medium at 30°C overnight. From this, a plasmid was extracted by use of QIAprep Spin Miniprep Kit. The anti-PD-1-1 scFv antibody expression cassette (containing Hu promoter to Hu terminator) of the extracted plasmid was subjected to a sequencing reaction by use of BigDye (registered trademark) Terminator v3.1 Cycle Sequencing Kit for determining the sequence thereof. The extracted plasmid was designated as pHuSP3L22-scFv-PD-1-1.

[0067]

(Transformation of bacterium of the genus *Bifidobacterium*)

Using the plasmid, pHuSP3L22-scFv-PD-1-1 (1000 ng) extracted from *E. coli* transformed above, *Bifidobacterium longum* 105-A strain was transformed by the electroporation system (Gene Pulser II, manufactured by Bio-Rad Laboratories, Inc.). Immediately after application of electric shock (2kV, 25 μ F, 200 Ω), a solution mixture of IMR liquid medium (800 μL) and a vitamin C-added solution

(50 μL) was poured in a cuvette (2 mm gap) and this was collected to a 2 mL-microtube sterilized. Individual tubes (2 mL) were prepared in the same manner and placed in an airtight container together with a de-oxygen and carbon dioxide gas generating agent (AnaeroPack (registered trademark) Kenki, manufactured by Mitsubishi Gas Chemical Company, Inc.) with the lids opened and kept warm in an incubator set at 37°C for 3 hours.

[0068]

Each of the resultant bacterium suspensions kept warm was spread onto a 75 μg/mL spectinomycin-containing IMR agar medium. These plates were placed in an airtight container together with a de-oxygen and carbon dioxide gas generating agent as mentioned above, and cultured in an incubator set at 37°C for 2 days.

[0069]

A part of the colonies formed on the spectinomycin-containing IMR agar medium was picked up, streaked on a 75 μg/mL spectinomycin-containing BL-bS agar medium (BL agar medium containing no equine defibrinated blood), placed in an airtight container together with a de-oxygen and carbon dioxide gas generating agent and cultured in an incubator set at 37°C for one day to obtain *Bifidobacterium longum* 105-A/pHuSP3L22-scFv-PD-1-1 strain.

[0070]

[Preparation of pHuSP7L20-scFv-PD-1-1]

(Preparation of anti-PD-1-1 scFv antibody insert)

An anti-PD-1-1 scFv antibody insert was prepared in the same procedure as in the above section: [Preparation

of plasmid, pHuSP3L20-scFv-PD-1-1], (Preparation of anti-PD-1-1 scFv antibody insert).

[0071]

(Preparation of vector fragment)

PCR amplification was performed by using a linearized vector fragment (500 pg) represented by SEQ ID No: 21 as a template and a primer set consisting of TGA_Hu_Terminator_F primer and vec-SP7L20-PD-1-1_R1 primer described in Table 1 and Table 2 above, setting each primer concentration at 0.2 μM and the reaction volume at 30 μL and using PrimeSTAR HS (Premix) kit. As an amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 72°C for 5 minutes was repeated 30 times. The PCR product obtained by the amplification was subjected to electrophoresis on 0.8% agarose gel and purified by QIAquick Gel Extraction Kit to prepare a 5'-Hu terminator-pTB6rep unit-SPCMr-pUCori-Hu promoter-SP7L20-3' vector fragment (1).

[0072]

The linearized vector fragment is the nucleotide sequence consisting of the 4th nucleotide to the 3845th nucleotide of the nucleotide sequence represented by SEQ ID No: 21 and constituted of:

Hu terminator: the nucleotide sequence consisting of the 4th nucleotide to the 117th nucleotide of the nucleotide sequence represented by SEQ ID No: 21;

Origin of replication pTB6 rep unit of bacterium of the genus *Bifidobacterium*: the nucleotide sequence consisting of the 124th nucleotide to the 1719th

nucleotide of the nucleotide sequence represented by SEQ ID No: 21;

Spectinomycin resistant gene, SPCMr: the nucleotide sequence consisting of the 1726th nucleotide to the 2804th nucleotide of the nucleotide sequence represented by SEQ ID No: 21;

Origin of replication of *E. coli*, pUCori: the nucleotide sequence consisting of the 2811st nucleotide to the 3478th nucleotide of the nucleotide sequence represented by SEQ ID No: 21; and

Hu promoter: the nucleotide sequence consisting of the 3485th nucleotide to the 3845th nucleotide of the nucleotide sequence represented by SEQ ID No: 21.

[0073]

(Infusion reaction)

An infusion reaction solution was prepared in the same procedure as in the infusion reaction in the above section: [Preparation of plasmid, pHuSP3L20-scFv-PD-1-1] except that the vector fragment and the anti-PD-1-1 scFv antibody insert prepared above were used.

[0074]

(Transformation of *E. coli* and sequencing of plasmid DNA)

E. coli HST08 competent cell was transformed and the anti-PD-1-1 scFv antibody expression cassette (containing Hu promoter to Hu terminator) of the extracted plasmid was subjected to a sequencing reaction in the same procedure as in the above section: [Preparation of plasmid, pHuSP3L20-scFv-PD-1-1], (Transformation of *E. coli* and sequencing of plasmid DNA) except that the infusion

reaction solution (5 μL) prepared above was used. The extracted plasmid was designated as pHuSP7L20-scFv-PD-1-1.

[0075]

(Transformation of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed in the same procedure in the above section: [Preparation of pHuSP3L22-scFv-PD-1-1], (Transformation of bacterium of the genus *Bifidobacterium*) except that DNA of a plasmid, pHuSP7L20-scFv-PD-1-1 (1000 ng) extracted from *E. coli* transformed above was used to obtain *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-1 strain.

[0076]

[Preparation pHuSP23L27-scFv-PD-1-1]

For preparing a plasmid, pHuSP23L27-scFv-PD-1-1, first, a plasmid, pHuSP23L20-scFv-PD-1-1 was prepared.

[0077]

[Preparation of plasmid, pHuSP23L20-scFv-PD-1-1]

(Preparation of anti-PD-1-1 scFv antibody insert)

An anti-PD-1-1 scFv antibody insert was prepared in the same procedure as in the above section: [Preparation of plasmid, pHuSP3L20-scFv-PD-1-1], (Preparation of an anti-PD-1-1 scFv antibody insert).

[0078]

(Preparation of vector fragment)

PCR amplification was performed by using the linearized vector fragment (500 pg) represented by SEQ ID No: 22 as a template and a primer set consisting of TGA_Hu_Terminator_F primer and vec-SP23L20-PD-1-1_R1 primer listed in Table 1 and Table 2 above, setting each

primer concentration at 0.2 μM and the reaction volume at 30 μL and using PrimeSTAR HS (Premix) kit. As an amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 72°C for 5 minutes was repeated 30 times. The PCR product obtained by the amplification was subjected to electrophoresis on 0.8% agarose gel and purified by QIAquick Gel Extraction Kit to prepare a 5'-Hu terminator-pTB6rep unit-SPCMr-pUCori-Hu promoter-SP23L20-3' vector fragment.

[0079]

The linearized vector fragment is the nucleotide sequence consisting of the 4th nucleotide to the 3845th nucleotide of the nucleotide sequence represented by SEQ ID No: 22 and constituted of:

Hu terminator: the nucleotide sequence consisting of the 4th nucleotide to the 117th nucleotide of the nucleotide sequence represented by SEQ ID No: 22;

Origin of replication pTB6 rep unit of bacterium of the genus *Bifidobacterium*: the nucleotide sequence from the 124th nucleotide to the 1719th nucleotide of the nucleotide sequence represented by SEQ ID No: 22;

Spectinomycin resistant gene, SPCMr: the nucleotide sequence consisting of the 1726th nucleotide to the 2804th nucleotide of the nucleotide sequence represented by SEQ ID No: 22;

Origin of replication of *E. coli*, pUCori: the nucleotide sequence consisting of the 2811st nucleotide to the 3478th nucleotide of the nucleotide sequence represented by SEQ ID No: 22; and

Hu promoter: the nucleotide sequence consisting of the 3485th nucleotide to the 3845th nucleotide of the nucleotide sequence represented by SEQ ID No: 22.

[0080]

(Infusion reaction)

An infusion reaction solution was prepared in the same procedure as in the infusion reaction in the above section: [Preparation of plasmid, pHuSP3L20-scFv-PD-1-1] except that the vector fragment and the anti-PD-1-1 scFv antibody insert prepared above were used.

[0081]

(Transformation of *E. coli* and sequencing of plasmid DNA)

E. coli HST08 competent cell was transformed and the anti-PD-1-1 scFv antibody expression cassette (containing Hu promoter to Hu terminator) of the extracted plasmid was subjected to a sequencing reaction (for determining the sequence) in the same procedure as in the above section: [Preparation of plasmid, pHuSP3L20-scFv-PD-1-1], (Transformation of *E. coli* and sequencing of plasmid DNA) except that the infusion reaction solution (5 μL) prepared above was used. The extracted plasmid was designated as pHuSP23L20-scFv-PD-1-1.

[0082]

[Preparation of plasmid, pHuSP23L27-scFv-PD-1-1]

(Preparation of phosphorylated primer)

Each of Ins_PD-1-1_F1 primer and SP23L27_R1 primer listed in Table 1 and Table 2 was phosphorylated by using T4 polynucleotide kinase in accordance with the instruction. Ins_PD-1-1_F1 phosphorylated primer and

SP23L27_R1 phosphorylated primer each were adjusted so as to have a final concentration of 20 μM, with 0.1 × TE buffer (1mM Tris-HCl, 0.1mM EDTA, pH7.5).

[0083]

(Preparation of linearized fragment of pHuSP23L27-scFv-PD-1-1 vector)

PCR amplification was performed by using a plasmid, pHuSP23L20-scFv-PD-1-1 (500 pg) obtained above as a template, a primer set consisting of Ins_PD-1-1_F1 phosphorylated primer and SP23L27_R1 phosphorylated primer, setting a final concentration at 0.2 μM and the reaction volume at 30 μL, and using PrimeSTAR HS (Premix) kit. As an amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 72°C for 5 minutes was repeated 30 times. The PCR product obtained by the amplification was subjected to electrophoresis on 0.8% agarose gel and purified by QIAquick Gel Extraction Kit to prepare a linearized fragment of pHuSP23L27-scFv-PD-1-1 vector.

[0084]

(Ligation reaction)

The vector fragment obtained above was subjected to a ligation reaction to prepare a ligation reaction solution in the same procedure as in the above section: [Preparation of plasmid, pHuSP3L22-scFv-PD-1-1], (Ligation reaction).

[0085]

(Transformation of *E. coli* and sequencing of plasmid DNA)

E. coli HST08 competent cell was transformed by using the above ligation reaction solution (10 μL) and subjected to a sequencing reaction in the same manner as in the above section: [Preparation of plasmid, pHuSP3L22-scFv-PD-1-1], (Transformation of *E. coli* and sequencing of plasmid DNA). The extracted plasmid was designated as pHuSP23L27-scFv-PD-1-1.

[0086]

(Transformation of bacterium of the genus *Bifidobacterium*)

Using 1000 ng of DNA of plasmid, pHuSP23L27-scFv-PD-1-1 extracted from *E. coli* transformed above, *Bifidobacterium longum* 105-A strain was transformed in the same procedure as in the above section: [Preparation of plasmid, pHuSP3L22-scFv-PD-1-1], (Transformation of bacterium of the genus *Bifidobacterium*) to obtain *Bifidobacterium longum* 105-A/pHuSP23L27-scFv-PD-1-1 strain.

[0087]

[Example 2]

(First Investigation of the presence or absence of secretion of scFv antibody in recombinant bacterium of the genus *Bifidobacterium*)

Whether three types of recombinant bacterium of the genus *Bifidobacterium* (*Bifidobacterium longum* 105-A/pHuSP3L22-scFv-PD-1-1 strain, *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-1 strain, *Bifidobacterium longum* 105-A/pHuSP23L27-scFv-PD-1-1 strain) transformed respectively with three types of anti-PD-1-1 scFv antibody secretory plasmids prepared in Example 1 secret anti-PD-1-

1 scFv antibody was checked by Western Analysis, as follows.

[0088]

A streak culture of each of the recombinant bacterium of the genus *Bifidobacterium* strains on a 75 μg/mL spectinomycin-containing BL-bS agar medium was inoculated in MRS (manufactured by Becton, Dickinson and Company) liquid medium (10 mL) supplemented with spectinomycin (a final concentration of 75 μg/mL), 100 μL of a vitamin C-added solution (a solution (100 mL) containing ascorbic acid (35 g), L-cysteine hydrochloride monohydrate (2 g) and sodium carbonate (11 g)), and anaerobically cultured at 37°C for 24 hours to obtain an activated culture solution. Subsequently, to a medium (20 mL) containing DMEM (Cat No. 11885-084: manufactured by Life Technologies) and MRS in a ratio of 9: 1, a vitamin C-added solution (100 μL) and spectinomycin (75 μg/mL) were added. The activated culture solution (100 μL) was inoculated to the medium. This medium was anaerobically cultured at 37°C for 18 hours.

[0089]

After completion of the anaerobic culture, the activated culture solution was centrifuged and the culture supernatant was collected. Protein in the culture supernatant was allowed to precipitate with trichloroacetic acid (TCA, manufactured by Wako Pure Chemical Industries Ltd.), washed with acetone, dissolved in SDS-PAGE buffer and subjected to a heat treatment at 95°C for 3 minutes to obtain a culture supernatant concentrate. *Bifidobacterium longum* 105-A/pBEshuttle

strain was subjected to the same operation and used as a negative control.

[0090]

The culture supernatant concentrate (corresponding to 1 mL of culture solution) was subjected to electrophoresis by Mini-PROTEAN (registered trademark) TGX™ gel (4 to 20%) (manufactured by Bio-Rad). The gel was transferred onto a PVDF membrane (iBlot Transfer Stacks, manufactured by Life Technologies) by use of an iBlot transfer device (manufactured by Life Technologies). After completion of blotting, the membrane was subjected to blocking (2% ECL Prime Blocking agent (manufactured by GE Healthcare Japan) in TTBS), and then to a reaction with a mouse histidine tag antibody (THE HIS Tag Antibody, mAb, Mouse, manufactured by GenScript Japan Inc.) used as a primary antibody and an ECL-peroxidase-labeled anti-mouse antibody (manufactured by GE Healthcare Japan) used as a secondary antibody, and allowed to emit light by use of Western Lightning Ultra (manufactured by PerkinElmer Co., Ltd.). The light-emission was analyzed by an imaging analyzer (Fluor SMax, manufactured by Bio-Rad). The results are shown in Figure 4. Lane M shows a marker; lane 1 shows a negative control; lane 2 shows an antibody secreted from a bacterium of the genus *Bifidobacterium* transformed with pHuSP3L22-scFv-PD-1-1 in lane 2; lane 3 shows an antibody secreted from a bacterium of the genus *Bifidobacterium* transformed with pHuSP7L20-scFv-PD-1-1; and lane 4 shows an antibody secreted from a bacterium of the genus *Bifidobacterium* transformed with pHuSP23L27-scFv-PD-1-1.

[0091]

(Results)

As is apparent from Figure 4, in the bacterium of the genus *Bifidobacterium* transformed with pHuSP7L20-scFv-PD-1-1, (which has an insert of a novel secretory signal peptide-linker conjugate, SP7L20, consisting of a secretory signal peptide sequence SP7 and a subsequent sequence of 20 amino acids), secretion of an anti-PD-1-1 scFv antibody was observed. In contrast, in the bacterium of the genus *Bifidobacterium* transformed with pHuSP3L22-scFv-PD-1-1 or pHuSP23L27-scFv-PD-1-1, no secretion of the anti-PD-1-1 scFv antibody was observed.

[0092]

[Example 3]

[Investigation on 33 types of secretory signal peptide-linker conjugate]

In the above pHuSP7L20-scFv-PD-1-1, secretory signal peptide-linker conjugate SP7L20 was replaced with each of 33 types of secretory signal peptide-linker conjugates (SP42L20 to SP46L20, SP48L20 to SP75L20). In this manner, 33 types of anti-PD-1-1 scFv antibody secretory plasmids were constructed. How to construct the plasmid is schematically shown in Figure 3. The primers used herein are more specifically shown in Table 4 and Table 5 below.

[0093]

[Table 4]

Insert	Forward primer	Reverse primer	PCR product (bp)
SP42L20	SP42-ins_F1	SP42-ins_R1	177
SP45L20	SP45-ins_F1	SP45-ins_R1	192
SP48L20	SP48-ins_F1	SP48-ins_R1	168
SP50L20	SP50-ins_F1	SP50-ins_R1	258
SP52L20	SP52-ins_F1	SP52-ins_R1	195
SP55L20	SP55-ins_F1	SP55-ins_R1	234
SP58L20	SP58-ins_F1	SP58-ins_R1	186
SP62L20	SP62-ins_F1	SP62-ins_R1	222
SP64L20	SP64-ins_F1	SP64-ins_R1	183
SP66L20	SP66-ins_F1	SP66-ins_R1	168
SP67L20	SP67-ins_F1	SP67-ins_R1	189
SP68L20	SP68-ins_F1	SP68-ins_R1	219
SP69L20	SP69-ins_F1	SP69-ins_R1	180

[0094]

[Table 5]

Primer Name	Sequence (5 -> 3)
PD-1-scFv_vec_F1	CAGGTCCAGCTGGTCCAGAG (SEQ ID NO: 53)
Hu_Vec_R1	AAAGCATCCTTCTTGGGTCAGG (SEQ ID NO: 54)
SP42-ins_F1	caagaaggatgctttGTGCCCGTGACTACGCG (SEQ ID NO: 55)
SP42-ins_R1	gaccagctggacctgTGCCAGCAAGGTGCAGAG (SEQ ID NO: 56)
SP45-ins_F1	caagaaggatgctttATGAAGCACCTCTCCCACCG (SEQ ID NO: 57)
SP45-ins_R1	gaccagctggacctgATCGCCGTCGCTTCCCT (SEQ ID NO: 58)
SP48-ins_F1	caagaaggatgctttGTGCTGATTCTCATCGTTCTCG (SEQ ID NO: 59)
SP48-ins_R1	gaccagctggacctgGAGCGCGTTCAAAGTGTCG (SEQ ID NO: 60)
SP50-ins_F1	caagaaggatgctttATGATCGTGGCCTACCCG (SEQ ID NO: 61)
SP50-ins_R1	gaccagctggacctgTTCCATATCGTTGTATGGAAACGC (SEQ ID NO: 62)
SP52-ins_F1	caagaaggatgctttATGAGTTTCCATGTATCCGCG (SEQ ID NO: 63)
SP52-ins_R1	gaccagctggacctgCAGTTCGTTATACGCGTGACCG (SEQ ID NO: 64)
SP55-ins_F1	caagaaggatgctttATGGTTCGTCGCGCCCA (SEQ ID NO: 65)
SP55-ins_R1	gaccagctggacctgCCAGGTGGCGTAGTCAACG (SEQ ID NO: 66)
SP58-ins_F1	caagaaggatgctttATGGCAATGGCACGGC (SEQ ID NO: 67)
SP58-ins_R1	gaccagctggacctgGGTGGTTTGCCCGTTGATG (SEQ ID NO: 68)
SP62-ins_F1	caagaaggatgctttATGACTCGTTCGGACGACGT (SEQ ID NO: 69)
SP62-ins_R1	gaccagctggacctgCATGTTGAACATCGAACGTTTGG (SEQ ID NO: 70)
SP64-ins_F1	caagaaggatgctttATGAAGTCACTAATCAGGAATGTAGCG (SEQ ID NO: 71)
SP64-ins_R1	gaccagctggacctgCTTCTTGCCGTTGAACGCG (SEQ ID NO: 72)
SP66-ins_F1	caagaaggatgctttGTGAAGCATTGGAAGAAGATGGC (SEQ ID NO: 73)
SP66-ins_R1	gaccagctggacctgCTTAGTAGTCTGCACTGTCGCGG (SEQ ID NO: 74)
SP67-ins_F1	caagaaggatgctttATGAAGATAAACAATAAGGGCAAGG (SEQ ID NO: 75)
SP67-ins_R1	gaccagctggacctgGGGCTGGAACTTGGTGTATGTC (SEQ ID NO: 76)
SP68-ins_F1	caagaaggatgctttATGGTTTATAACATTCACATATTGCAAAC (SEQ ID NO: 77)
SP68-ins_R1	gaccagctggacctgCTTCCAACCATTAAGATCGTCTTCG (SEQ ID NO: 78)
SP69-ins_F1	caagaaggatgctttATGAATTATTTACGACAAAAAATTTCGG (SEQ ID NO: 79)
SP69-ins_R1	gaccagctggacctgACCGCTATCAGTCGTGGTGTAAC (SEQ ID NO: 80)

[0095]

[Selection of secretory signal peptide sequence and subsequent linker peptide sequence]

The whole amino acid sequence of a protein derived from *Bifidobacterium longum* NCC2705 strain registered on the NCBI genomic database (http://www.ncbi.nlm.nih.gov/genome/) was analyzed by signal sequence estimation program Signal P 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) for use in

estimating the presence or absence of a secretory signal. As a result of the analysis, proteins estimated to have a secretory signal peptide sequence at the N terminal were selected and a part of the amino acid sequences of the proteins was subjected to a topology analysis using program TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) estimating a transmembrane region (TM). As a result of the topology analysis, 33 types of proteins were selected as the proteins estimated to have TM at the N terminal. These 33 types of proteins were selected as secretory signal peptides derived from *Bifidobacterium longum* NCC2705 strain and estimated to have TM. In the amino acid sequences of *Bifidobacterium longum* 105A strain corresponding to the amino acid sequences of the 33 types of peptides selected, the sequence from the N terminal of each of the amino acid sequences (of the proteins) up to the amino acid which is estimated as a protease cleavage putative site by Signal P analysis was determined as a secretory signal peptide sequence. Furthermore, 20 amino acid residues following from the C terminal of each of the signal sequences was specified as the linker peptide sequence.

[0096]

[Preparation of plasmid, pHuSPxL20-scFv-PD-1-1]

(Preparation of SPxL20 insert)

PCR amplification was performed by using *Bifidobacterium longum* 105-A strain (120 pg) as a template and each of the primer sets listed in Table 4 and Table 5. Individual primer sequences were designed such that an

insert fragment and a vector fragment were overlapped at the end of 15 bp. The PCR amplification was performed by setting a primer concentration at 0.2 μM and the reaction volume at 20 μL and using PrimeSTAR HS (Premix) kit. As an amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 65°C for 5 seconds and a reaction at 72°C for 20 seconds was repeated 30 times, followed by an elongation reaction at 72°C for 30 seconds. In this manner, inserts consisting of 33 types of secretory signal peptide-linker conjugates were obtained by amplification.

[0097]

(Preparation of vector)

PCR amplification was performed by using pHuSP7L20-scFv-PD-1-1 (2.5 ng) mentioned above as a template, and a primer set consisting of PD-1-scFv_vec_F1 primer and Hu_Vec_R1 primer listed in Table 4 and Table 5 above, setting a final concentration at 0.2 μM and the reaction volume at 50 μL, and using PrimeSTAR HS (Premix) kit. As an amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 65°C for 5 seconds and a reaction at 72°C for 4 minutes and 45 seconds was repeated 30 times followed by an elongation reaction at 72°C for 30 seconds. In this manner, a vector fragment, 5'-scFv-PD-1-1-Hu terminator-pTB6rep unit-SPCMr-pUCori-Hu promoter-3', was prepared.

[0098]

(Infusion reaction)

The vector fragment prepared in the above and each of 33 types secretory signal peptide-linker conjugate

inserts were ligated by use of In-Fusion (registered trademark) HD Cloning Kit. More specifically, in a microtube, the vector and an insert were added at a molar ratio of 1: 2, and 5 × In-Fusion HD Enzyme premix (2 μL) provided in the kit was added. The volume of the mixture was adjusted with 0.1 × TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH7.5) so as to be 10 μL. This solution was kept warm at 37°C for 15 minutes and further kept warm at 50°C for 15 minutes. The procedure as described in the instruction of the kit except the aforementioned procedure was performed to prepare 33 types of infusion reaction solutions.

[0099]

(Transformation of *E. coli* and sequencing of plasmid DNA)

Using 33 types of infusion reaction solutions (0.8 μL) mentioned above, *E. coli* HST16CR competent cells (manufactured by Takara Bio Inc.) were separately transformed. After transformation, each of the bacterial suspensions was spread onto a 75 μg/mL spectinomycin-containing LB agar medium and subjected to static culture at 37°C overnight. The transformation was performed in accordance with the conditions described in the instruction of *E. coli* HST16CR competent cell as outlined below. *E. coli* colonies formed on the agar medium were cultured in a 75 μg/mL spectinomycin-containing LB liquid medium at 37°C overnight. From the culture medium, a plasmid was extracted by use of QIAprep Spin Miniprep Kit. The anti-PD-1-1 scFv antibody expression cassette (containing Hu promoter to Hu terminator) of the extracted

plasmid was subjected to a sequencing reaction by BigDye (registered trademark) Terminator v3.1 Cycle Sequencing Kit for determining the sequence thereof. The extracted plasmid was designated as pHuSPxL20-scFv-PD-1-1 (where x = 42 to 46, 48 to 75).

[0100]

(Transformation of bacterium of the genus *Bifidobacterium*)

Using DNA (500 ng) of each of the plasmids represented by pHuSPxL20-scFv-PD-1-1 (where x = 42 to 46, 48 to 75) extracted from the *E. coli* transformed above, *Bifidobacterium longum* 105-A strain was transformed in the same manner as in the above section: [Preparation of plasmid, pHuSP3L22-scFv-PD-1-1], (Transformation of bacterium of the genus *Bifidobacterium*) to obtain *Bifidobacterium longum* 105-A/pHuSPxL20-scFv-PD-1-1 (where x =42 to 46, 48 to 75) strain.

[0101]

[Example 4]

(Second Investigation of the presence or absence of secretion of scFv antibody in recombinant bacterium of the genus *Bifidobacterium*)

Whether each of the recombinant *Bifidobacterium* bacterial strains obtained in Example 3, and represented by *Bifidobacterium longum* 105-A/pHuSPxL20-scFv-PD-1-1 (where x =42 to 46, 48 to 75) secrets anti-PD-1-1 scFv antibody was checked by Western Analysis in the same manner as in the above (First investigation of the presence or absence of secretion of scFv antibody in

recombinant bacterium of the genus *Bifidobacterium*). The results are shown in Figure 5.

[0102]

As is apparent from Figure 5, secretion was observed in *Bifidobacterium longum* 105-A strains transformed with anti-PD-1-1 scFv antibody secretory plasmids in which 11 types of secretory signal peptide-linker conjugates (SP7L20, SP45L20, SP50L20, SP52L20, SP55L20, SP58L20, SP64L20, SP66L20, SP67L20, SP68L20, SP69L20) were respectively inserted. In contrast, secretion of an anti-PD-1-1 scFv antibody was not observed in bacterium of the genus *Bifidobacterium* transformed with plasmids in which secretory signal peptide-linker conjugates (SP42L20, SP48L20, SP62L20, (which respectively consist of secretory signal peptide sequences (SP42, SP48, SP62) and subsequent linker peptide sequences of 20 amino acid residues) were inserted.

[0103]

From the above investigation, it was found that the bacteria of the genus *Bifidobacterium* that can secrete an scFv antibody were only those transformed by using the expression cassettes containing SP45L20, SP50L20, SP52L20, SP55L20, SP58L20, SP64L20, SP66L20, SP67L20, SP68L20 and SP69L20, and that secretion was not observed or insufficient in the other cases (23 types of sequences). Examples of secretory signal peptide-linker conjugates as to which secretion was not observed or insufficient are SP42L20, SP48L20 and SP62L20 and the sequences of them are represented by SEQ ID Nos: 17, 18 and 19.

[0104]

[Example 5]

[Preparation of pHuSP7L20-scFv-PD-1-2]

Using the anti-PD-1-2 scFv antibody as a heterogeneous peptide, an anti-PD-1-2 scFv antibody secretory plasmid, pHuSP7L20-scFv-PD-1-2 having a secretory signal peptide-linker conjugate, SP7L20, was constructed. The details of the construction are as follows.

[0105]

(Preparation of anti-PD-1-2 scFv antibody insert)

PCR amplification was performed by using the artificial plasmid, pUC57-scFv-PD-1-2 (500 pg) synthesized above as a template and a primer set consisting of Ins_PD-1-2_F1 primer and Ins_PD-1-2_R1 primer listed in Table 1 and Table 2 above. The primer sequences were designed such that an insert fragment and a vector fragment were mutually overlapped at the end of 15 bp. The PCR reaction solution used herein was prepared so as to contain a primer in a concentration of 0.2 μM in a reaction volume of 30 μL by using PrimeSTAR HS (Premix) kit. As an amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 72°C for 60 seconds was repeated 30 times. The PCR product obtained by the amplification was subjected to electrophoresis on 2% agarose gel and purified by QIAquick Gel Extraction Kit to prepare an anti-PD-1-2 scFv antibody insert.

[0106]

(Preparation of vector fragment)

PCR amplification was performed by using a linearized vector fragment (500 pg) represented by SEQ ID No: 23 as a template and a primer set consisting of TGA_Hu_Terminator_F primer and vec-SP7L20-PD-1-2_R1 primer listed in Table 1 and Table 2 above, setting a final concentration at 0.2 μM and the reaction volume at 30 μL and using PrimeSTAR HS (Premix) kit. As an amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 72°C for 5 minutes was repeated 30 times. The PCR product obtained by the amplification was subjected to electrophoresis on 0.8% agarose gel and purified by QIAquick Gel Extraction Kit to prepare a 5'-Hu terminator-pTB6rep unit-SPCMr-pUCori-Hu promoter-SP7L20-3' vector fragment (2).

[0107]

The linearized vector fragment is the nucleotide sequence consisting of the 4th nucleotide to the 3845th nucleotide of the nucleotide sequence represented by SEQ ID No: 23 and constituted of:

Hu terminator: the nucleotide sequence consisting of the 4th nucleotide to the 117th nucleotide of the nucleotide sequence represented by SEQ ID No: 23;

Origin of replication pTB6 rep unit of bacterium of the genus *Bifidobacterium*: the nucleotide sequence consisting of the 124th nucleotide to the 1719th nucleotide of the nucleotide sequence represented by SEQ ID No: 23;

Spectinomycin resistant gene, SPCMr: the nucleotide sequence consisting of the 1726th nucleotide to the 2804th

nucleotide of the nucleotide sequence represented by SEQ ID No: 23;

Origin of replication of *E. coli*, pUCori: the nucleotide sequence consisting of the 2811st nucleotide to the 3478th nucleotide of the nucleotide sequence represented by SEQ ID No: 23; and

Hu promoter: the nucleotide sequence consisting of the 3485th nucleotide to the 3845th nucleotide of the nucleotide sequence represented by SEQ ID No: 23.

[0108]

(Infusion reaction)

An infusion reaction solution was prepared in the same procedure as in the infusion reaction in the above section: [Preparation of plasmid, pHuSP3L20-scFv-PD-1-1] except that the vector fragment and anti-PD-1-2 scFv antibody insert prepared above were used.

[0109]

(Transformation of *E. coli* and sequencing of plasmid DNA)

E. coli HST08 competent cell was transformed in the same procedure as in Example 1, [Preparation of plasmid, pHuSP3L20-scFv-PD-1-1], (Transformation of *E. coli* and sequencing of plasmid DNA) except that the infusion reaction solution (5 μL) prepared above was used. The anti-PD-1-2 scFv antibody expression cassette (containing Hu promoter to Hu terminator) of the plasmid extracted was subjected to a sequencing reaction for determining the sequence thereof. The extracted plasmid was designated as pHuSP7L20-scFv-PD-1-2.

[0110]

(Transformation of bacterium of the genus *Bifidobacterium*)

Using DNA of a plasmid, pHuSP7L20-scFv-PD-1-2 (1000 ng) extracted from *E. coli* transformed above, *Bifidobacterium longum* 105-A strain was transformed in the same procedure as in the above section: [Preparation of plasmid, pHuSP3L22-scFv-PD-1-1], (Transformation of bacterium of the genus *Bifidobacterium*) to obtain *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-2 strain.

[0111]

[Example 6]

[Preparation of pHuSP7L20-scFv-PD-1-3]

An anti-PD-1-3 scFv antibody was selected as a heterogeneous peptide and an anti-PD-1-3 scFv antibody secretory plasmid, pHuSP7L20-scFv-PD-1-3 having a secretory signal peptide-linker conjugate, SP7L20 was constructed. The details of the construction are as follows.

[0112]

(Preparation of anti-PD-1-3 scFv antibody insert)

An anti-PD-1-3 scFv antibody insert was prepared in the same procedure as in the above section: [Preparation of pHuSP7L20-scFv-PD-1-2], (Preparation of an anti-PD-1-2 scFv antibody insert) except that the artificial plasmid, pUC57-scFv-PD-1-3 (500 pg) synthesized above was used as a template and a primer set consisting of Ins_PD-1-3_F1 primer and Ins_PD-1-3_R1 primer listed in Table 1 and Table 2 was used.

[0113]

(Preparation of vector fragment)

A 5'-Hu terminator-pTB6rep unit-SPCMr-pUCori-Hu promoter-SP7L20-3' vector fragment (3) was prepared in the same procedure in [Preparation of pHuSP7L20-scFv-PD-1-2], (Preparation of vector fragment) except that the linearized vector fragment (500 pg) represented by SEQ ID No: 24 was used as a template and a primer set consisting of TGA_Hu_Terminator_F primer and vec-SP7L20-PD-1-3_R1 primer listed in Table 1 and Table 2 above was used.

[0114]

The linearized vector fragment is the nucleotide sequence consisting of the 4th nucleotide to the 3845th nucleotide of the nucleotide sequence represented by SEQ ID No: 24 and constituted of:

Hu terminator: the nucleotide sequence consisting of the 4th nucleotide to the 117th nucleotide of the nucleotide sequence represented by SEQ ID No: 24;

Origin of replication pTB6 rep unit of bacterium of the genus *Bifidobacterium*: the nucleotide sequence consisting of the 124th nucleotide to the 1719th nucleotide of the nucleotide sequence represented by SEQ ID No: 24;

Spectinomycin resistant gene, SPCMr: the nucleotide sequence consisting of the 1726th nucleotide to the 2804th nucleotide of the nucleotide sequence represented by SEQ ID No: 24;

Origin of replication of *E. coli*, pUCori: the nucleotide sequence consisting of the 2811st nucleotide to the 3478th nucleotide of the nucleotide sequence represented by SEQ ID No: 24; and

Hu promoter: the nucleotide sequence consisting of the 3485th nucleotide to the 3845th nucleotide of the nucleotide sequence represented by SEQ ID No: 24.

[0115]

(Infusion reaction)

An infusion reaction solution was prepared in the same procedure as in the infusion reaction in the above section: [Preparation of plasmid, pHuSP3L20-scFv-PD-1-1] except that the vector fragment and the anti-PD-1-3 scFv antibody insert prepared above were used.

[0116]

(Transformation of *E. coli* and sequencing of plasmid DNA)

E. coli HST08 competent cell was transformed in the same procedure as in Example 1, [Preparation of plasmid, pHuSP3L20-scFv-PD-1-1], (Transformation of *E. coli* and sequencing of plasmid DNA) except that the infusion reaction solution (5 μL) prepared above was used. The anti-PD-1-3 scFv antibody expression cassette (containing Hu promoter to Hu terminator) of the plasmid extracted was subjected to a sequencing reaction for determining the sequence thereof. The extracted plasmid was designated as pHuSP7L20-scFv-PD-1-3.

[0117]

(Transformation of bacterium of the genus *Bifidobacterium*)

Using DNA of a plasmid, pHuSP7L20-scFv-PD-1-3 (1000 ng) extracted from *E. coli* transformed above, *Bifidobacterium longum* 105-A strain was transformed in the same procedure as in the above section: [Preparation of

plasmid, pHuSP3L22-scFv-PD-1-1], (Transformation of bacterium of the genus *Bifidobacterium*) to obtain *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-3 strain.

[0118]

[Example 7]

[Preparation of pHuSP7L20-scFv-CTLA-4-1]

An anti-CTLA-4-1 scFv antibody secretory plasmid, pHuSP7L20-scFv-CTLA-4-1, having a secretory signal peptide-linker conjugate, SP7L20, was constructed.

How to prepare the anti-CTLA-4 scFv antibody secretory plasmid is schematically shown in Figure 2. On the left side of Figure 2, scFv-gene is CTLA-4. In preparation of pHuSP7L20-scFv-CTLA-4-1, x = 7 and m = 1 and in the preparation of pHuSP7L20-scFv-CTLA-4-2, x = 7 and m = 2. The details of the construction are as follows.

[0119]

[Expression cassette for anti-CTLA-4 scFv antibody]

An Expression cassette for anti-CTLA-4 scFv antibody is, as shown in Figure 1(b), constituted of Hu promoter DNA (derived from *Bifidobacterium longum*), a DNA encoding a secretory signal peptide, a DNA encoding a linker peptide following the secretory signal peptide, a DNA encoding an anti-CTLA-4 scFv antibody (containing a heavy chain sequence, linker $((GGGGS)_3)$ and a light chain sequence), a His tag sequence and a Hu terminator (derived from *Bifidobacterium longum*). As the anti-CTLA-4 scFv antibody (containing nucleotide sequence containing a heavy chain sequence, a linker $((GGGGS)_3)$ and a light chain sequence) in each of individual expression cassettes, two types of anti-human CTLA-4 scFv antibodies as shown

below was used, with reference to the document listed in Table 6 below.

[0120]

[Table 6]

Antibody		Reference document
scFv-CTLA-4-1	SEQ ID No: 4 (765 nucleotide sequence) SEQ ID No: 141 (254 amino acids) Heavy chain sequence (1-378 nucleotides) $(GGGGS)_3$ linker (379-423 nucleotides) Light chain sequence (424-744 nucleotides) Histidine tag (745-762 nucleotides)	Japanese Patent No. 3793693
scFv-CTLA-4-2	SEQ ID No: 5 (747 nucleotide sequence) SEQ ID No: 142 (248 amino acids) Heavy chain sequence (1-357 nucleotides) $(GGGGS)_3$ linker (358-402 nucleotides) Light chain sequence (403-726 nucleotides) Histidine tag (727-744 nucleotides)	Japanese Patent No. 4093757

[0121]

The scFv antibody genes, namely, scFv-CTLA-4-1 represented by SEQ ID No: 4 and scFv-CTLA-4-2 represented by SEQ ID No: 5, were separately subcloned in plasmids for *E. coli* pUC57 by GenScript Japan Inc. In this manner, artificial plasmids pUC57-scFv-CTLA-4-1 and pUC57-scFv-CTLA-4-2 were obtained by gene synthesis.

[0122]

(Preparation of anti-CTLA-4-1 scFv antibody insert)

PCR amplification was performed by using the plasmid, pUC57-scFv-CTLA-4-1 (500 pg) as a template, and a primer set consisting of Ins_CTLA-4-1_F1 primer and Ins_CTLA-4-1_R1 primer listed in Table 1 and Table 2 above. The primer sequences were designed such that an insert

fragment and a vector fragment were mutually overlapped at the end of 15 bp. The amplification was performed by PrimeSTAR HS (Premix) (manufactured by Takara Bio Inc.) kit using a PCR reaction solution, which was prepared so as to satisfy a primer concentration of 0.2 μM and a reaction volume of 30 μL. As an amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 72°C for 60 seconds was repeated 30 times. The insert PCR product obtained by the amplification was subjected to electrophoresis on 2% agarose gel and purified by QIAquick Gel Extraction Kit to prepare an anti-CTLA-4-1 scFv antibody insert.

[0123]

(Preparation of vector fragment)

A 5'-Hu terminator-pTB6rep unit-SPCMr-pUCori-Hu promoter-SP7L20-3' vector fragment (1) was prepared in the same procedure as in the above section: [Preparation of pHuSP7L20-scFv-PD-1-1], (Preparation of vector fragment).

[0124]

(Infusion reaction)

An infusion reaction solution was prepared in the same procedure as in the infusion reaction in the above section: [Preparation of plasmid, pHuSP3L20-scFv-PD-1-1] except that the vector fragment and anti-CTLA-4-1 scFv antibody insert prepared above were used.

[0125]

(Transformation of *E. coli* and sequencing of plasmid DNA)

E. coli HST08 competent cell was transformed in the same procedure as in the above section: [Preparation of plasmid, pHuSP3L20-scFv-PD-1-1], (Transformation of *E. coli* and sequencing of plasmid DNA) except that the infusion reaction solution (5 μL) prepared above was used. The anti-CTLA-4-1 scFv antibody expression cassette (containing Hu promoter to Hu terminator) of the plasmid extracted was subjected to a sequencing reaction for determining the sequence thereof. The extracted plasmid was designated as pHuSP7L20-scFv-CTLA-4-1.

[0126]

(Transformation of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed in the same procedure as in the above section: [Preparation of pHuSP3L22-scFv-PD-1-1], (Transformation of bacterium of the genus *Bifidobacterium*) except that DNA of a plasmid, pHuSP7L20-scFv-CTLA-4-1 (1000 ng), extracted from *E. coli* transformed above was used to obtain *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-CTLA-4-1 strain.

[0127]

[Example 8]

[Preparation of pHuSP7L20-scFv-CTLA-4-2]

An anti-CTLA-4-2 scFv antibody secretory plasmid, pHuSP7L20-scFv-CTLA-4-2, having a secretory signal peptide-linker conjugate (SP7L20) was constructed. The details of the construction are as follows.

[0128]

(Preparation of anti-CTLA-4-2 scFv antibody insert)

An anti-CTLA-4-2 scFv antibody insert was purified in the same procedure as in the above section: (Preparation of anti-CTLA-4-1 scFv antibody insert) except that the plasmid, pUC57-scFv-CTLA-4-2 obtained above was used as a template and a primer set consisting of Ins_CTLA-4-1_F1 primer and Ins_CTLA-4-2_R1 primer set listed in Table 1 and Table 2 above was used.

[0129]

(Preparation of vector)

A 5'-Hu terminator-pTB6rep unit-SPCMr-pUCori-Hu promoter-SP7L20-3' vector fragment (1) was prepared in the same procedure as in the above section: [Preparation of pHuSP7L20-scFv-PD-1-1], (Preparation of vector fragment).

[0130]

(Infusion reaction)

An infusion reaction solution was prepared in the same procedure as in the infusion reaction in the above section: [Preparation of plasmid, pHuSP3L20-scFv-PD-1-1] except that the vector fragment and the anti-CTLA-4-2 scFv antibody insert prepared above were used.

[0131]

(Transformation of *E. coli* and sequencing of plasmid DNA)

E. coli HST08 competent cell was transformed in the same procedure as in Example 1, [Preparation of plasmid, pHuSP3L20-scFv-PD-1-1], (Transformation of *E. coli* and sequencing of plasmid DNA) except that the infusion reaction solution (5 μL) prepared above was used. The anti-CTLA-4-2 scFv antibody expression cassette (containing Hu promoter to Hu terminator) of the plasmid

extracted was subjected to a sequencing reaction for determining the sequence thereof. The extracted plasmid was designated as pHuSP7L20-scFv-CTLA-4-2.

[0132]

(Transformation of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed in the same procedure as in the above section: [Preparation of pHuSP3L22-scFv-PD-1-1], (Transformation of bacterium of the genus *Bifidobacterium*) except that a plasmid, pHuSP7L20-scFv-CTLA-4-2 (1000 ng) extracted from *E. coli* transformed above was used to obtain *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-CTLA-4-2 strain.

[0133]

[Example 9]

(Third Investigation of the presence or absence of secretion of scFv antibody in recombinant bacterium of the genus *Bifidobacterium*)

Whether the recombinant *Bifidobacterium* bacteria transformed with an anti-PD-1 scFv antibody secretory plasmid and an anti-CTLA-4 scFv antibody secretory plasmid, namely *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-1 strain, *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-2 strain, *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-3 strain, *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-CTLA-4-1 strain and *Bifidobacterium longum* and 105-A/pHuSP7L20-scFv-CTLA-4-2 strain, secrete anti-PD-1 scFv antibody and anti-CTLA-4 scFv antibody was checked by Western Analysis in the same manner as in the above (First investigation of

the presence or absence of secretion of scFv antibody in recombinant bacterium of the genus *Bifidobacterium*). Note that culture time was set at 15 hours. The results are shown in Figure 6.

[0134]

(Results)

As is apparent from Figure 6, an anti-PD-1-1 scFv antibody was secreted in the culture supernatant of *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-1 strain; an anti-PD-1-2 scFv antibody was secreted in the culture supernatant of *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-2 strain; an anti-PD-1-3 scFv antibody was secreted in the culture supernatant of *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-3 strain; anti-CTLA-4-1 scFv antibody was secreted in the culture supernatant of *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-CTLA-4-1 strain; and anti-CTLA-4-2 scFv antibody was secreted in the culture supernatant of *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-CTLA-4-2 strain.

[0135]

[Example 10]

[Purification of anti-PD-1 antibody and anti-CTLA-4 scFv antibody from recombinant bacterium of the genus *Bifidobacterium* strain]

A scFv antibody was purified from each of *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-1 strain, *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-2 strain, *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-3 strain, *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-CTLA-4-1

strain and *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-CTLA-4-2 strain, by the method shown below.

[0136]

Each of the recombinant bacterium of the genus *Bifidobacterium* strains was inoculated in MRS culture medium (10 mL) supplemented with spectinomycin (a final concentration of 75 μg/mL) and a vitamin C-added solution (100 μL) and anaerobically cultured at 37°C for 24 hours. A culture medium containing DMEM: MRS (9: 1) was prepared by adding spectinomycin (a final concentration of 75 μg/mL) and a vitamin C-added solution (500 μL per culture medium 100 mL). To this medium, the culture solution obtained above (0.5% of the amount of the medium) was inoculated and anaerobically cultured at 37°C for 18 hours.

After the anaerobic culture, the culture supernatant was obtained by centrifugation. To the culture supernatant, ammonium sulfate was added little by little while stirring the culture supernatant so as to obtain a saturation of 80%. The solution obtained was stirred at 4°C overnight to perform salting out. The solution was centrifuged and the precipitate was recovered. A histidine tag fused protein was purified by a purification kit for a histidine tagged protein (TALON resin, manufactured by Takara Bio Inc.). The solution of the purified protein was concentrated by ultrapurification (Amicon Ultra-0.5, manufactured by Merck Millipore Corporation). The concentration of the purified protein was measured by the Bradford method (Coomassie Plus Protein Assay manufactured by Thermo Scientific). The purified scFv antibody was aliquoted, subjected to SDS-

PAGE and then stained with Coomassie brilliant blue (SimplyBlue (registered trademark) Safe Stain, manufactured by Life Technologies). As a result, it was verified that each of the scFv antibodies, namely, scFv-PD-1-1, scFv-PD-1-2, scFv-PD-1-3, scFv-CTLA4-1 and scFv-CTLA4-2, is purified with a purity of about 90%.

[0137]

[Example 11]

[Verification of the presence or absence of binding between human PD-1 and anti-PD-1 scFv antibody or anti-CTLA-4 scFv antibody]

Whether the anti-PD-1-2 scFv antibody, which was purified from the culture supernatant of *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-2 strain, binds to human PD-1 (hPD-1) was checked by ELISA.

[0138]

To 96-well plates, 100 μL of hPD-1 (Recombinant Human PD-1 Fc Chimera, manufactured by R&D Systems, Inc.) adjusted to be 1 μg/mL with 1 × PBS was dispensed and incubated at 4°C overnight to immobilize. After the solution was removed, 350 μL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing. In the above plates, 350 μL of a 1% BSA solution was dispensed and incubated at room temperature for 2 hours to carry out blocking. After removing the solution, 350 μL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0139]

The anti-PD-1-2 scFv antibody was adjusted so as to have a concentration of 1000 ng/mL, 100 ng/mL, 10 ng/mL and 1 ng/mL with a signal enhancing reagent (Signal Enhancer HIKARI, manufactured by Nacalai Tesque). Each solution (100 µL) was dispensed in the plates to which blocking treatment was already applied. An anti-CTLA-4-1 scFv antibody and an anti-CTLA-4-2 scFv antibody purified from a bacterium of the genus *Bifidobacterium* were subjected to the same operation as above and used as a negative control(s). To a blank well, a signal enhancing reagent (100 µL) alone was dispensed. After the plates were sealed and incubated at room temperature for 2 hours, immobilized hPD-1 was allowed to react with each of the scFv antibodies. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0140]

A secondary antibody (The His-Tag Antibody, manufactured by GenScript) was diluted 2500 fold with a signal enhancing reagent. The dilution solution (100 µL) was dispensed in the plates. The plates were sealed and incubated at room temperature for 2 hours. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0141]

A tertiary antibody (Biotin anti-mouse IgG, manufactured by Biolegend) was diluted 20,000 fold with a signal enhancing reagent. The dilution solution (100 µL)

was dispensed in the plates. The plates were sealed and incubated at room temperature for 30 minutes. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing. An avidin-biotinylated enzyme complex (Vectastain ABC Kit, manufactured by Vector), more specifically, three drops of each of Solution A and Solution B, were added to a signal enhancing reagent (7.5 mL). This solution (100 µL) was dispensed in the plates and the plates were sealed and incubated at room temperature for 30 minutes. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0142]

Color Solution A and Color Solution B (manufactured by R&D Systems) serving as a detection reagent were mixed in equal amounts. This solution (200 µL) was dispensed in the plates. The plates were sealed and incubated at room temperature for 20 minutes. Accurately 20 minutes later, 50 µL of a Stop solution (manufactured by R&D Systems) was each added to terminate a color reaction. Absorbance at 450 nm was measured, whereas absorbance at 570 nm was measured as a control. The results of the presence or absence of binding of the anti-PD-1-2 scFv antibody, anti-CTLA-4-1 scFv antibody and anti-CTLA-4-2 scFv antibody to a human PD-1 immobilized plates are shown in Table 7 below and Figure 7.

[0143]

[Table 7]

Antibody concentration	Anti-PD-1-2 scFv antibody (scFvPD−1−2)	Anti-CTLA-4-1 scFv antibody (scFvCTLA−4−1)	Anti-CTLA-4-2 scFv antibody (scFvCTLA−4−2)
1000ng/mL	2.816	-0.038	-0.022
100ng/mL	3.062	-0.117	-0.151
10ng/mL	0.912	-0.106	-0.153
1ng/mL	0.058	-0.092	-0.161

[0144]

(Results)

As is apparent from Table 7 and Figure 7, the anti-PD-1-2 scFv antibody purified from a bacterium of the genus *Bifidobacterium* bound to hPD-1 in a concentration dependent manner. In contrast, the anti-CTLA-4-1 scFv antibody and anti-CTLA-4-2 scFv antibody similarly purified from a bacterium of the genus *Bifidobacterium* did not bind to hPD-1. From the results, it was verified that the anti-PD-1-2 scFv antibody (anti-human PD-1 antibody) secreted from the bacterium of the genus *Bifidobacterium* specifically binds to hPD-1.

[0145]

[Example 12]

[Verification of the presence or absence of binding between human CTLA-4 and anti-PD-1 scFv antibody or anti-CTLA-4 scFv antibody]

Using the anti-PD-1-2 scFv antibody, an anti-CTLA-4-1 scFv antibody and an anti-CTLA-4-2 scFv antibody purified from the above culture supernatants, whether the antibodies bind to human CTLA-4 (hCTLA-4) (Recombinant

Human CTLA-4-Fc Chimera, carrier-free, manufactured by BioLegend, Inc.) was checked in the same procedure as in the above section: [Verification of the presence or absence of binding between human PD-1 and anti-PD-1 scFv antibody or anti-CTLA-4 scFv antibody]. The results of the presence or absence of binding of the anti-PD-1-2 scFv antibody, anti-CTLA-4-1 scFv antibody and anti-CTLA-4-2 scFv antibody to human CTLA-4 immobilized plates are shown in Table 8 below and Figure 8.

[0146]

[Table 8]

Antibody concentration	Anti-PD-1-2 scFv antibody (scFv PD-1-2)	Anti-CTLA-4-1 scFv antibody (scFv CTLA-4-1)	Anti-CTLA-4-2 scFv antibody (scFv CTLA-4-2)
1000ng/mL	0.649	2.240	2.967
100ng/mL	0.085	1.528	1.643
10ng/mL	-0.006	0.365	0.404
1ng/mL	-0.012	0.059	0.028

[0147]

(Results)

As is apparent from Table 8 and Figure 8, the anti-CTLA-4-1 scFv antibody and anti-CTLA-4-2 scFv antibody bound to hCTLA-4 in a concentration dependent manner. In contrast, the anti-PD-1-2 scFv antibody similarly purified from the bacterium of the genus *Bifidobacterium* did not bind to hCTLA-4. From the results, it was verified that the anti-CTLA-4-1 scFv antibody and anti-CTLA-4-2 scFv antibody (both are anti-human CTLA-4 antibodies) secreted

from a bacterium of the genus *Bifidobacterium* specifically bind to hCTLA-4.

[0148]

[Example 13]

(First verification of the presence or absence of binding between mouse PD-1 and anti-PD-1-3 scFv antibody)

Whether the anti-PD-1-3 scFv antibody purified from the culture supernatant of the *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-3 strain binds to mouse PD-1 (mPD-1) was checked by ELISA.

[0149]

To 96 well plates, 100 µL of mPD-1 (Recombinant Mouse PD-1 Fc Chimera, manufactured by R&D Systems) adjusted to be 1 µg/mL with 1 × PBS was dispensed and incubated at 4°C overnight to immobilize. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing. In the above plates, 350 µL of a 1% BSA solution was dispensed and incubated at room temperature for 2 hours to perform blocking. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0150]

The anti-PD-1-3 scFv antibody purified from a bacterium of the genus *Bifidobacterium* was diluted with a signal enhancing reagent (Signal Enhancer HIKARI, manufactured by Nacalai Tesque) to prepare solutions having a concentration of 1000 ng/mL, 100 ng/mL and 10 ng/mL. Each solution (100 µL) was dispensed in the plates

to which blocking treatment was already applied. An anti-PD-1-2 scFv antibody purified from a bacterium of the genus *Bifidobacterium* and anti-CTLA-4-2 scFv antibody purified from a bacterium of the genus *Bifidobacterium* were subjected to the same operation as above and used as a control. To blank wells, a signal enhancing reagent (100 μL) alone was dispensed. After the plates were sealed and incubated at room temperature for 2 hours, immobilized mPD-1 was allowed to react with the anti-PD-1-3 scFv antibody. After the solution was removed, 350 μL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0151]

A secondary antibody (Anti-His-tag mAb-Biotin, MBL) was diluted 2,000 fold with a signal enhancing reagent. The dilution solution (100 μL) was dispensed to the plates. The plates were sealed and incubated at room temperature for 2 hours. After the solution was removed, 350 μL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0152]

Using the avidin-biotinylated enzyme complex, Solution A and Solution B of a kit each (three drops) were added to a signal enhancing reagent (7.5 mL). This solution (100 μL) was dispensed in the plates. The plates were sealed and incubated at room temperature for 30 minutes. After the solution was removed, 350 μL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0153]

Color Solution A and Color Solution B (manufactured by R&D Systems) serving as a detection reagent were mixed in equal amounts. This mixture (200 µL) was dispensed in the plates. The plates were sealed and incubated at room temperature for 20 minutes. Accurately 20 minutes later, 50 µL of a Stop solution (manufactured by R&D Systems) was added to terminate a color reaction.

[0154]

Absorbance at 450 nm was measured, whereas absorbance at 570 nm was measured as a control. The results of the presence or absence of binding of the anti-PD-1-2 scFv antibody, anti-PD-1-3 scFv antibody and anti-CTLA-4-2 scFv antibody to mouse PD-1 immobilized plates are shown in Table 9 and Figure 9.

[0155]

[Table 9]

Antibody concentration	Anti-PD-1-2 scFv antibody (scFv PD-1-2)	Anti-PD-1-3 scFv antibody (scFv PD-1-3)	Anti-CTLA-4-2 scFv antibody (scFv CTLA-4-2)
1000ng/mL	0.014	3.107	0.090
100ng/mL	-0.010	0.561	-0.038
10ng/mL	0.075	0.312	-0.036

[0156]

(Results)

The anti-PD-1-3 scFv antibody bound to mPD-1 in a concentration dependent manner. In contrast, the anti-PD-1-2 scFv antibody and anti-CTLA-4-2 scFv antibody similarly purified did not bind to mPD-1. From the results, it was verified that the anti-PD-1-3 scFv

antibody (anti-mouse PD-1 antibody) secreted from the bacterium of the genus *Bifidobacterium* specifically binds to mPD-1.

[0157]

[Example 14]

(Second verification of the presence or absence of binding between mouse PD-1 and anti-PD-1-3 scFv antibody)

Whether the anti-PD-1-3 scFv antibody purified from the culture supernatant of the *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-3 strain specifically binds to mouse PD-1 (mPD-1) was verified. The same experiment as in [Example 13] was repeated except that hCTLA-4 (Recombinant Human CTLA-4-Fc Chimera carrier-free, manufactured by BioLegend) was used as the protein to be immobilized to the plate. The results are shown in Table 10 and Figure 10.

[0158]

(Results)

As is apparent from Table 10 and Figure 10, a positive control, i.e., anti-CTLA-4-2 scFv antibody bound to hCTLA-4 in a concentration dependent manner but the anti-PD-1-3 scFv antibody did not bind. An anti-human PD-1 antibody, i.e., anti-PD-1-2 scFv antibody, did not bind. From these results, it was also verified that the binding between mPD-1 and the anti-PD-1-3 scFv antibody found in [Example 13] is specific.

[0159]

[Table 10]

Antibody concentration	Anti-PD-1-2 scFv antibody (scFv PD-1-2)	Anti-PD-1-3 scFv antibody (scFv PD-1-3)	Anti-CTLA-4-2 scFv antibody (scFv CTLA-4-2)
1000ng/mL	0.045	0.166	2.984
100ng/mL	-0.031	0.030	1.035
10ng/mL	-0.010	0.004	0.117

[0160]

[Example 15]

(Verification of the presence or absence of binding between anti-PD-1-2 scFv antibody and human PD-1 by flow cytometric analysis)

Binding between an anti-PD-1-2 scFv antibody and human PD-1 over-expressing cells

Experiment was conducted by using Jurkat cells and HEK293T cells (provided by Prof. Shunichiro Taniguchi, Dept. Molecular Oncology, Shinshu University) over-expressing the human PD-1. Human PD-1 over-expressing Jurkat cells were seeded in a 100-mm Petri dish (Ina-optika corporation) at a rate of 5 × 10^6 cells/10 mL culture medium (RPMI1640 culture medium containing inactivated 10% fetal bovine serum and 50 μM 2-mercaptoethanol and 8 mM HEPES)/dish; whereas, human PD-1 over-expressing HEK293T cells were seeded in a 100 mm-dish (manufactured by Greiner Japan) at a rate of 2 × 10^6 cells/10 mL culture medium (DMEM culture medium containing inactivated 10% fetal bovine serum). The following day, human PD-1 over-expressing Jurkat cells were collected in a 15-mL Falcon tube (manufactured by Becton, Dickinson and

Company) and the number of cells were counted by a counting chamber. The cells were dispensed in 1.5 mL-tubes (manufactured by Ina-optika corporation) so as to contain 1×10^5 cells/mL/tube. The human PD-1 over-expressing HEK293T cells, culture supernatant was removed. Cells were washed twice with PBS (Ca^{2+}, Mg^{2+} -free phosphate buffer). 1 mL of a trypsin/EDTA solution (manufactured by Wako Pure Chemical Industries Ltd.) diluted 10 fold with PBS was added. cells were incubated at room temperature for one minute. To this,DMEM culture medium (10 mL) containing inactivated 10% fetal bovine serum, was added. Cells were transferred to a 15 mL Falcon tube, spun by a low-speed centrifuge (manufactured by TOMY SEIKO CO., LTD.) at 1000 rpm for 5 minutes. Thereafter, the supernatant was removed. To this, 1 mL of DMEM culture medium containing inactivated 10% fetal bovine serum was added and the number of cells was counted. DMEM culture medium was further added to prepare a cell suspension having 1×10^5 cells/mL and dispensed to 1.5 mL tubes (Ina-optika corporation) so as to contain 1×10^5 cells/mL/tube.

[0161]

PD-1 over-expressing Jurkat cell and HEK293T cells dispensed to the 1.5 mL tubes were spun by a micro refrigerated centrifuge (manufactured by TOMY SEIKO CO., LTD.) at 5000 rpm and 4°C for one minute. After centrifugation, the supernatants were removed. The remaining cell pellets in the tube were washed twice with 0.5 mL of PBS. An anti-PD-1-2 scFv antibody and LEAF purified anti-human PD-1 antibody (clone: EH12.2H7)

(manufactured by BioLegend) serving as a positive control were respectively added in a concentration of 10 μg/mL and in an amount of 50 μL. Cells were incubated on ice for 30 minutes. FACS buffer (PBS containing 1% BSA and 0.1% NaN_3) was added to a 500 μL per tube and spun by a micro refrigerated centrifuge mentioned above at 5000 rpm and 4°C for one minute. After centrifugation, the supernatants were removed. Biotinylated protein L (50 μL)(manufactured by Pierce) diluted with sterilized ultrapure water to 1 μg/mL was added to the 1.5 mL tube, stirred well by pipetting and incubated on ice for 30 minutes. Thirty minutes later, 500 μL of FACS buffer (PBS containing 1% BSA and 0.1% NaN_3) was added to the tube and spun by the micro refrigerated centrifuge at 5000 rpm and 4°C for one minute. After centrifugation, the supernatants were removed. Brilliant violet 421 streptavidin (50 μL) (manufactured by BioLegend) diluted with FACS buffer to 5 μg/mL was added in a 1.5 mL tube, stirred well by pipetting and incubated on ice for 15 minutes. Fifteen minutes later, centrifugation was performed by the micro refrigerated centrifuge at 5000 rpm and 4°C for one minute. After centrifugation, the supernatants were removed and 500 μL of FACS buffer was added. Then, the cells suspended with FACS buffer were transferred to a 5 mL polystyrene round-bottom tubes (manufactured by Becton, Dickinson and Company). A propidium iodide solution (5 μL) diluted with FACS buffer to 5 μg/mL was added, analysis was performed by BD FACS cantoII flow cytometer (manufactured by Becton, Dickinson and Company) and flow cytometric analysis software Kaluza

ver 1.2 (manufactured by Beckman Coulter, Inc.). The results are shown in Figure 11 and Figure 12.

[0162]

(Results)

As is apparent from Figure 11, it was verified that the anti-PD-1-2 scFv antibody binds to Jurkat cells over-expressing PD-1. In contrast, binding of the anti-PD-1-2 scFv antibody to Jurkat-Mock cells not expressing PD-1 was not observed. Also, it is apparent from Figure 12, binding of the anti-PD-1-2 scFv antibody and anti-human PD-1 antibody (clone: EH12.2H7) to HEK293T-PD-1 cells over-expressing human PD-1 was verified. In contrast, binding of the anti-PD-1-2 scFv antibody and anti-human PD-1 antibody (clone: EH12.2H7) to HEK293T-Mock cell not expressing PD-1 was not verified.

[0163]

[Example 16]

(Verification of competitive inhibitory activity of anti-CTLA-4 scFv antibody against binding of human CTLA-4 and CD80 and CD86 by ELISA)

The anti-CTLA-4-1 scFv antibody and anti-CTLA-4-2 scFv antibody respectively secreted from recombinant bacteria of the genus *Bifidobacterium* prepared by Examples 7 and 8 were used to check the competitive inhibitory activity of these antibodies against binding between human CD80 (hCD80) or human CD86 (hCD86) to human CTLA-4 (hCTLA-4). As a negative control, an anti-PD-1-2 scFv antibody was used.

[0164]

To 96 well plates, 100 μL of hCTLA-4 (Recombinant Human CTLA-4-Fc Chimera, carrier-free, manufactured by BioLegend) adjusted to be 1 μg/mL with 1 × PBS was dispensed and incubated at 4°C overnight to immobilize. After the solution was removed, 350 μL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0165]

In the above plates, 350 μL of 1% BSA solution was dispensed. The plates were incubated at room temperature for 2 hours to carry out blocking. After the solution was removed, 350 μL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing. The concentrations of hCD80 (Recombinant Human B7-1/CD80 Fc Chimera, manufactured by R&D Systems) and hCD86 (Recombinant Human B7-2/CD86 Fc Chimera, manufactured by R&D Systems) were adjusted with a signal enhancing reagent (Signal Enhancer HIKARI, manufactured by Nacalai Tesque) to be 2000 ng/mL.

[0166]

The anti-CTLA-4-1 antibody and anti-CTLA-4-2 scFv antibody purified from bacteria of the genus *Bifidobacterium* each were adjusted with a signal enhancing reagent to have a concentration of 20000 ng/mL, 2000 ng/mL, 200 ng/mL, 20 ng/mL and 2ng/mL and mixed with hCD80 and hCD86 (each was controlled to be 110 μL) in equal amounts. Thereafter, 100 μL of each of the solutions was dispensed to the plates to which blocking treatment was already applied. The anti-PD-1-2 scFv antibody purified from a bacterium of the genus

Bifidobacterium and serving as a negative control was similarly adjusted as mentioned above. To blank wells, 100 µL of a signal enhancing reagent alone was dispersed. The plates were sealed, incubated at room temperature for 2 hours. With the immobilized hCTLA-4, hCD80 and hCD86 mixed with the anti-CTLA-4 scFv antibody and anti-CTLA-4-2 scFv antibody were reacted. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing. A secondary antibody (Human B7-1/CD80 Biotinylated Antibody, manufactured by R&D Systems) against hCD80 and a secondary antibody (Biotinylated Anti-human B7-2 Antibody, manufactured by R&D Systems) against hCD86 were adjusted to have a concentration of 2.5 µg/mL and 0.5 µg/mL with a signal enhancing reagent. This (100 µL) was dispensed. The plates were sealed and incubated at room temperature for 30 minutes. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0167]

An avidin-biotinylated enzyme complex, more specifically, Solution A and Solution B, each (three drops) were added to a signal enhancing reagent (7.5 mL). This (100 µL) was dispensed in the plates and the plates were sealed and incubated at room temperature for 30 minutes. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0168]

Color Solution A and Color Solution B (manufactured by R&D Systems) serving as a detection reagent were mixed in equal amounts. This mixture (200 µL) was dispensed to the plates. The plates were sealed and incubated at room temperature for 20 minutes. Accurately 20 minutes later, 50 µL of a Stop solution (manufactured by R&D Systems) was added to terminate a color reaction. Absorbance at 450 nm was measured, whereas absorbance at 570 nm was measured as a control. The measurement results are shown in Table 11 and Table 12 and the inhibitory rates are shown in Figure 13 and Figure 14.

[0169]

[Table 11]

Competitive binding (antagonistic inhibitory rate) of anti-CTLA-4-1 scFv antibody and anti-CTLA-4-2 scFv antibody to human CTLA-4 (immobilized) with hCD80 by ELISA

Antibody concentration	Absorbance: 450-570 nm (binding inhibition rate %)		
	Anti-CTLA-4-1 scFv antibody (scFv CTLA4-1)	Anti-CTLA-4-2 scFv antibody (scFv CTLA4-2)	Anti-PD-1-2 scFv antibody (scFv PD1-2)
0.001µg/mL	1.807 (10.3%)	1.862 (7.5%)	1.299 (35.5%)
0.01µg/mL	1.546 (23.2%)	2.118 (-5.2%)	1.559 (22.6%)
0.1µg/mL	1.682 (16.5%)	1.116 (44.6%)	1.343 (33.3%)
1µg/mL	0.772 (61.7%)	0.541 (73.1%)	1.466 (27.2%)
10µg/mL	0.119 (94.1%)	0.015 (99.3%)	1.633 (18.9%)
Non-scFv	2.014 (0%)		

[0170]

[Table 12]

Competitive binding (antagonistic inhibitory rate) of anti-CTLA-4-1 scFv antibody and anti-CTLA-4-2 scFv antibody to human CTLA-4 (immobilized) with hCD86 by ELISA

Antibody concentration	Absorbance: 450-570 nm (binding inhibition rate %)		
	Anti-CTLA-4-1 scFv antibody (scFv CTLA4-1)	Anti-CTLA-4-2 scFv antibody (scFv CTLA4-2)	Anti-PD-1-2 scFv antibody (scFv PD1-2)
0.001μg/mL	1.200 (39.6%)	1.534 (22.8%)	1.301 (34.5%)
0.01μg/mL	1.610 (18.9%)	1.266 (36.3%)	1.174 (40.9%)
0.1μg/mL	1.293 (34.9%)	1.136 (42.8%)	1.206 (39.3%)
1μg/mL	0.324 (83.7%)	0.181 (90.9%)	1.395 (29.8%)
10μg/mL	0.079 (96.0%)	0.045 (97.7%)	1.716 (13.6%)
Non-scFv	1.986 (0%)		

[0171]

(Results)

The inhibitory rate of binding of hCD80 to hCTLA-4 was 61.7% and 73.1% in the case of 1 μg/mL anti-CTLA-4-1 scFv antibody and anti-CTLA-4-2 scFv antibody, respectively, and 94.1% and 99.3%, respectively in the case of 10 μg/mL. The inhibitory rates of binding of hCD86 to hCTLA-4 by 1 μg/mL anti-CTLA-4-1 scFv antibody and anti-CTLA-4-2 scFv antibody were 83.7% and 90.9%, respectively. The inhibitory rates thereof by 10 μg/mL of anti-CTLA-4-1 scFv antibody and anti-CTLA-4-2 scFv antibody were 96.0% and 97.7%, respectively. From the above results, it was demonstrated that the anti-CTLA-4-1 scFv antibody and anti-CTLA-4-2 scFv antibody both competitively inhibit binding of hCD80 and hCD86 (1 μg/mL) to hCTLA-4 in a concentration of 1 μg/mL or more.

[0172]

[Example 17]

[Competitive inhibitory activity of anti-mouse PD-1 scFv antibody to the binding reaction between mouse PD-1 and mouse PD-L1]

Using the anti-mouse PD-1 scFv antibody secreted from the *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-3 strain prepared in Example 6, competitive inhibition activity to the binding between mouse PD-1 (mPD-1) and mouse PD-L1 (mPD-L1) was checked by ELISA. As a negative control, an anti-human CTLA-4-2 scFv antibody was used.

To 96 well plates, 100 μL of mPD-1 adjusted to be 1 μg/mL with 1 × PBS was dispensed, incubated at 4°C overnight so as to immobilize. After the solution was removed, 350 μL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing. To the above plates, 350 μL of a 1% BSA solution was dispensed. The plates were incubated at room temperature for 2 hours to carry out blocking. After the solution was removed, 350 μL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0173]

The mPD-L1 (Recombinant Mouse B7-H1/PD-L1 Fc Chimera, manufactured by R&D Systems) was diluted with a signal enhancing reagent (Signal Enhancer HIKARI, manufactured by Nacalai Tesque) to prepare a solution having a concentration of 2000 ng/mL. The anti-mouse PD-1 scFv antibody purified from *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-3 strain was diluted with a signal

enhancing reagent to prepare solutions having a concentration of 20000 ng/mL, 2000 ng/mL, 200 ng/mL and 20 ng/mL and mixed with the mPD-L1 in the equal amounts. The solutions each (100 µL) were dispensed to the plates to which blocking treatment was already applied. The anti-human CTLA-4-2 scFv antibody purified from a bacterium of the genus *Bifidobacterium* was similarly prepared as mentioned above as a negative control. To blank wells, 100 µL of a signal enhancing reagent alone was added. The plates were sealed and incubated at room temperature for 2 hours. With the immobilized mPD-1, mPD-L1 mixed with the anti-mouse PD-1 scFv antibody was reacted. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0174]

A secondary antibody (Biotin anti-mouse CD274, manufactured by Biolegend) against mPD-L1 was diluted with a signal enhancing reagent to prepare a solution having a concentration of 10 ng/mL. This solution (100 µL) was dispensed. The plates were sealed and incubated at room temperature for 30 minutes. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing. The avidin-biotinylated enzyme complex (Vectastain ABC Kit, manufactured by Vector), more specifically, Solution A and Solution B, each (a single drop) were added to a signal enhancing reagent (2.5 mL). This solution (100 µL) was dispensed to the plates. The plates were sealed and incubated at room temperature for

30 minutes. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0175]

Color Solution A and Color Solution B (manufactured by R&D Systems) serving as a detection reagent were mixed in equal amounts. This mixture (200 µL) was dispensed to the plates. The plates were sealed and incubated at room temperature for 20 minutes. Accurately 20 minutes later, 50 µL of a Stop solution (manufactured by R&D Systems) was added to terminate a color reaction. Absorbance at 450 nm was measured, whereas absorbance at 570 nm was measured as a control. The measurement results are shown in Table 13 below and Figure 15.

[0176]

[Table 13]

Competitive inhibitory activity of anti-mouse PD-1 scFv antibody to binding reaction between mouse PD-1 and mouse PD-L1

	Absorbance: 450-570 nm (inhibition rate %)	
Antibody concentration	Anti-mouse PD-1 scFv antibody (mPD-1 scFv)	Anti-human CTLA-4-2 scFv antibody (hCTLA-4 scFv02)
0.01µg/mL	2.104 (8.3%)	2.100 (8.5%)
0.1µg/mL	2.195 (4.4%)	2.059 (10.3%)
1µg/mL	2.152 (6.2%)	2.124 (7.5%)
10µg/mL	1.116 (51.4%)	2.443 (-6.4%)
Non-scFv	2.295 (0%)	

[0177]

(Results)

As is apparent from Table 13 and Figure 15, in the case where 1 μg/mL of mPD-L1 was added to immobilized mPD-1, competitive inhibitory rate of the scFv antibody to the binding reaction thereof was 51.4% at 10 μg/mL anti-mouse PD-1 scFv antibody. In the case of the anti-human CTLA-4-2 scFv antibody serving as a control, competitive inhibition was not observed (-6.4%). From the above results, it was verified that the anti-mouse PD-1 scFv antibody (10 μg/mL or more) exhibits competitive (binding) inhibitory activity against mPD-L1 (1 μg/mL).

[0178]

[Example 18]

[Verification of the presence or absence of binding between anti-mouse PD-1 scFv antibody and mouse T-cell (CD3/CD28-stimulated CD4 positive cell)]

The presence or absence of binding between anti-mouse PD-1 scFv antibody (which was purified from the culture supernatant of an anti-mouse PD-1 scFv antibody secreting bacterium of the genus *Bifidobacterium*) and mPD-1 expressing cell was investigated by flow cytometric analysis using a CD4 positive cell expressing mPD-1 by stimulation-induction with CD3 and CD28 (hereinafter referred to also as "CD3/CD28-stimulated CD4 positive cells").

[0179]

(Preparation of CD3/CD28-stimlated CD4 positive cells)

The spleen was excised out from Balb/c mouse and spleen cells were collected. Thereafter, CD4 positive cells were labeled with Biotin anti-mouse CD4 Antibody

(Biolegend). After excessive antibody was washed away, Streptavidin Particles Plus-DM (manufactured by Becton, Dickinson and Company, Japan) was added and magnetic beads were allowed to bind to the CD4 positive cells. The magnetic beads were separated by a magnet and the cells attracted by the magnet were collected as CD4 positive cells and suspended in RPMI-1640 culture medium containing 10% fetal bovine serum, 8 mM HEPES and 50 μM 2-mercaptoethanol to prepare CD4 positive cells (2×10^6 cells/mL).

[0180]

A purified anti-mouse CD3ε antibody (manufactured by Biolegend, hereinafter referred to also as "anti-CD3 antibody") adjusted to be 2 μg/mL was dispensed to 48-well plates at a rate of 110 μL/well and incubated at 4°C overnight to immobilize. The CD4 positive cells prepared above were dispensed to immobilized plates at a rate of 500 μL/well. Further, Functional Grade Anti-Mouse CD28 (manufactured by eBioscience, hereinafter referred to also as "anti-CD28 antibody") adjusted to be 1 mg/mL was added at a rate of 1 μL/well to obtain a CD3/CD28 stimulated CD4 positive cells. Similarly, to wells to which the anti-CD3 antibody was not immobilized, the CD4 positive cells were dispensed to obtain a non-stimulated CD4-positive cells. The plate was cultured at 37°C in the conditions of 5% CO_2 for two days.

[0181]

The cell obtained by culturing for two days were removed by pipetting, transferred to a 1.5 mL tube and centrifuged at 4°C and 5000 rpm for one minute, and then

the supernatants were removed. PBS (1 mL) was added and the resultant solution was centrifuged and the supernatant was removed. After this operation was repeated twice for washing, 0.9 mL of FACS buffer (PBS containing 1% BSA) was added. The solution was centrifuged and the supernatants were removed in the same manner as above. Twenty μL of TruStain fcX (manufactured by Biolegend) diluted 50 fold with Sorting buffer (PBS containing 0.5% BSA) was added, stirred and incubated on ice for 10 minutes. To this solution, 20 μL of Alexa Fluor 647 anti-mouse CD4 Antibody (manufactured by Biolegend) diluted 200 fold with FACS buffer was added. The solution was stirred and incubated on ice for 20 minutes. FACS buffer (1 mL) was added and centrifuged and the supernatants were removed. This operation was repeated twice for washing. Subsequently, 20 μL of PE anti-mouse CD69 Antibody (manufactured by Biolegend) diluted 200 fold with FACS buffer was added. The solution was stirred and incubated on ice for 20 minutes. An operation of adding 1 mL of FACS buffer, centrifuging the solution and removing the supernatants were repeated twice for washing to obtain CD3/CD28-stimulated CD4 positive cells. The cells were divided into two portions. One of the portions was used for verification of the binding to the anti-mouse PD-1 scFv antibody and the other portion was used for verification of expression of PD-1.

[0182]

(Verification of binding of anti-mouse PD-1 scFv antibody to cells)

The binding of anti-mouse PD-1 scFv antibody to the cells was checked by using the non-stimulated CD4-positive cells obtained from the mouse spleen cell and the CD3/CD28-stimulated CD4 positive cell. The cells used herein were separately collected and 1 mL of 1 × PBS buffer was added and centrifuged, and the supernatants were removed. This operation was repeated twice to wash away the medium components. To the resultant cell, further FACS buffer was added and, centrifuged, and the supernatants were removed. To this, 20 μL of TruStain fcX (manufactured by Biolegend) diluted 50 fold with FACS buffer was added. The solution obtained was stirred well and then incubated on ice for 10 minutes. Subsequently, 20 μL of Alexa Fluor 647 anti-mouse CD4 Antibody (manufactured by Biolegend) diluted 200 fold with FACS buffer was added. The solution was stirred well and then incubated on ice for 20 minutes. After the reaction, an operation of adding 1 mL of FACS buffer, centrifuging the solution and removing the supernatant was repeated twice for washing. Next, 20 μL of PE anti-mouse CD69 Antibody (manufactured by Biolegend) diluted 200 fold with FACS buffer was added. The solution was stirred well and incubated on ice for 20 minutes. After the reaction, an operation of adding 1 mL of FACS buffer, centrifuging the solution and removing the supernatants was repeated twice for washing.

The anti-mouse PD-1 scFv antibody (20 μL) controlled to have a concentration of 10 μg/mL with FACS buffer was added. The solution was stirred and incubated on ice for 20 minutes. An operation of adding 1 mL of FACS buffer,

centrifuging the solution and removing the supernatant was repeated twice for washing. Subsequently, 20 µL of Anti-His-Tag Alexa Fluor 488 Antibody (MBL) diluted 1000 fold with FACS buffer was added. The solution was incubated on ice for 20 minutes. An operation of adding 1 mL of FACS buffer, centrifuging the solution and removing the supernatant was repeated twice for washing. FACS buffer (1 mL) was added and then the cells suspended with FACS buffer were transferred to a 5 mL-polystyrene round-bottom tube (manufactured by Becton, Dickinson and Company).

[0183]

(Verification of PD-1 expression on cells)

Whether or not PD-1 is expressed on the cell used herein was verified by using a non-stimulated CD4-positive cell obtained from a mouse spleen cell and a CD3/CD28-stimulated CD4 positive cell, in which CD4 and CD69 were labelled with a fluorescent antibody. To this cells, 20 µL of FITC anti-mouse CD279 (PD-1) antibody (manufactured by Biolegend) diluted 100 fold with FACS buffer was added. The solution obtained was incubated on ice for 20 minutes. To this, 1 mL of FACS buffer was added. The solution was centrifuged and the supernatants were removed. This operation was repeated twice for washing. FACS buffer (1 mL) was added and then the cell suspended with FACS buffer were transferred to a 5 mL-polystyrene round-bottom tube (manufactured by Becton, Dickinson and Company).

[0184]

To the suspension solution of the fluorescent labeled cells, 5 µL of a propidium iodide solution diluted to a 5 µg/mL with FACS buffer was added. The obtained

solution was subjected to analysis performed by BD FACS canto II flow cytometer (Becton, Dickinson and Company) and flow cytometric analysis software Kaluza ver 1.2 (manufactured by Beckman Coulter, Inc.). The forward scattering (FSC) and side scattering (SSC) of the cell suspension solution were checked by the flow cytometer and gating of a cell population was performed depending upon the size and shape of the cells. Subsequently, the cell population was subjected to gating with PI and dead cells were removed to obtain a cell population. Furthermore, CD4 positive cells were subjected to gating at a wavelength of 647 nm and whether the cells express CD69 and are stimulated with CD3/CD28 was checked by PE. Expression of mPD-1 was checked and binding of the anti-mouse PD-1 scFv antibody to mPD-1 was verified by an increase of FITC positive cells (means that mPD-1 is expressed or anti-mouse PD-1 scFv antibody is bound) in the CD4 positive cell population. The results of binding of the anti-mouse PD-1 scFv antibody (which was purified from an anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium*) to a CD3/CD28-stimulated CD4 positive cells are shown in Figure 16C and Figure 16D, and mPD-1 expression on the cells is shown in Figure 16A and Figure 16B.

[0185]

(Results)

As is apparent from Figure 16A and Figure 16B, the anti-mPD-1 antibody specifically binds to the CD3/CD28 stimulated CD-positive cells and expression of PD-1 on the cell surface was verified. Also, as shown in Figure 16C

and Figure 16D, it was verified that anti-mouse PD-1 scFv antibody purified from the anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium* specifically binds to the mPD-1 expressing cells (CD3/CD28-stimulated CD4 positive cell).

[0186]

[Example 19]

[Colonization of anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium* within tumor of a cancer-bearing mouse and secretion of anti-mouse PD-1 scFv antibody]

To a cancer-bearing mouse having a mouse large-intestine cancer cell strain CT26, *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-3 strain was intravenously administered and colonization of the strain in the tumor and local secretion of an anti-mouse PD-1 scFv antibody were verified by immunohistochemical staining.

[0187]

Mouse large-intestine cancer cell strain CT-26 (ATCC) was cultured in RPMI1640 culture medium (manufactured by Wako Pure Chemical Industries Ltd.) containing 10% of FBS (manufactured by EQUITECH-BIO, INC.) and grafted to 6 weeks old, female BALB/c mice (manufactured by Japan SLC, Inc.) to prepare cancer-bearing mice. The cancer-bearing mice having a tumor size of 30.18 to 138.16 mm^3 were divided into 3 groups (Day 1, each consisting of 3 mice) as shown in the Table below. The first group and second group were used for verifying secretion of an anti-mouse PD-1 scFv antibody from a recombinant bacterium of the genus *Bifidobacterium* in a

tumor tissue; whereas, the third group was used for verifying colonization of the recombinant bacterium of the genus *Bifidobacterium* in the tumor tissue. To the second group and third group, a briefly prepared frozen product of an anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium*, namely, *Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-3 strain, was administered in an amount of 1.0×10^9 cfu from the tail vein. Note that, 1 mL of a 10% maltose solution was administered at a frequency of two times a day for 5 days. Seven days after the administration of the bacterium of the genus *Bifidobacterium*, a tumor was excised out. The tumor of the third group was cryopreserved and then viable bacterial count was obtained. The tumors of the first group and second group were embedded with O.C.T. compound (manufactured by Sakura Finetek Japan Co. Ltd.) and frozen. These embedded and frozen tissues were sliced by a cryostat microtome, Leica CM1900 (manufactured by Leica) and thin-slice slide samples were prepared and subjected to histological staining.

[0188]

(Viable bacterial count)

To the tumor tissue frozen, an anaerobic diluting solution was added and homogenized. The homogenate was appropriately diluted with the anaerobic diluting solution, spread onto BLFS agar medium (BL agar medium containing 250 μg/mL 5-fluorouracil and 30 μg/mL spectinomycin) and cultured in anaerobic conditions at 37°C for 3 days. The number of colonies formed on the BLFS agar medium was counted and the viable bacterial count of the recombinant

bacterium of the genus *Bifidobacterium* in the tumor was calculated. The results are shown in the Table 14 below.

[0189]

[Table 14]

Group	Administration	Evaluation method	Tumor volume (mm^3) (upon excision)	Tumor weight (g) (upon excision)	Viable bacterial count (cfu/g)
1	Non-treatment	Immunohistochemical staining (control)	370.60±379.82	0.31±0.36	-
2	bacterium of the genus *Bifidobacterium*	Immunohistochemical staining	202.52±201.33	0.18±0.20	-
3	bacterium of the genus *Bifidobacterium*	Determination of viable bacterial count	265.56±273.73	0.31±0.37	2.7E+06±7.0E+05

[0190]

(Results)

As is apparent from Table 14, vial cells of 2.7 × 10^6 cfu per tumor (1 g) were observed.

[0191]

(Immunohistochemical staining)

The thin-slice slides were dried in air and soaked in a 4% PFA (manufactured by Wako Pure Chemical Industries Ltd.) for 10 minutes for fixation. After fixation, the thin slice slides were washed with pure water for one minute and washed three times with 1 × PBS (-) for 5 minutes. Water around the tissue was wiped out and the tissue was enclosed by a line drawn by Dako pen (manufactured by Dako) and then 3% BSA-PBS was added dropwise to the tissue and allowed to react for 60 minutes to inhibit non-specific binding. An Anti-His-tag mAb-Alexa Fluor (registered trademark) 488 (manufactured by

MBL) antibody reaction solution was diluted 1000 fold with 3% BSA-PBS, added dropwise onto the tissue and allowed to react at 4°C overnight. After the antibody reaction, the tissue was washed with 1 × PBS (-) for 5 minutes three times and mounted with VECTASHIELD (R) Mounting Medium with DAPI. The stained slice was observed by a microscope DM5000B (Manufactured by Leica) and an image thereof was photographed. The resultant images are shown in Figure 17.

[0192]

(Results)

As is apparent from Figure 17, in the mouse administered with an anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium* (Figure 17A), the tumor tissue was diffusely positive to the anti-mouse PD-1 scFv antibody (stained in green).

[0193]

[Example 20]

[Investigation of pharmacological (anti-tumor) effect of anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium* on CT26 cancer-bearing model mouse]

CT26 cancer-bearing model mice were prepared in the same manner as in Example 19. The cancer-bearing mice having a tumor size of 40.51 to 88.03 mm^3 were divided into 5 groups (Day 0, each consisting of 7 mice) as shown in the Table 15 below. The first group is a non-administration control group; the second group was a single agent administration (i.v.) group with an anti-mouse PD-1 scFv antibody-secreting strain (*Bifidobacterium longum* 105-A/pHuSP7L20-scFv-PD-1-3 strain); the third

group was a single agent administration (i.t.) group with an anti-mPD-1 antibody (manufactured by BioXcell, Clone: RPM1-14); the fourth group was a single agent administration (i.t.) group with an anti-mCTLA-4 antibody (manufactured by BioXcell, Clone: 9D9); and the fifth group was a combined administration of two agents group with the anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium* (i.v.) and the anti-mCTLA-4 antibody (i.t.). The date of drug administration, dosage and administration route are shown in Table 15 below. Furthermore, to mice of the second group and fifth group, a 10% maltose solution (1 mL) was intraperitoneally administrated at a frequency of two times a day from Day 1 to Day 5 and Day 8 to Day 12. The diameter of tumors was periodically measured (Day 0, 3, 7, 10, 14, 17, 22) in all groups. The tumors were excised out on Day 22 and weight of the tumors was measured. A change of tumor volume is shown in Table 16 below. The results of a statistical test of tumor volumes in CT26 cancer-bearing mice are shown in Table 17, and changes in CT26 tumor volume of single agent administration groups and the non-administration group are shown in Figure 18.

[0194]

[Table 15]

Group	Administration agent	Dosage	Administration route	Administration date	Number of mice
1	None	-	-	-	7
2*	Anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium*	5×10^8 cfu twice a day	i.v.	Day 1, 4, 8, 11	7
3	Anti-mPD-1 antibody	100 μg once a day	i.t.	Day 1, 4, 8, 11	7
4	Anti-mCTLA-4 antibody	100 μg once a day	i.t.	Day 1, 4, 8, 11	7
5*	Anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium*, Anti-mCTLA-4 antibody	5×10^8 cfu twice a day	i.v.	Day 1, 4, 8, 11	7
	Anti-mCTLA-4 antibody	100 μg once a day	i.t.	Day 1, 4, 8, 11	

i.v.: Intravenous administration

i.t.: Intratumoral (local) administration

[0195]

[Table 16]

Mean tumor volume of CT26 cancer-bearing mice on single agent administration and combined administration of two agents

Day	Tumor volume (mm^3)				
	Non-administration	Anti-mouse PD-1 scFv antibody-secreting *bacterium of the genus Bifidobacterium*	Anti-mPD-1 antibody	Anti-mCTLA-4 antibody	Anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium* + Anti-mCTLA-4 antibody
0	60.30 ± 13.46	60.59 ± 13.86	61.31 ± 13.74	61.08 ± 13.84	61.21 ± 14.94
3	148.48 ± 36.43	69.96 ± 25.13	78.88 ± 21.81	108.99 ± 30.86	72.87 ± 34.89
7	466.83 ± 167.61	153.42 ± 77.79	115.71 ± 49.80	175.70 ± 79.77	116.26 ± 67.78
10	902.05 ± 352.64	209.58 ± 119.71	138.55 ± 86.62	200.04 ± 118.46	88.04 ± 61.75
14	1571.61 ± 636.62	364.42 ± 246.94	195.31 ± 202.52	241.99 ± 192.36	78.58 ± 54.86*
17	2506.78 ± 993.48	615.38 ± 380.16	282.07 ± 328.92	322.65 ± 296.33	98.33 ± 85.98*
22	3718.65 ± 1064.17	1259.90 ± 737.04	556.60 ± 599.70	475.74 ± 516.73	175.93 ± 215.91*

The mean values ± S.D. of seven mice.

*: The mean values ± S.D. of six mice.

[0196]

[Table 17]

Statistical test on tumor volume of CT26 cancer-bearing mice

Day	T-test P (T<=f)					
	First group vs Second group	First group vs Third group	First group vs Fourth group	First group vs Fifth group	Second group vs Fifth group	Fourth group vs Fifth group
3	0.000 **	0.000 **	0.049 *	0.002 **	0.861	0.063
7	0.002 **	0.001 **	0.002 **	0.001 **	0.359	0.159
10	0.002 **	0.001 **	0.002 **	0.001 **	0.034 *	0.047 *
14	0.002 **	0.001 **	0.001 **	0.001 **	0.021 *	0.069
17	0.002 **	0.001 **	0.001 **	0.001 **	0.010 *	0.098
22	0.000 **	0.000 **	0.000 **	0.000 **	0.007 **	0.199

*: P value < 0.05

**: P value < 0.01

First group: Non-administration

Second group: Anti-mouse PD-1 scFv antibody-secreting *bacterium of the genus Bifidobacterium* administration group#

Third group: Anti-mPD-1 antibody administration group

Fourth group: Anti-mCTLA-4 antibody administration group

Fifth group: Two agent combined administration group: anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium* # + anti-mCTLA-4 antibody

#: *Bifidobacterium longum* 105-A/pHuSP7L20-mPD-1scFv

[0197]

[Table 18]

T/C of mean tumor volume of CT26 cancer-bearing mouse on single agent administration and combined administration of two agents

Day	T/C (Ratio of administration group to non-administration group)			
	Anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium*	Anti-mPD-1 antibody	Anti-mCTLA-4 antibody	Anti-mouse PD-1 scFv antibody-bacterium of the genus *Bifidobacterium* + Anti-mCTLA-4 antibody
0	1.00	1.02	1.01	1.02
3	0.47	0.53	0.73	0.49
7	0.33	0.25	0.38	0.25
10	0.23	0.15	0.22	0.10
14	0.23	0.12	0.15	0.05
17	0.25	0.11	0.13	0.04
22	0.34	0.15	0.13	0.05

[0198]

(Results)

As is apparent from Table 17 and Figure 18, in the anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium* administration group (second group), the volume of a tumor is lower than the non-administration group (first group) at all time points and a significant growth suppression effect was observed at all time points (Table 17). Furthermore, the tumor growth suppression expressed by the ratio of the administration group relative to the non-administration group (T/C) was 0.34 or less on and after Day 10.

In the anti-mPD-1 antibody single agent administration group (third group), a significantly high suppression effect on tumor volume was observed at all time points than in the non-administration group (first group) and the T/C ratio was 0.15 or less on and after Day

10. Note that in the anti-mCTLA-4 antibody intratumoral single agent administration group (fourth group), a significantly higher inhibitory effect on tumor volume than in the non-administration group was found at all time points and the T/C ratio was 0.22 or less on and after Day 10.

[0199]

In contrast, in combined administration of two agents group (the anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium* and the anti-mCTLA-4 antibody) (fifth group), a significantly higher inhibitory effect on tumor volume than in the non-administration group was found at all time points and the T/C ratio was 0.10 or less, which was the strongest anti-tumor effect, on and after Day 10. When the combined administration of two agents group was compared to the single agent administration groups, a significantly strong inhibitory effect was produced in each single agent administration group on Day 10; and a significantly strong inhibitory effect was produced in the group administered with the anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium* on and after Day 14 (see Table 16, Table 17, Table 18, Figure 19, Figure 20). Note that in either case of the anti-mCTLA-4 antibody administration group and the combined administration of two agents group, complete repression of a tumor was observed in a single mouse.

[0200]

The same results as in tumor volume were obtained with respect to tumor weight. A significant difference

was observed in all groups compared to the non-administration group. The T/C ratios of the anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium* administration group, anti-mPD-1 antibody administration group, anti-mCTLA-4 antibody administration group and combined administration of two agents group were 0.31, 0.12, 0.10 and 0.04, respectively. The tumor weight value of the combined administration of two agents group was the lowest (see Table 19 and Table 20).

[0201]

[Table 19]

Anti-tumor effect of on single agent administration and combined administration of two agents on CT26 cancer-bearing mice (mean tumor weight and T/C ratio)

	Day 22	
Administration agent	Tumor weight (g)	T/C ratio
Non-treatment (C)	5.20±2.16	-
Anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium*#	1.62±0.92	0.31
Anti-mPD-1 antibody	0.60±0.70	0.12
Anti-mCTLA-4 antibody	0.54±0.61	0.10
Combined administration of two agents (Anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium*# + Anti-mCTLA-4 antibody)	0.21±0.26*	0.04

The mean values ± S.D. of seven animals.

*: The mean values ± S.D. of six animals.

T/C ratios = mean tumor weight of administration group/mean tumor weight of non-administration group

-: Not determined

#: *Bifidobacterium longum* 105A/pHuSP7L20-mPD-1scFv

[0202]

[Table 20]

Statistical test on tumor weight of CT26 cancer-bearing mice

VS	Day 22 T-test P (T<=f)
First group vs Second group	0.004**
First group vs Third group	0.001**
First group vs Fourth group	0.001**
First group vs Fifth group	0.001**
Second group vs Fifth group	0.006**
Fourth group vs Fifth group	0.231

**: P value < 0.01

First group: Non-administration

Second group: Anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium* administration group#

Third group: Anti-mPD-1 antibody administration group

Fourth group: Anti-mCTLA-4 antibody administration group

Fifth group: Anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium*# + Anti-mCTLA-4 antibody combined administration group of two agents

#: *Bifidobacterium longum* 105A/pHuSP7L20-mPD-1scFv

[0203]

(Results)

From the above results, it was demonstrated that the anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium* colonizes within a tumor of CT-26 cancer-bearing mouse by intravenous administration (Example 19) and secrets an anti-mouse PD-1 scFv antibody to produce a tumor growth suppression effect; and that the effect is almost the same obtained when an reagent antibody, i.e., an anti-mPD-1 antibody, was directly administered into the tumor. It was also demonstrated that the anti-mouse PD-1 scFv antibody secreted from a bacterium of the genus *Bifidobacterium* has an activity of competitively inhibiting binding between mPD-1 and mPD-L1

in the in-vitro test (Example 17), and inhibits the negative signal transduction between PD-1 on a T cell and PD-L1 on a tumor cell in the same mechanism also within a tumor to activate the T cell, with the result that an anti-tumor effect is presumably produced. Furthermore, the effect of combined administration of the anti-mouse PD-1 scFv antibody-secreting bacterium of the genus *Bifidobacterium* and the anti-mCTLA-4 antibody was verified. From this, usefulness of a bacterium of the genus *Bifidobacterium* simultaneously expressing both a scFv antibody inhibiting CTLA-4 and a scFv antibody inhibiting PD-1 was suggested.

[0204]

[Example 21]

[Preparation of anti-human PD-1scFv03 scFv antibody-secreting bacterium of the genus *Bifidobacterium* having a linker different in length]

(Outline)

An anti-human PD-1scFv03 secretory plasmid, pHuSP7L20-hPD-1scFv03 having a secretory signal SP7 and a subsequent linker consisting of 20 amino acid residues was constructed. The secretory signal and the subsequent linker in this plasmid were replaced respectively with SP69 and a subsequent linker consisting of 20 amino acid residues corresponding to SP69 to construct an anti-human PD-1scFv03 secretory plasmid, pHuSP69L20-hPD-1scFv03. Furthermore, plasmids were constructed in the same manner as above except that the length of the linker was changed from 20 amino acid residues to L0 to 10 and 15 amino acid

residues (L0, L1, L2, L3, L4, L5, L6, L7, L8, L9, L10 and L15).

[0205]

[Preparation of plasmid, pHuSP7L20-hPD-1scFv03]

(Outline)

A plasmid, pHuSP7L20-hPD-1scFv03 containing an expression cassette (anti-human PD-1scFv03 secretion expression cassette) secreting anti-hPD-1scFv03 and serving as an *E. coli*-bacterium of the genus *Bifidobacterium* shuttle vector, was prepared. The primers used herein are shown in Table 21 below.

[0206]

[Table 21]

Primer Name	DNA Sequence (5'→3')	SEQ ID NO:
Ins-hPD-1scFv03-F1	CAGGTCCAGCTGGTCGAATCGGGCGGCGGC	SEQ ID No: 82
Ins-hPD-1scFv03-R1	ACGAGCAGAAGGTCAGTGGTGGTGATGATG GTGCTT	SEQ ID No: 83
TGA-Hu-Terminator-F	TGACCTTCTG CTCGTAGCGA TTAC	SEQ ID No: 84
vec-SP7L20-R1	GACCAGCTGGACCTGCACCGAACTCGCCTTCGGGAA	SEQ ID No: 85

[0207]

(Constitution of anti-human PD-1scFv03 secretion expression cassette)

The nucleotide sequence of DNA encoding the anti-human PD-1scFv03 secretion expression cassette, which is a cassette comprising (1) to (5) DNA: (1) Hu promoter DNA, (2) a DNA encoding a signal peptide-linker conjugate, SP7L20, (3) a DNA encoding the amino acid sequence of anti-hPD-1scFv03 (containing a heavy chain sequence, a linker $(GGGGS)_3$ and a light chain sequence), (4) a DNA encoding a His tag sequence and (5) Hu terminator DNA,

sequentially in this order ((1) to (5)) from the upstream side (the 5' end) to the downstream side (the 3' end) was constructed. The nucleotide sequence of DNA encoding a heavy chain sequence, a linker $(GGGGS)_3$ and a light chain sequence in the amino acid sequence of the aforementioned anti-hPD-1scFv03) was prepared with reference to the document listed in the Table 22 below.

[0208]

(Artificial synthesis of DNA of anti-hPD-1scFv03)

The gene of the anti-hPD-1scFv03 represented by SEQ ID No: 86 was subcloned in a plasmid for *E. coli*, i.e., pUC57 by GenScript Japan Inc. and artificially synthesized as a plasmid, namely, pUC57-hPD-1scFv03.

[0209]

[Table 22]

Document regarding anti-hPD-1scFv03

Antibody		Reference document
Anti-hPD-1scFv03	SEQ ID No: 86 (729 nucleotides sequence) Heavy chain sequence(1-342 nucleotides) $(GGGGS)_3$ linker (343-387 nucleotides) Light chain sequence (388-708 nucleotides) Histidine tag (709-726 nucleotides)	Japanese Patent No. 5028700

[0210]

(Preparation 1: Anti-hPD-1scFv03 insert fragment)

PCR amplification was performed by using the plasmid, pUC57-hPD-1scFv03 (500 pg) as a template and a primer set of Ins-hPD-1scFv03-F1 primer (forward) and Ins-hPD-1scFv03-R1 primer (reverse) listed in Table 21 above. The primer sequences were designed such that an insert

fragment and a vector fragment were mutually overlapped at the end of 15 bp. The PCR amplification was performed by setting each primer concentration at 0.2 μM and the reaction volume at 30 μL and using PrimeSTAR HS (Premix) kit (manufactured by Takara Bio Inc.). As an amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 72°C for 60 seconds was repeated 30 times. The PCR product obtained by the amplification was subjected to electrophoresis on 2% agarose gel and purified by QIAquick Gel Extraction Kit (manufactured by QIAGEN) to prepare about 0.7 kbp of anti-hPD-1scFv03 insert fragment (1).

[0211]

(Preparation of vector fragment (1) containing DNA encoding the amino acid sequence of SP7L20)

PCR amplification was performed by using the linearized vector fragment (500 pg) represented by SEQ ID No: 87 as a template and a primer set of TGA-Hu-Terminator-F primer (forward) and vec-SP7L20-R1 primer (reverse) listed in Table 21 above. The PCR amplification was performed by setting each primer concentration at 0.2 μM and the reaction volume at 30 μL and using PrimeSTAR HS (Premix) kit. As an amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 72°C for 5 minutes was repeated 30 times. The PCR product obtained by the amplification was subjected to electrophoresis on 0.8% agarose gel and purified by QIAquick Gel Extraction Kit to prepare about 4.0 kbp of 5'-Hu terminator-pTB6rep

unit-SPCMr-pUCori-Hu promoter-SP7L20-3' vector fragment (1).

[0212]

(Infusion reaction)

The vector fragment (1) prepared above and the anti-hPD-1scFv03 insert fragment (1) were ligated by use of In-Fusion (registered trademark) HD Cloning kit (manufactured by Takara Bio Inc.). More specifically, the above vector within the kit and the insert were added in a molar ratio of 1: 5 to a microtube and then 2 μL of 5 × In-Fusion (registered trademark) HD Enzyme premix (manufactured by Takara Bio Inc.) was added. The reaction solution, after the volume of the solution was adjusted to be 10 μL, was kept at 50°C for 15 minutes. The procedure described in the instruction of the kit except the aforementioned procedure was performed to prepare an infusion reaction solution 1.

[0213]

(Verification of transformation of *E. coli* and DNA sequence of pHuSP7L20-hPD-1scFv03)

E. coli HST16CR competent cell was transformed by use of the infusion reaction solution (5 μL) prepared above in accordance with the instruction. After the transformation, a bacterial suspension was spread onto a 75 μg/mL spectinomycin-containing LB agar medium and subjected to a static culture performed at 37°C overnight. Colonies formed on the agar medium were subjected to shaking culture performed in a 75 μg/mL spectinomycin-containing LB liquid medium at 30°C overnight. From the culture, a plasmid was extracted by use of QIAprep Spin

Miniprep Kit (manufactured by QIAGEN). In order to sequence the anti-human PD-1scFv03 secretion expression cassette (5'-Hu promoter-SP7L20-anti-hPD-1scFv03-His tag-Hu terminator-3') of the extracted plasmid, a sequencing reaction using BigDye (registered trademark) Terminator v3.1 Cycle Sequencing Kit (manufactured by Applied Biosystems) was carried out. The plasmid sequenced was designated as pHuSP7L20-hPD-1scFv03. The sequence of pHuSP7L20-hPD-1scFv03 is represented by SEQ ID No: 88.

[0214]

[Preparation of plasmid, pHuSP69L20-hPD-1scFv03]

A plasmid, pHuSP69L20-hPD-1scFv03, which is obtained by replacing the signal peptide sequence and the subsequent linker peptide sequence of an anti-human PD-1scFv03 secretory plasmid, pHuSP7L20-hPD-1scFv03 from SP7L20 to SP69L20, was constructed as follows.

[0215]

(Preparation of vector fragment and insert fragment)

A vector fragment was prepared by PCR amplification using a plasmid, pHuSP7L20-hPD-1scFv03 as a template and a primer set of hPD-1scFv03_Vec_F1 primer and Hu-mCCL21_Vec_R1 primer listed in Table 23 (V1) and Table 24 below at a primer concentration of 0.2 μM and a reaction volume of 50 μL and using PrimeSTAR HS (Premix) kit. As an amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 65°C for 5 seconds and a reaction at 72°C for 4 minutes and 45 seconds was repeated 30 times. The PCR product obtained by amplification was designated as vector fragment V1.

[0216]

PCR amplification was performed by using *Bifidobacterium longum* 105-A genomic DNA (800 pg) as a template and a primer set of SP69-ins_F1 primer and SP69-ins_R1_hPD1_03 primer listed in Table 23 (Ins-1) and Table 24 below. The primer sequences were designed such that an insert fragment and a vector fragment were mutually overlapped at the end of 15 bp. The PCR amplification was performed in the same manner as in the above vector. However, the elongation reaction time at 72°C was set at 20 seconds. The PCR product obtained by amplification was designated as insert fragment Ins-1.

[0217]

[Table 23]

Template and primer in PCR amplification

PCR product	Template	Forward Primer	Reverse primer	PCR product (bp)
V1	pHuSP7L20-hPD-1 scFv03	hPD-1 scFv03_Vec-F1	Hu-mCCL21_Vec_R1	4568 bp
Ins-1	*Bifidobacterium longum* 105-A	SP69-ins_F1	SP69-ins_R1_hPD1_03	180 bp
Ins-L0	pHuSP69L20-hPD-1 scFv03	SP69-ins_F1	SP69L0-ins_R1_hPD1_03	120 bp
Ins-L1	pHuSP69L20-hPD-1 scFv03	SP69-ins_F1	SP69L1-ins_R1_hPD1_03	123 bp
Ins-L2	pHuSP69L20-hPD-1 scFv03	SP69-ins_F1	SP69L2-ins_R1_hPD1_03	126 bp
Ins-L3	pHuSP69L20-hPD-1 scFv03	SP69-ins_F1	SP69L3-ins_R1_hPD1_03	129 bp
Ins-L4	pHuSP69L20-hPD-1 scFv03	SP69-ins_F1	SP69L4-ins_R1_hPD1_03	132 bp
Ins-L5	pHuSP69L20-hPD-1 scFv03	SP69-ins_F1	SP69L5-ins_R1_hPD1_03	135 bp
Ins-L6	pHuSP69L20-hPD-1 scFv03	SP69-ins_F1	SP69L6-ins_R1_hPD1_03	138 bp
Ins-L7	pHuSP69L20-hPD-1 scFv03	SP69-ins_F1	SP69L7-ins_R1_hPD1_03	141 bp
Ins-L8	pHuSP69L20-hPD-1 scFv03	SP69-ins_F1	SP69L8-ins_R1_hPD1_03	144 bp
Ins-L9	pHuSP69L20-hPD-1 scFv03	SP69-ins_F1	SP69L9-ins_R1_hPD1_03	147 bp
Ins-L10	pHuSP69L20-hPD-1 scFv03	SP69-ins_F1	SP69L10-ins_R1_hPD1_03	150 bp
Ins-L15	pHuSP69L20-hPD-1 scFv03	SP69-ins_F1	SP69L15-ins_R1_hPD1_03	165 bp

[0218]

[Table 24]

Primer sequence in PCR amplification

Primer Name	Sequence (5' → 3')	SEQ ID NO:
Hu-mCCL21_Vec_R1	AAAGCATCCTTCTTGGGTCAGG	SEQ ID No: 89
hPD-1 scFv03_Vec_F1	CAGGTCCAGCTGGTCGAATC	SEQ ID No: 90
SP69-ins_F1	CAAGAAGGATGCTTTATGAATTATTTACGACAAAAAATTTCGG	SEQ ID No: 91
SP69-ins_R1_hPD1_03	GACCAGCTGGACCTGACCGCTATCAGTCGTGGTGTAAC	SEQ ID No: 92
SP69L5-ins_R1_hPD1_03	GACCAGCTGGACCTGTGGCGTTGAATCATCCGC	SEQ ID No: 93
SP69L10-ins_R1_hPD1_03	GACCAGCTGGACCTGCGATGGCGTTGAAGATGG	SEQ ID No: 94
SP69L15-ins_R1_hPD1_03	GACCAGCTGGACCTGGGTGTAACTGCCATCCGATG	SEQ ID No: 95
SP69L0-ins_R1_hPD1_03	GACCAGCTGGACCTGCGCAAAGACCGGCATTG	SEQ ID No: 96
SP69L1-ins_R1_hPD1_03	GACCAGCTGGACCTGATCCGCAAAGACCGGCAT	SEQ ID No: 97
SP69L2-ins_R1_hPD1_03	GACCAGCTGGACCTGATCATCCGCAAAGACCGG	SEQ ID No: 98
SP69L3-ins_R1_hPD1_03	GACCAGCTGGACCTGTGAATCATCCGCAAAGACCG	SEQ ID No: 99
SP69L4-ins_R1_hPD1_03	GACCAGCTGGACCTGCGTTGAATCATCCGCAAAGAC	SEQ ID No: 100
SP69L6-ins_R1_hPD1_03	GACCAGCTGGACCTGAGATGGCGTTGAATCATCCG	SEQ ID No: 101
SP69L7-ins_R1_hPD1_03	GACCAGCTGGACCTGTGAAGATGGCGTTGAATCATCC	SEQ ID No: 102
SP69L8-ins_R1_hPD1_03	GACCAGCTGGACCTGCGTTGAAGATGGCGTTGAATC	SEQ ID No: 103
SP69L9-ins_R1_hPD1_03	GACCAGCTGGACCTGTGGCGTTGAAGATGGCG	SEQ ID No: 104

[0219]

(Infusion reaction)

Vector fragment V1 and insert fragment Ins-1 prepared above were ligated by use of In-Fusion HD Cloning Kit. More specifically, in a microtube, the vector fragment and the insert fragment were added in a molar ratio of 1: 2 and then 5 × In-Fusion (registered trademark) HD Enzyme premix (2 μL) and Cloning Enhancer (1 μL) were added. The reaction solution, after the volume of the solution was adjusted to be 10 μL, was kept at 37°C for 15 minutes and then at 50°C for 15 minutes. The procedure described in the instruction of the kit except

the aforementioned procedure was performed to prepare an infusion reaction solution.

[0220]

(Transformation of *E. coli* and sequencing of plasmid DNA)

E. coli HST16CR competent cell (manufactured by Takara Bio Inc.) was transformed by using the infusion reaction solution (1 μL) in accordance with the instruction. After the transformation, a bacterial suspension was spread onto a 75 μg/mL spectinomycin-containing LB agar medium and subjected to a static culture performed at 37°C overnight. Colonies formed on the agar medium were subjected to shaking culture performed in a 75 μg/mL spectinomycin-containing LB liquid medium at 37°C overnight. From the culture, a plasmid was extracted by use of QIAprep Spin Miniprep Kit (manufactured by QIAGE N Genomics Inc.). In order to sequence the anti-human PD-1scFv03 expression cassette (containing Hu promoter to Hu terminator) of the extracted plasmid, a sequencing reaction using BigDye (registered trademark) Terminator v3.1 Cycle Sequencing Kit (manufactured by Applied Biosystems) was carried out. The plasmid sequenced was designated as pHuSP69L20-hPD-1scFv03.

[0221]

[Preparation of pHuSP69Ly-hPD-1scFv03 (y = 0 to 10 and 15)]

A plasmid prepared by shortening the linker of pHuSP69L20-hPD-1scFv03 from the 3' end thereof to prepare DNA encoding 0 to 10 and 15 amino acids, namely,

pHuSP69Ly-hPD-1scFv03 (y = 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15), as follows.

[0222]

(Preparation of vector fragment and insert fragment)

As a vector fragment, vector fragment V1 as prepared above was used. An insert fragment was prepared in the same manner as in preparation of insert fragment Ins-1 mentioned above except that a plasmid, pHuSP69L20-hPD-1scFv03 was used as a template and a primer set listed in Table 23 (Ins-L0 to 10, Ins-L15) were used. The PCR product amplified were designated as insert fragments Ins-L0, Ins-L1, Ins-L2, Ins-L3, Ins-L4, Ins-L5, Ins-L6, Ins-L7, Ins-L8, Ins-L9, Ins-L10 and Ins-L15.

[0223]

(Infusion reaction)

Vector fragment V1 and insert fragments Ins-Ly (y = 0 to 10 and 15) prepared above were ligated by an infusion reaction in the same manner as in preparing pHuSP69L20-hPD-1scFv03 as mentioned above.

[0224]

(Transformation of *E. coli* and sequencing of plasmid DNA)

E. coli was transformed by using the infusion reaction solution prepared above and plasmid DNA was sequenced in the same manner as in preparing pHuSP69L20-hPD-1scFv03.

[0225]

[Transformation of bacterium of the genus *Bifidobacterium*]

Using 13 types of plasmids, pHuSP69Ly-hPD-1scFv03 (L0, L1, L2, L3, L4, L5, L6, L7, L8, L9, L10, L15 and L20) prepared above, *Bifidobacterium longum* 105-A strain was transformed by an electroporation system (Gene Pulser II, manufactured by Bio-Rad Laboratories). After electric shock (2kV, 25 μF, 200Ω) was applied, a solution mixture of IMR liquid medium (800 μL) and a vitamin C-added solution (50 μL) was immediately added in a cuvette (2 mm gap) and this was collected in a sterilized 2 mL-microtube. Individual tubes were subjected to the same operation. These 2 mL-tubes with the cap loosen were placed in an airtight container together with a de-oxygen and carbon dioxide gas generating agent (AnaeroPack (registered trademark)-Kenki, manufactured by Mitsubishi Gas Chemical Company, Inc.) and kept in an incubator set at 37°C for 3 hours. After keeping warm, each of the bacterial suspensions was spread onto a 75 μg/mL spectinomycin-containing IMR agar medium. These plates were placed in an airtight container together with the above de-oxygen and carbon dioxide gas generating agent and cultured in an incubator set at 37°C for 2 days.

[0226]

Colonies formed on the spectinomycin-containing IMR agar medium was picked up, streaked on a 75 μg/mL spectinomycin-containing BL-bS agar medium (BL agar medium containing no equine defibrinated blood), placed in an airtight container together with de-oxygen and carbon dioxide gas generating agent and cultured in an incubator set at 37°C for one day to obtain *Bifidobacterium longum*

105-A/pHuSP69Ly-hPD-1scFv03 strains (y = 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 and 20).

[0227]

[Example 22]

[Expression analysis of scFv of anti-human PD-1scFv03 secreting bacterium of the genus *Bifidobacterium* different in linker length]

Since anti-hPD-1scFv03 secreted from *Bifidobacterium longum* 105-A/pHuSP69Ly-hPD-1scFv03 strain (y = 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 and 20) prepared in Example 21 had a histidine tag fused to the C terminal, scFv antibody secretion was analyzed by Western blotting using an antibody against the anti-histidine tag.

[0228]

(Culture of bacterium of the genus *Bifidobacterium*)

A streak culture of each of the recombinant bacterium of the genus *Bifidobacterium* strains on the 75 μg/mL spectinomycin-containing BL-bS agar medium was inoculated in MRS (manufactured by Becton, Dickinson and Company) liquid medium (10 mL) supplemented with spectinomycin (a final concentration of 75 μg/mL), 100 μL of vitamin C-added solution (a solution (100 mL) containing ascorbic acid (35 g), L-cysteine hydrochloride monohydrate (2 g) and sodium carbonate (11 g)) and anaerobically cultured at 37°C for 24 hours to obtain an activated culture solution. Next, to a culture medium (20 mL) containing DMEM (Cat No. 12320-032: manufactured by Life Technologies) and MRS in a ratio of 9: 1, a vitamin C-added solution (100 μL) and spectinomycin were added so as to obtain a spectinomycin concentration of 75 μg/mL and

the activated culture solution (100 µL) was added for inoculation. This was anaerobically cultured at 37°C for 18 hours.

After the anaerobic culture, the culture solution was centrifuged and the culture supernatant was collected. Protein in the culture supernatant was allowed to precipitate with trichloroacetic acid (TCA, manufactured by Wako Pure Chemical Industries Ltd.), washed with acetone, dissolved in SDS-PAGE buffer and subjected to a heat treatment at 95°C for 3 minutes to obtain a culture supernatant concentrate.

[0229]

The culture supernatant concentrate (corresponding to 1 mL of culture solution) was subjected to electrophoresis on by Mini-PROTEAN (registered trademark) TGX™ gel (4 to 20%) (manufactured by Bio-Rad). The gel was transferred onto a PVDF membrane (iBlot Transfer Stacks, manufactured by Life Technologies) by use of Trans-Blot Turbo (manufactured by Bio-Rad). After completion of blotting, the membrane was subjected to a blocking treatment (2% ECL Prime Blocking agent (manufactured by GE Healthcare Japan) in TTBS), subjected to a reaction using a mouse histidine tag antibody (THE HIS Tag Antibody, mAb, Mouse, manufactured by GenScript Japan Inc.) as a primary antibody and an ECL-peroxidase-labeled anti-mouse antibody (manufactured by GE Healthcare Japan) as a secondary antibody and allowed to emit light by use of Western Lightning Ultra (manufactured by PerkinElmer Co., Ltd.). This was analyzed by an imaging analyzer (myECL Imager manufactured by Thermo Scientific

or Fluor S Max, manufactured by Bio-Rad). The results are shown in Figure 21.

[0230]

(Results)

As is apparent from Figure 21, anti-hPD-1scFv03 was detected in all strains except *Bifidobacterium longum* 105-A/pHuSP69L0-hPD-1scFv03 strain having a linker L0. In addition, the amount of anti-hPD-1scFv03 secretion tended to decrease as the length of a linker reduced.

[0231]

[Example 23]

[Verification of binding of anti-hPD-1scFv03 having a linker different in length to human PD-L1 by ELISA]

(Purification of anti-hPD-1scFv03)

Two types of bacterium of the genus *Bifidobacterium* s different in linker length, namely, *Bifidobacterium longum* 105-A/pHuSP69L20-hPD-1scFv03 strain and *Bifidobacterium longum* 105-A/pHuSP69L5-hPD-1scFv03, were cultured in the same manner as in Example 22. Note that the scale of culture was increased to 200 mL.

[0232]

After the anaerobic culture, the culture supernatant was obtained by centrifugation. To the culture supernatant, ammonium sulfate was added little by little while stirring the culture supernatant so as to obtain a saturation of 80%. The solution obtained was stirred at 4°C overnight to perform salting out. The solution was centrifuged and the precipitate was recovered. A histidine-tag fused protein was purified by a purification kit for a histidine-tagged protein (TALON resin,

manufactured by Takara Bio Inc). The solution of the purified protein was concentrated by ultrapurification (Amicon Ultra-0.5, NMWL: 10,000, manufactured by Merck Millipore Corporation). The concentration of the purified protein was measured by the Bradford method (Coomassie Plus Protein Assay manufactured by Thermo Scientific). Albumin was used as the concentration standard. The purified scFv antibody was aliquoted, subjected to SDS-PAGE and stained with Coomassie brilliant blue (SimplyBlue™ Safe Stain, Life Technologies). As a result, it was verified that each of the anti-hPD-1scFv03 scFv antibodies is purified with a purity of about 90%.

[0233]

(Verification of binding of anti-hPD-1scFv03 to human PD-1)

Binding of anti-hPD-1scFv03, which was derived from each of *Bifidobacterium longum* 105-A/pHuSP69L20-hPD-1scFv03 strain and *Bifidobacterium longum* 105-A/pHuSP69L5-hPD-1scFv03, to human PD-1 (hPD-1) was verified by ELISA.

[0234]

To 96 well plates, 100 μL of hPD-1 (Recombinant Human PD-1 Fc Chimera, manufactured by R&D Systems, Inc.) adjusted to be 1 μg/mL with 1 × PBS was dispensed and incubated at 4°C overnight to immobilize. After the solution was removed, 350 μL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing. To the above plates, 350 μL of a 1% BSA solution was dispensed and incubated at room temperature for 2 hours to carry out blocking. After the solution was removed, 350 μL of 1 × PBS was dispensed

and the solution was removed. This operation was repeated three times to carry out washing.

[0235]

The anti-hPD-1scFv03 was diluted with a signal enhancing reagent (Signal Enhancer HIKARI, manufactured by Nacalai Tesque) to prepare solutions having a concentration of 5ng/mL, 10 ng/mL, 20 ng/mL and 40 ng/mL. Each solution (100 µL) was dispensed to the plates to which blocking treatment was already applied. To blank wells, a signal enhancing reagent (100 µL) alone was added. After the plates were sealed and incubated at room temperature for 2 hours, immobilized hPD-1 was allowed to react with each of scFv antibodies. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0236]

A secondary antibody (Anti-His-tag-Biotin, manufactured by MBL) was diluted 2000 fold with a signal enhancing reagent. The dilution solution (100 µL) was dispensed to the plates. The plates were sealed and incubated at room temperature for 30 minutes. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing. An avidin-biotinylated enzyme complex (Vectastain ABC Kit, manufactured by Vector), more specifically, Solution A and Solution B, each (three drops) were added to a signal enhancing reagent (7.5 mL). This solution mixture (100 µL) was dispensed to the plates and the plates were sealed and

incubated at room temperature for 30 minutes. After the solution was removed, 350 µL of 1 × PBS was dispensed to the plates and the solution was removed. This operation was repeated three times to carry out washing. Color Solution A and Color Solution B (manufactured by R&D Systems) serving as a detection reagent were mixed in equal amounts. This solution (200 µL) was dispensed to the plates. The plates were sealed and incubated at room temperature for 20 minutes. Accurately 20 minutes later, 50 µL of a Stop solution (manufactured by R&D Systems) was added to the plates to terminate a color reaction. Absorbance at 450 nm was measured, whereas absorbance at 570 nm was measured as a control.

[0237]

(Results)

The results of ELISA on binding of anti-hPD-1scFv03 are shown in Figure 22. Anti-hPD-1scFv03 bound to human PD-1 regardless of the length of linkers (L5 and L20), and that the binding amount varied depending upon the concentration of scFv.

[0238]

[Example 24]

[Verification of competitive (binding) inhibitory activity of anti-hPD-1scFv03 different in linker length against the binding reaction between human PD-1 and PD-L1]

Using anti-hPD-1scFv03 different in linker length purified in Example 23, competitive (binding) inhibitory activity against the binding of human PD-L1 to human PD-1 was verified by ELISA. As a negative control, an anti-human CTLA-4-2 scFv antibody was used.

[0239]

To 96 well plates, 100 μL of hPD-1 (Recombinant Human PD-1 Fc Chimera, manufactured by R&D Systems, Inc.) adjusted to be 1 μg/mL with 1 × PBS was dispensed and incubated at 4°C overnight to immobilize. After the solution was removed, 350 μL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing. To the above plates, 350 μL of a 1% BSA solution was dispensed and incubated at room temperature for 2 hours to carry out blocking. After the solution was removed, 350 μL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing. The concentration of hPD-L1 (manufactured by R&D Systems, Recombinant Human B7-H1, hPDL1) was controlled to be 40 nM with a signal enhancing reagent (Signal Enhancer HIKARI, manufactured by Nacalai Tesque).

[0240]

The concentrations of the anti-human PD-1 scFv antibody purified from each bacterium of the genus *Bifidobacterium* were adjusted to be 1280 nM, 640 nM, 320 nM, 160 nM, 80 nM, 40 nM, 20 nM and 10 nM with a signal enhancing reagent. Each of the solutions prepared was mixed with 40nM hPD-L1 in equal amounts. The scFv/hPD-L1 mixed solution (100 μL) was dispensed to the plates to which blocking treatment was already applied. As a negative control, an anti-human CTLA-4-2 scFv antibody purified from a bacterium of the genus *Bifidobacterium* was used and subjected to the same operation as above. Furthermore, to a scFv-free (no competitive inhibition is

shown) well, a hPD-L1 solution (100 µL) controlled to be 20 nM with a signal enhancing reagent was dispensed. To a blank well, a signal enhancing reagent (100 µL) alone was dispensed. After the plates were sealed and incubated at room temperature for 2 hours, immobilized hPD-1 was allowed to react with hPD-L1 mixed with anti-hPD-1scFv. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0241]

A secondary antibody (Biotin-anti-human CD274, PD-L1, manufactured by Biolegend) against hPD-L1 was adjusted to be 200 ng/mL with a signal enhancing reagent. This solution (100 µL) was dispensed to the plates. The plates were sealed and incubated at room temperature for 30 minutes. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing. An avidin-biotinylated enzyme complex (Vectastain ABC Kit, manufactured by Vector), more specifically, Solution A and Solution B, each (four drops) were added to a signal enhancing reagent (10 mL). This solution (100 µL) was dispensed to the plates and the plates were sealed and incubated at room temperature for 30 minutes. After the solution was removed, 350 µL of 1 × PBS was dispensed and the solution was removed. This operation was repeated three times to carry out washing.

[0242]

Color Solution A and Color Solution B (manufactured by R&D Systems) serving as a detection reagent were mixed

in equal amounts. This solution (200 µL) was dispensed to the plates. The plates were sealed and incubated at room temperature for 20 minutes. Accurately 20 minutes later, 50 µL of a Stop solution (manufactured by R&D Systems) was added to terminate a color reaction. Absorbance at 450 nm was measured, whereas absorbance at 570 nm was measured as a control. From the measured values, a regression formula was obtained by use of free software Image J and the antibody concentration (IC50) at which 50% of binding between PD-1 and PD-L1 is inhibited, was obtained. Measurement values and analysis results are shown in Table 25 below and Figure 23.

[0243]

[Table 25]

Competitive inhibitory activity of various anti-human PD-1-scFv antibodies different in linker length to the binding reaction between hPD-1 and hPD-L1

scFv Concentration nM	SP69L5 hPD-1 scFv03		SP69L20 hPD-1 scFv03		SP69L20 hCTLA4 scFv02	
	A450-570	Inhibition (%)	A450-570	Inhibition (%)	A450-570	Inhibition (%)
0	2.571	0	2.249	0	2.249	0
5	2.021	21.4	2.469	-9.8	2.47	-9.8
10	1.857	27.8	2.398	-6.6	2.579	-14.7
20	1.248	51.5	2.02	10.2	2.582	-14.8
40	0.697	72.9	1.492	33.7	2.558	-13.7
80	0.378	85.3	0.939	58.2	2.489	-10.7
160	0.183	92.9	0.587	73.9	2.375	-5.6
320	0.131	94.9	0.31	86.2	2.535	-12.7
640	0.079	96.9	0.211	90.6	2.469	-9.8
IC_{50}	18.7 nM		51.7 nM			

[0244]

(Results)

As is apparent from Table 25 and Figure 23, anti-hPD-1scFv03 exhibited competitive inhibitory activity against binding of human PD-L1 to human PD-1, regardless of the length of linkers (L5 and L20). The IC_{50} values of anti-hPD-1scFv03 antibodies having SP69L5 and SP69L20 were 18.7 nM and 51.7 nM, respectively. The antibody having SP69L5 having a shorter exhibited competitive inhibitory activity at a lower concentration. Note that, in the negative control, i.e., anti-human CTLA-4-2 scFv antibody, competitive inhibition was not observed.

[0245]

[Example 25]

[Expression analysis of scFv of anti-human PD-1scFv03 secreting bacteria of the genus *Bifidobacterium* having different signal peptides integrated]

Since anti-hPD-1scFv03 secreted from each of *Bifidobacterium longum* 105-A/pHuSPxL20-hPD-1-scFv03 strains (where x =7, 45, 50, 52, 55, 58, 64, 66, 67, 68 and 69) has a histidine tag fused to the C terminal, scFv antibody secretion was analyzed by Western Blotting using an anti-histidine tag attached antibody in the same manner as in [Preparation of plasmid, pHuSP7L20-hPD-1scFv03] in Example 21. The results are shown in Figures 24(a) and 24(b).

[0246]

As is apparent from Figures 24(a) and 24(b), secretion of anti-hPD-1scFv03 was verified in *Bifidobacterium longum* 105-A strains transformed with vectors containing expression cassettes in which anti-hPD-1scFv03 was integrated downstream of the following 11

types of secretory signal peptide-linker conjugates (SP7L20, SP45L20, SP50L20, SP52L20, SP55L20, SP58L20, SP64L20, SP66L20, SP67L20, SP68L20, SP69L20).

[0247]

Of them, in the above *Bifidobacterium longum* 105-A strains in which secretory signal peptide-linker conjugates of SP50L20, SP64L20, SP68L20 and SP69L20 were inserted, the amount of secretion was relatively large.

[0248]

[Example 26]

[Verification of binding of anti-hPD-1scFv03 to human PD-1 by ELISA]

Binding of the anti-hPD-1-scFv03 antibody, which was purified from each of the culture supernatants of *Bifidobacterium longum* 105-A/pHuSPxL20-hPD-1-scFv03 strains, to human PD-1 was verified. Absorbance at 450 nm was measured, whereas absorbance at 570 nm was measured as a control. The ELISA results of binding of anti-hPD-1scFv03 are shown in Table 26 below.

[0249]

[Table 26]

SP/Linker	Binding by ELISA
	A450-570
SP7L20	3.171
SP45L20	2.605
SP50L20	3.481
SP52L20	2.427
SP55L20	1.449
SP58L20	3.017
SP64L20	3.055
SP66L20	1.928
SP67L20	3.411
SP68L20	2.187
SP69L20	1.827

[0250]

(Results)

As is apparent from Table 26, anti-hPD-1scFv03 antibodies bound to human PD-1 regardless of the type of signal peptide. Of them, in the cases of SP7, SP50, SP64 and SP67, the amount of binding was large.

[0251]

[Example 27]

[Verification of competitive binding inhibitory activity of anti-hPD-1scFv03 to binding reaction between human PD-1 and PD-L1]

Various types of anti-hPD-1scFv03 antibodies purified from the above *Bifidobacterium longum* 105-A/pHuSPxL20-hPD-1-scFv03 strains were used. To a microplate on which human PD-1 was immobilized, a mixture of each of the various types of anti-hPD-1scFv03 antibodies (10 μg/mL) and human PD-L1 (1 μg/mL) was added and the amount of PD-L1 bound to human PD-1 was measured. The competitive inhibition rate (%) to PD-1/PD-L1 binding when anti-hPD-1scFv03 antibody was co-present, was calculated provided that PD-L1 binding inhibition rate was regarded as 0% when anti-hPD-1scFv03 antibody was not added. The results are shown in Table 27 below.

[0252]

[Table 27]

SP/Linker	Competitive inhibition rate
	%
SP7L20	75.5
SP45L20	73.7
SP50L20	81.4
SP52L20	74.4
SP55L20	64.2
SP58L20	88.1
SP64L20	84.9
SP66L20	87.5
SP67L20	88.4
SP68L20	90.4
SP69L20	84.6

[0253]

(Results)

As is apparent from Table 27, the competitive inhibition rate was not greatly varied by the type of signal peptide; however, when SP55L20 was used, the competitive inhibition rate was relatively low.

[0254]

[Example 28]

[Investigation on linker length]

Investigation on length (Ly) of various linkers was carried out with respect to *Bifidobacterium longum* 105-A/pHuSPxLy-hPD-1-scFv03 strains (where x =7, 45, 50, 52, 55, 58, 64, 66, 67, 68 and 69).

[0255]

Eleven types of secretory signal peptides (SP7, SP45, SP50, SP52, SP55, SP58, SP64, SP66, SP67, SP68 and SP69) were used with respect to anti-hPD-1-scFv03 antibody. Anti-hPD-1scFv03 secretory plasmids were constructed by inserting secretory signal peptide-linker conjugates different in linker length. *Bifidobacterium longum* 105-A strains transformed with these plasmids were prepared.

[0256]

Whether each of the *Bifidobacterium longum* 105-A strains secrets an anti-hPD-1-scFv03 antibody was analyzed by Western Blotting using an anti-histidine tagged antibody. The analysis results of the scFv antibody secretion are shown in Figures 25(a) to 25(d).

[0257]

(Results)

As is apparent from Figures 25(a) to 25(d), a scFv antibody was secreted from *Bifidobacterium longum* 105-A strains transformed with an anti-hPD-1scFv03 secretory plasmids in which secretory signal peptide-linker conjugates, namely, SP7L5, SP7L10, SP7L15,SP7L20, SP45L5, SP45L10, SP45L15,SP45L20, SP50L5, SP50L10, SP50L15,SP50L20, SP52L5, SP52L10, SP52L15,SP52L20, SP55L5, SP55L10, SP55L15,SP55L20, SP58L15,SP58L20, SP64L5, SP64L10, SP64L15,SP64L20, SP66L5, SP66L10, SP66L15,SP66L20, SP67L5,

SP67L10, SP67L15,SP67L20, SP68L5, SP68L10, SP68L15,SP68L20, SP69L5, SP69L10, SP69L15,SP69L20 except SP58L5 and SP58L10, were inserted.

[0258]

As the detection results, the secretion products from SP7 series had the same molecular size regardless of the linker length designed; the secretion products from SP45 series had molecular sizes in accordance with the linker length designed; and the secretion products from SP50 series had molecular sizes in accordance with the linker length designed. In SP58 series, even if the linker length designed was L20, the secretion product had a small molecular size and no product was secreted in shorter linker length of (L10, L5) (see, Figure 25(c)). The molecular size of a scFv antibody determined by Western Analysis was not always consistent with the linker length designed.

[0259]

[Example 29]

[Verification of binding of anti-hPD-1scFv03 antibody to human PD-1 by ELISA]

The anti-hPD-1scFv03 antibody (1 μg/mL) purified from each of the culture supernatants of *Bifidobacterium longum* 105-A/pHuSPxLy-hPD-1-scFv03 strains (where x =7, 68, 69) was added and the binding amount of the antibody to human PD-1 (hPD-1) was checked by ELISA. Absorbance at 450 nm was measured, whereas absorbance at 570 nm was measured as a control. The binding amounts of anti-hPD-1scFv03 antibodies to a human PD-1-immobilized plate by ELISA are shown in Table 28 below.

[0260]

[Table 28]

SPx	Ly		Binding amount
			OD450−570
SP7	L20	SP7L20−hPD−1scFv03	1.728
SP7	L5	SP7L5−hPD−1scFv03	2.861
SP68	L20	SP68L20−hPD−1scFv03	2.358
SP68	L5	SP68L5−hPD−1scFv03	3.119
SP69	L20	SP69L20−hPD−1scFv03	2.332
SP69	L5	SP69L5−hPD−1scFv03	3.643

[0261]

(Results)

As is apparent from Table 28, in any case of SP7, SP68 and SP69, the binding amount of anti-hPD-1scFv03 antibody to the human PD-1-immobilized plate was larger in linker length 5 (L5) than in linker length 20 (L20). From this, it was demonstrated that the binding amount of anti-hPD-1scFv03 antibody to human PD-1 can be increased by optimizing the length of the linker.

[0262]

[Example 30]

(Investigation on linker length L = 0 to 10)

With respect to five types of signal peptides, namely, SP45, SP50, SP64, SP68 and SP69, investigation on linker length was conducted. *Bifidobacterium longum* 105-A strains were prepared by transforming them with anti-hPD-1scFv03 secretory plasmids in which secretory signal peptide-linker conjugates represented by SPxLy (note that,

if x = 45, y = 0, 1, 2, 3, 5; if x = 50, 64, 68, y = 0, 1, 2, 3, 4, 5; and if x = 69, y = 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10) were inserted. Secretion of the scFv antibodies was analyzed by Western Blotting using an anti-histidine tag attached antibody. The results are shown in Figure 26.

[0263]

(Results)

As is apparent from Figure 26, no secretion was observed in *Bifidobacterium longum* 105-A strain transformed with an anti-hPD-1scFv03 secretory plasmid in which SP69L0 secretory signal peptide-linker conjugate was inserted (see, Figure 26(c)). Except this case, secretion was observed. In other words, secretion was observed in *Bifidobacterium longum* 105-A strains transformed with an anti-hPD-1scFv03 secretory plasmids in which secretory signal peptide-linker conjugates SP45L0, SP45L1, SP45L2, SP45L3, SP45L5, SP50L0, SP50L1, SP50L2, SP50L3, SP50L4, SP50L5, SP64L0, SP64L1, SP64L2, SP64L3, SP64L4, SP64L5, SP68L0, SP68L1, SP68L2, SP68L3, SP68L4, SP68L5, SP69L1, SP69L2, SP69L3, SP69L4, SP69L5, SP69L6, SP69L7, SP69L8, SP69L9 and SP69L10 were inserted. In the cases where SP68L0 and SP68L1 were used as a secretory signal peptide-linker conjugate, the amount of secretion was low (see, Figure 26(b)).

[0264]

[Example 31]

Bifidobacterium longum 105-A strains were prepared by transforming them with anti-hPD-1scFv03 secretory plasmids in which secretory signal peptide-linker conjugates, namely, SP50Ly (note that, y = 0, 1, 5),

SP64Ly (note that, y = 0, 5), SP67Ly (note that, y = 10), SP68Ly (note that, y = 1, 5), and SP69Ly (note that, y = 1, 7) were inserted. Secretion of the scFv antibodies was analyzed by Western Blotting using an anti-histidine tag attached antibody. The results are shown in Figure 27.

[0265]

(Results)

As is apparent from Figure 27, in any one of the strains transformed with anti-hPD-1scFv03 secretory plasmids in which secretory signal peptide-linker conjugates, namely, SP50L0, SP50L1, SP50L5, SP64L0, SP64L5, SP67L10, SP68L1, SP68L5, SP69L1, SP69L7 were inserted, secretion of an anti-hPD-1-scFv03 antibody was verified.

[0266]

[Example 32]

(Verification of competitive (binding) inhibitory activity of anti-hPD-1scFv03 to binding reaction between human PD-1 and PD-L1)

In the same manner as in Example 24, competitive (binding) inhibitory activity of scFv antibodies purified from hPD-1scFv03 secreting bacteria of the genus *Bifidobacterium* to PD-1/PD-L1 binding was verified. Absorbance at 450 nm was measured, whereas absorbance at 570 nm was measured as a control. Provided that the binding amount of PD-L1 in the absence of scFv was regarded as 100%, scFv concentration (IC50) at which 50% of binding between PD-1 and PD-L1 was inhibited was computationally obtained. The measurement results are shown in Table 29 below.

[0267]

[Table 29]

SP／Linker	IC50* (nM)
SP50L5	10. 9
SP64L5	23. 4
SP67L10	26. 9
SP68L1	21. 7
SP68L5	41. 1
SP69L1	21. 8
SP69L7	28. 8

[0268]

(Results)

As is apparent from Table 29, in the case of SP50L5, the binding between PD-1 and PD-L1 was inhibited at the lowest concentration. Comparison between SP68L1 and SP68L5 and between SP69L1 and SP69L7 having the same types of signal peptides (SP) and different linkers in length demonstrated that the signal peptide (SP) having a shorter linker length (L1) inhibits binding between PD-1 and PD-L1 at a lower concentration.

[0269]

[Example 33]

(Binding affinity)

Binding affinity of an anti-PD-1 antibody was analyzed by Biacore analysis (Biacore AB, Uppsala, Sweden). More specifically, the binding affinity of 7 samples (No.

1 to No. 7, n = 5) shown in Table 30 below was analyzed. As a ligand, human PD-1 Fc chimera (manufactured by R&D) was used and dissolved in acetate pH4.5 (manufactured by GE Healthcare) to prepare a solution having a concentration of a 5 µg/mL. The solution was applied and immobilized onto a sensor chip having a gold film-surface coated with dextran having a CM group introduced therein by a standard protein immobilization method (amine coupling method). The anti-human PD-1 scFv antibody serving as an analyte was diluted with HBS-EP buffer (manufactured by GE Healthcare) to prepare a solution having a concentration of 12.5, 25, 50, 100 or 200 nM. Measurement of kinetics was started from a low concentration and carried out at an addition time of 60 seconds and at a dissociation time at 60 seconds and a flow rate at 30 µL/minute, in accordance with a single cycle method. The dissociation conditions of samples after measurement were the addition time of Glycin 1.5 (manufactured by GE Healthcare): 60 seconds; and the flow rate: 30 µL/ minutes. The running buffer in this experiment was HBS-EP buffer. The results are shown in Table 30 below.

[0270]

[Table 30]

Sample	Name	ka (1/Ms)	kd (1/s)	KD (M)
No1	SP50L5 PD-1 scFv	417037.2	0.002666	6.39E-09
No2	SP64L5 PD-1 scFv	274449.6	0.003960	1.44E-08
No3	SP67L10 PD-1 scFv	359502.6	0.003186	8.86E-09
No4	SP68L1 PD-1 scFv	398089.3	0.003388	8.51E-09
No5	SP68L5 PD-1 scFv	291332.5	0.005457	1.87E-08
No6	SP69L1 PD-1 scFv	393708.8	0.003379	8.58E-09
No7	SP69L7 PD-1 scFv	238025.4	0.003652	1.53E-08

[0271]

(Results)

As is apparent from Table 30, in view of function as the antibody, SP50L5 was the most excellent since it had strong binding force (KD value is low) and slow dissociation rate (kd value is low) and subsequently SP67L10, SP68L1, SP69L1 (Sample No. 3, 4, 6) were excellent.

[0272]

[Example 34]

[Expression analysis of scFv of anti-hCTLA-4scFv02-FLAG secretion bacteria of the genus *Bifidobacterium* having signal peptides inserted]

Bifidobacterium longum 105-A/pP30SPxL20-hCTLA-4scFv02 strains (where x =7, 45, 50, 52, 55, 58, 64, 66, 67, 68 and 69) each was cultured for 18 hours. From the culture solution, a sample was prepared. Using a culture supernatant (80 μL), scFv antibody secretion was analyzed by Western Blotting using an anti-FLAG tag antibody. The results are shown in Figure 28.

[0273]

As is apparent from Figure 28, in *Bifidobacterium longum* 105-A strains transformed with anti-hCTLA-4scFv02 secretory plasmids in which 11 types of secretory signal peptide-linker conjugates ((SP7L20, SP45L20, SP50L20, SP52L20, SP55L20, SP58L20, SP64L20, SP66L20, SP67L20, SP68L20 and SP69L20) were inserted, secretion of antibodies was verified.

[0274]

Of them, in *Bifidobacterium longum* 105-A strains transformed with anti-hCTLA-4scFv02 secretory plasmids in which 11 types of secretory signal peptide-linker conjugates (SP50L20, SP52L20, SP64L20, SP67L20, SP68L20, SP69L20) were inserted, the amounts of secretion were relatively large.

[0275]

[Example 35]

[Verification of binding of anti-hCTLA-4scFv02 to human CTLA-4 by ELISA]

Binding of anti-hCTLA-4scFv02 purified from each of the culture supernatants of *Bifidobacterium longum* 105-A/pP30SPxL20-hCTLA-4scFv02 strains to human CTLA-4 was verified. Absorbance at 450 nm was measured, whereas absorbance at 570 nm was measured as a control. The ELISA results of binding to human CTLA-4 are shown in Table 31 below.

[0276]

[Table 31]

SP/Linker	Binding by ELISA
	A450-570
SP7L20	1.281
SP45L20	0.379
SP50L20	0.655
SP52L20	0.388
SP55L20	0.402
SP58L20	0.715
SP64L20	0.697
SP66L20	0.306
SP67L20	0.981
SP68L20	0.218
SP69L20	0.344

[0277]

(Results)

As is apparent from Table 31, the binding amount of scFv varied depending upon the type of SP/Linker. SP7L20 and SP67L20 showed relatively high values.

[0278]

[Example 36]

[Verification of competitive (binding) inhibitory activity of anti-hCTLA-4scFv02 against binding of human CTLA-4/human CD80 or human CTLA-4/human CD86]

To a microplate having human CTLA-4 immobilized thereto, a mixture of purified scFv (10 μg/mL) and human CD80 or human CD86 (1 μg/mL) was added. The amount of human CD80 or human CD86 bound to human CTLA-4 was measured when anti-hCTLA-4scFv02 obtained from a *Bifidobacterium longum* 105-A/pP30SPxL20-hCTLA-4scFv02 strain was used. Provided that the binding inhibition rate against human CD80 or human CD86 in the absence of anti-hCTLA-4scFv02 was regarded as 0%, the competitive inhibition rate against binding of human CTLA-4/human CD80 or binding of human CTLA-4/human CD86 in the presence of anti-hCTLA-4scFv02 was computationally obtained. The results are shown in Table 32 below.

[0279]

[Table 32]

SP/Linker	Competitive inhibition rate (%)	
	hCD80	hCD86
SP7L20	98.0	94.8
SP45L20	72.3	81.9
SP50L20	80.2	88.5
SP52L20	77.9	85.6
SP55L20	80.8	88.8
SP58L20	97.3	98.7
SP64L20	97.0	98.8
SP66L20	91.4	94.8
SP67L20	100	100
SP68L20	92.3	87.8
SP69L20	97.9	96.2

[0280]

(Results)

In the case where the linker length was 20, no big difference was observed between the signal peptides.

[0281]

[Example 37]

In the case of an anti-hCTLA-4scFv02 antibody, the length of a linker to be used with each of signal peptides

of SP45, SP50, SP64, SP68, SP69 was investigated. *Bifidobacterium longum* 105-A strains, which were transformed with anti-hCTLA-4scFv02 secretory plasmids in which secretory signal peptide-linker conjugates represented by SPxLy (note that, if x = 45, 50, 64, 68, y = 0, 1, 2, 3, 4, 5; and if x = 69, y = 0, 1, 2, 3, 4, 5,6,7,8,9,10) were inserted, were prepared and cultured for 18 hours. From the culture solutions, samples were prepared. Secretion of scFv antibody was analyzed by Western Blotting using an anti-FLAG tag antibody by using the culture supernatant of 100 μL. The results are shown in Figures 29(a) to 29(c).

[0282]

(Results)

As is apparent form Figure 29, secretion of anti-hCTLA-4scFv02 was verified in *Bifidobacterium longum* 105-A strains transformed with vectors containing expression cassettes in which anti-hCTLA-4scFv02 was integrated downstream secretory signal peptides and secretory signal peptide-linker conjugates represented by SP45L0, SP45L1, SP45L2, SP45L3, SP45L4, SP45L5, SP50L0, SP50L1, SP50L2, SP50L3, SP50L4, SP50L5, SP64L0, SP64L1, SP64L2, SP64L3, SP64L4, SP64L5, SP68L1, SP68L2, SP68L3, SP68L4, SP68L5, SP69L0, SP69L1, SP69L2, SP69L3, SP69L4, SP69L5, SP69L6, SP69L7, SP69L8, SP69L9 and SP69L10 except S68L0.

Industrial Applicability

[0283]

The bacterium of the genus *Bifidobacterium* and pharmaceutical composition of the present invention are useful in the field of medicines.

CLAIMS

1. An expression cassette expressed in a bacterium of the genus *Bifidobacterium*, sequentially comprising the following DNAs (1) to (4).

(1) a promoter DNA functioning in the bacterium of the genus *Bifidobacterium*;

(2) a DNA encoding a secretory signal peptide consisting of an amino acid sequence represented by the following a) or b);

a) an amino acid sequence represented by any one of SEQ ID Nos. 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16;

b) an amino acid sequence obtained by deletion, replacement or addition of one or several amino acids in the amino acid sequence represented by any one of SEQ ID Nos: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16, wherein a peptide consisting of the amino acid sequence functions as a secretory signal peptide in the bacterium of the genus *Bifidobacterium*;

(3) a DNA encoding a heterogeneous polypeptide; and

(4) a terminator DNA functioning in the bacterium of the genus *Bifidobacterium*.

2. The expression cassette according to claim 1, wherein a DNA encoding a linker peptide is linked downstream of a DNA encoding a secretory signal peptide.

3. The expression cassette according to claim 2, wherein the linker peptide consists of each amino acid sequence obtained by deletion of 0 to 29 amino acid residues from a

C terminal of an amino acid sequence represented by any one of SEQ ID Nos: 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 and 35.

4. The expression cassette according to claim 2, wherein the linker peptide consists of each amino acid sequence obtained by deletion of 10 to 29 amino acid residues from a C terminal of an amino acid sequence represented by any one of SEQ ID Nos: 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 and 35.

5. The expression cassette according to any one of claims 1 to 4, wherein the heterogeneous polypeptide is a single-chain antibody.

6. The expression cassette according to claim 5, wherein the single-chain antibody is an anti-PD-1 antibody.

7. The expression cassette according to claim 5, wherein the single-chain antibody is an anti-CTLA-4 antibody.

8. A vector comprising the expression cassette according to any one of claims 1 to 7.

9. A bacterium of the genus *Bifidobacterium* transformed with the vector according to claim 8.

10. The bacterium of the genus *Bifidobacterium* according to claim 9, which is *Bifidobacterium longum*.

11. A pharmaceutical composition comprising the bacterium of the genus *Bifidobacterium* according to claim 9 or 10.

12. A secretory signal peptide consisting of the amino acid sequence represented by any one of SEQ ID Nos: 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16.

13. A DNA encoding the secretory signal peptide according to claim 12.

14. A secretory signal peptide-linker conjugate consisting of each amino acid sequence obtained by deletion of 0 to 29 amino acid residues from a C terminal of an amino acid sequence represented by any one of SEQ ID Nos: 105, 106, 107, 108, 109, 110, 111, 112, 113, 114 and 115.

15. The secretory signal peptide-linker conjugate consisting of each amino acid sequence obtained by deletion of 10 to 29 amino acid residues from a C terminal of an amino acid sequence represented by any one of SEQ ID Nos: 105, 106, 107, 108, 109, 110, 111, 112, 113, 114 and 115.

16. A DNA encoding the secretory signal peptide-linker conjugate according to claim 14 or 15.

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aag ctg tgg atc tac cgt acc tcg aac ctg gcc tcc ggc gtg ccg tcc      576
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cgc ttc tcg ggc tcc ggc tcg ggc acc agc tac tgc ctg acc atc aac      624
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Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly
        115                 120                 125

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    130                 135                 140

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Ser Leu Ser Ala Ser Val Gly Glu Thr Val Thr Leu Thr Cys Arg Ala
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Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Gln Tyr Ser Leu
        195                 200                 205

aag atc aac tcc ctg cag ccg gaa gac ttc ggc tcg tac tac tgc cag      672
Lys Ile Asn Ser Leu Gln Pro Glu Asp Phe Gly Ser Tyr Tyr Cys Gln
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Thr Ile Arg Asp Val Arg Ala Glu Asp Glu Gly Asp Tyr Tyr Cys Phe
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Met Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly
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cgt tcc ctg cgt ctg tcg tgc gcc gcc tcg ggc ttc acc ttc tcc agc       96
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Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser
            20                  25                  30

tac ggc atg cac tgg gtg cgt cag gcc ccg ggc aag ggc ctg gag tgg      144
Tyr Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp
        35                  40                  45

gtg gcc gtc atc tgg tac gac ggc tcc aac aag tac tac gcc gat tcc      192
Val Ala Val Ile Trp Tyr Asp Gly Ser Asn Lys Tyr Tyr Ala Asp Ser
    50                  55                  60

gtc aag ggc cgc ttc acc atc tcg cgt gac aac tcc aag aac acc ctg      240
Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu
65                  70                  75                  80

tac ctg cag atg aac tcc ctg cgt gcc gaa gac acc gcc gtg tac tac      288
Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr
                85                  90                  95

tgc gcc cgc gat ccg cgt ggc gcc acc ctg tac tac tac tac tac ggc      336
Cys Ala Arg Asp Pro Arg Gly Ala Thr Leu Tyr Tyr Tyr Tyr Tyr Gly
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atg gat gtc tgg ggc cag ggc acc acc gtg acc gtc tcg tcc ggc ggc      384
Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly
        115                 120                 125

ggc ggc tcc ggc ggc ggc ggc agc ggc ggc ggc ggc tcc gac atc cag      432
Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln
    130                 135                 140

atg acc cag tcc ccg agc tcg ctg agc gcc tcg gtg ggc gat cgc gtc      480
Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val
145                 150                 155                 160

acc atc acc tgc cgt gcc tcc cag agc atc aac tcc tac ctg gac tgg      528
Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Asn Ser Tyr Leu Asp Trp
                165                 170                 175

tac cag cag aag ccg ggc aag gcc ccg aag ctg ctg atc tac gcc gcc      576
Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Ala Ala
            180                 185                 190

tcc agc ctg cag agc ggc gtg ccg tcg cgc ttc tcg ggc tcc ggc agc      624
Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser
        195                 200                 205

ggc acc gac ttc acc ctg acc atc tcg tcc ctg cag ccg gag gat ttc      672
Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe
    210                 215                 220

gcc acc tac tac tgc cag cag tac tac tcc acc ccg ttc acc ttc ggc      720
Ala Thr Tyr Tyr Cys Gln Gln Tyr Tyr Ser Thr Pro Phe Thr Phe Gly
225                 230                 235                 240

ccg ggc acc aag gtc gag atc aag cat cat cac cat cat cac tga          765
Pro Gly Thr Lys Val Glu Ile Lys His His His His His His
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<223> scFv-CTLA-4-2

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Met Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly
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cgt agc ctg cgt ctg tcg tgc gcc gcc tcg ggc ttc acc ttc tcc agc       96
Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser
            20                  25                  30

tac acc atg cac tgg gtg cgt cag gcc ccg ggc aag ggc ctg gag tgg      144
Tyr Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp
        35                  40                  45

gtc acc ttc atc tcc tac gac ggc aac aac aag tac tac gcc gat tcc      192
Val Thr Phe Ile Ser Tyr Asp Gly Asn Asn Lys Tyr Tyr Ala Asp Ser
    50                  55                  60

gtg aag ggc cgc ttc acc atc tcc cgt gac aac agc aag aac acc ctg      240
Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu
65                  70                  75                  80

tac ctg cag atg aac tcc ctg cgc gcc gaa gac acc gcc atc tac tac      288
Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Ile Tyr Tyr
                85                  90                  95

tgc gcc cgt acc ggc tgg ctg ggc ccg ttc gat tac tgg ggc cag ggc      336
Cys Ala Arg Thr Gly Trp Leu Gly Pro Phe Asp Tyr Trp Gly Gln Gly
            100                 105                 110

acc ctg gtg acc gtc tcg tcg ggc ggc ggc ggc tcg ggc ggc ggc ggc      384
Thr Leu Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly
        115                 120                 125

tcc ggc ggc ggc ggc agc gag atc gtg ctg acc cag tcc ccg ggc acc      432
Ser Gly Gly Gly Gly Ser Glu Ile Val Leu Thr Gln Ser Pro Gly Thr
    130                 135                 140

ctg tcg ctg tcc ccg ggc gaa cgc gcc acc ctg tcc tgc cgt gcc agc      480
Leu Ser Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser
145                 150                 155                 160

cag tcg gtc ggc agc tcg tac ctg gcc tgg tac cag cag aag ccg ggc      528
Gln Ser Val Gly Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly
                165                 170                 175

cag gcc ccg cgt ctg ctg atc tac ggc gcc ttc tcc cgt gcc acc ggc      576
Gln Ala Pro Arg Leu Leu Ile Tyr Gly Ala Phe Ser Arg Ala Thr Gly
            180                 185                 190

atc ccg gac cgt ttc tcc ggc agc ggc tcg ggc acc gat ttc acc ctg      624
Ile Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu
        195                 200                 205

acc atc tcc cgc ctg gag ccg gaa gat ttc gcc gtc tac tac tgc cag      672
Thr Ile Ser Arg Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln
    210                 215                 220

cag tac ggc tcc agc ccg tgg acc ttc ggc cag ggc acc aag gtg gaa      720
Gln Tyr Gly Ser Ser Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
225                 230                 235                 240

atc aag cat cat cat cat cat cac tga                                  747
Ile Lys His His His His His His
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<220>
<223> SP7

<400> 6

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Ala

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<211> 34
<212> PRT
<213> Bifidobacterium spp.

<220>
<223> SP45

<400> 7

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Ser Thr Leu Ser Leu Ala Leu Ala Val Ile Ser Leu Pro His Gln Ala
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Tyr Ala

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<212> PRT
<213> Bifidobacterium spp.

<220>
<223> SP50

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Arg Lys Gly Arg Met Met Ile Thr Thr Trp Arg Gln Arg Gly Met Ala
20 25 30

Ile Val Ala Met Leu Thr Gly Leu Ile Ile Met Val Gly Val Val Phe
35 40 45

Gly Ser Ala Asn Thr Ala Tyr Ala
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<212> PRT
<213> Bifidobacterium spp.

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<400> 9

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Ala Met Ala
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<212> PRT
<213> Bifidobacterium spp.

<220>
<223> SP55

<400> 10

Met Val Arg Arg Ala Gln Ala Ala Leu Arg Ser Val Glu His Thr Ser
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Val Arg Gln Leu Arg Val Leu Ala Ala Ile Val Phe Leu Phe Ala Met
20 25 30

Val Ile Ala Thr Val Ala Pro Ala Val Thr Ala Pro Met Ala Phe Ala
35 40 45

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Thr Met Leu Gly Ile Ala Gly Leu Gly Ala Thr Thr Ala Ser Ala
20 25 30

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<213> Bifidobacterium spp.

<220>
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<400> 13

Met Lys His Trp Lys Lys Met Ala Ala Ser Leu Ile Ala Ile Ser Thr
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Met Val Ala Val Val Pro Thr Thr Tyr Ala
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<220>
<223> SP67

<400> 14

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Ala Ala Ala Thr Leu Leu Ser Cys Gly Leu Ala Ala Ala Ser Ala Ser
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Ala

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<212> PRT
<213> Bifidobacterium spp.

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<223> SP68

<400> 15

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Val Ala Ala Ala Ala Ala Ser Val Leu Cys Cys Met Gly Ala Val Phe
20 25 30

Pro Ala Thr Ile Gly Val Thr Ala Ala Ser Ala
35 40

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<212> PRT
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<223> SP69

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Ser Thr Cys Gly Leu Ile Leu Ala Pro Met Pro Val Phe Ala
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<212> DNA
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gcc agt ttc atg tgc atg gcg caa acc tcg ttc ttc gcc cag aat ttt 96
Ala Ser Phe Met Cys Met Ala Gln Thr Ser Phe Phe Ala Gln Asn Phe
20 25 30

gcg cag gat tcc gca gga tac acg ctg atg gtc ctc tgc acc ttg ctg 144
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35 40 45

gca 147
Ala

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<212> DNA
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<222> (1)..(138)
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1 5 10 15

ctg tta ctg ccg cag gtc agc tcc gac gcg gcc aaa atc acc ggt ggc 96
Leu Leu Leu Pro Gln Val Ser Ser Asp Ala Ala Lys Ile Thr Gly Gly
20 25 30

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Tyr Ala Ala Thr Gly Ser Ala Ala Asp Thr Leu Asn Ala Leu
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<212> DNA
<213> Bifidobacterium spp.

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<222> (1)..(192)
<223> SP62L20

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Met Thr Arg Ser Asp Asp Val Val Asn Glu Asp Gly His Asp Val Ile
1 5 10 15

gcc gca cac act cgc gtt gtc agg ggt atg gct gcg gca ctt gcc gcg 96
Ala Ala His Thr Arg Val Val Arg Gly Met Ala Ala Ala Leu Ala Ala
20 25 30

ata gcc gcc aca gga gcc ggc gtc aca gcc gcg gcc gcc aac gtc atg 144
Ile Ala Ala Thr Gly Ala Gly Val Thr Ala Ala Ala Ala Asn Val Met
35 40 45

ttc acg ttc gcg ctg gac acc aag gcc aaa cgt tcg atg ttc aac atg 192
Phe Thr Phe Ala Leu Asp Thr Lys Ala Lys Arg Ser Met Phe Asn Met
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<220>
<223> Linker peptide (SP7)

<400> 25

Asp Ser Ala Gln Thr Ser Ala Val Val Ser Ser Arg Ser Phe Pro Lys
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Ala Ser Ser Val Lys Lys Asn Leu Phe Ala Glu Ser Thr Ser
20 25 30

<210> 26
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<212> PRT
<213> Bifidobacterium spp.

<220>
<223> Linker peptide (SP45)

<400> 26

Val Asp Gly Thr Asp Gly Thr Ser Gly Thr Asn Ser Thr Ser Gln Gly
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Ser Asp Gly Asp Ser Ala Pro Ile Ala Gly Pro Val Pro Asn
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<213> Bifidobacterium spp.

<220>
<223> Linker peptide (SP50)

<400> 27

Ala Thr Leu Thr Pro Ala Asp Glu Arg Tyr His Val Ala Phe Pro Tyr

1 5 10 15

Asn Asp Met Glu Tyr Tyr Val Gly Val Ala Gly Leu Asp Ala
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<220>
<223> Linker peptide (SP52)

<400> 28

Ala Asp Ser Ala Thr Ala Asp Asn Ala Pro Ser Val Ala Gly His Ala
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Tyr Asn Glu Leu Pro Tyr Asn Asn Pro Asp Val Thr Val Thr
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<400> 29

Asp Ser Ser Thr Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Val Asp
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Tyr Ala Thr Trp Ala Glu Val Ser Lys Ala Met Asp Lys Gln
20 25 30

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Gly Ser Phe Ala Phe Thr Ala Arg Lys Ser Val Ala Leu Asn Ile Asn
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Gly Gln Thr Thr Gln Val Thr Thr Tyr Ala Met Thr Ala Thr
20 25 30

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Glu Asp Ala Thr Gly Thr Leu Thr Val Thr Ser Ser Asp Ala Ala Phe
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Asn Gly Lys Lys Val Asn Ala Tyr Gln Met Phe Ser Ala Ser
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Met Glu Ser Glu Asp Ser Gln Pro Gln Thr Thr Asp Thr Ala Thr Val
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Gln Thr Thr Lys Ala Ala Glu Pro Thr Leu Leu Ala Ser Trp
20 25 30

<210> 33
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<220>
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<400> 33

Ala Gly Val Asp Tyr Leu Pro Thr Ile Gly Gln Val Pro Thr Tyr Thr
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Lys Phe Gln Pro Thr Ala Asp Pro Gly Lys Asn Ala Ser Asp
20 25 30

<210> 34
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<220>
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<400> 34

Asp Glu Pro Val Glu Leu Val Val Asn Gly Gly Phe Glu Asp Asp Leu
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Asn Gly Trp Lys Ser Gly Thr Val Trp Asn Ser Ser Ala Ser
20 25 30

<210> 35
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<213> Bifidobacterium spp.

<220>
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1 5 10 15

Thr Asp Ser Gly Asp Gly Thr Tyr Ser Ile Pro Met Leu Asn
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Ile Ile Ala Thr Gly Ala Met Phe Ala Cys Val Ala Pro Phe Ala Ser
20 25 30

gcc gat tcc gcg cag acg agt gct gtg gtg tcc tca cgt tct ttc ccg 144
Ala Asp Ser Ala Gln Thr Ser Ala Val Val Ser Ser Arg Ser Phe Pro
35 40 45

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gggccgcttc accatctcgc gcgacaactc caagaacacc ctgttcctgc agatgaactc 780

cctgcgcgcc gaagacaccg ccgtgtacta ctgcgccacc aacgatgact actggggcca 840

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cggcggcagc gaaatcgtgc tgacccagtc cccggccacc ctgtccctgt ccccgggcga 960

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gaaacgcagg ctgtcgggct tcgtgttcac attcgcccgc gagacccctc cggtgatcga 2460

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gtatttggta tctgcgctct gctgaagcca gttaccttcg gaaaaagagt tggtagctct 4620

tgatccggca aacaaaccac cgctggtagc ggtggttttt ttgtttgcaa gcagcagatt 4680

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<210> 89
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 89
aaagcatcct tcttgggtca gg 22

<210> 90
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 90
caggtccagc tggtcgaatc 20

<210> 91
<211> 43
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 91
caagaaggat gctttatgaa ttatttacga caaaaaattt cgg 43

<210> 92
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 92
gaccagctgg acctgaccgc tatcagtcgt ggtgtaac 38

<210> 93
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 93
gaccagctgg acctgtggcg ttgaatcatc cgc 33

<210> 94
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 94
gaccagctgg acctgcgatg gcgttgaaga tgg 33

<210> 95
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 95
gaccagctgg acctgggtgt aactgccatc cgatg 35

<210> 96
<211> 32
<212> DNA
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<220>
<223> primer

<400> 96
gaccagctgg acctgcgcaa agaccggcat tg 32

<210> 97
<211> 33
<212> DNA
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<220>
<223> primer

<400> 97
gaccagctgg acctgatccg caaagaccgg cat 33

<210> 98
<211> 33
<212> DNA
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<220>
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<400> 98
gaccagctgg acctgatcat ccgcaaagac cgg 33

<210> 99
<211> 35
<212> DNA
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<220>
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<400> 99
gaccagctgg acctgtgaat catccgcaaa gaccg 35

<210> 100
<211> 36
<212> DNA
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<220>
<223> primer

<400> 100
gaccagctgg acctgcgttg aatcatccgc aaagac 36

<210> 101
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 101
gaccagctgg acctgagatg gcgttgaatc atccg 35

<210> 102
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 102
gaccagctgg acctgtgaag atggcgttga atcatcc 37

<210> 103
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 103
gaccagctgg acctgcgttg aagatggcgt tgaatc 36

<210> 104
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 104
gaccagctgg acctgtggcg ttgaagatgg cg 32

<210> 105
<211> 63
<212> PRT
<213> Bifidobacterium spp.

<220>
<223> SP7L30

<400> 105

Met Ala Leu Met Met Ser Val Lys Thr Ile Ile Ser Thr Ser Val Ala
1 5 10 15

Ile Ile Ala Thr Gly Ala Met Phe Ala Cys Val Ala Pro Phe Ala Ser
20 25 30

Ala Asp Ser Ala Gln Thr Ser Ala Val Val Ser Ser Arg Ser Phe Pro
35 40 45

Lys Ala Ser Ser Val Lys Lys Asn Leu Phe Ala Glu Ser Thr Ser
50 55 60

<210> 106
<211> 64
<212> PRT
<213> Bifidobacterium spp.

<220>
<223> SP45L30

<400> 106

Met Lys His Leu Ser His Arg Thr Ile Ala Ile Ile Val Ala Leu Leu
1 5 10 15

Ser Thr Leu Ser Leu Ala Leu Ala Val Ile Ser Leu Pro His Gln Ala
20 25 30

Tyr Ala Val Asp Gly Thr Asp Gly Thr Ser Gly Thr Asn Ser Thr Ser
35 40 45

Gln Gly Ser Asp Gly Asp Ser Ala Pro Ile Ala Gly Pro Val Pro Asn

50 55 60

<210> 107
<211> 86
<212> PRT
<213> Bifidobacterium spp.

<220>
<223> SP50L30

<400> 107

Met Ile Val Ala Tyr Pro His Thr Val Gln Tyr Ala Gly Lys Arg Thr
1 5 10 15

Arg Lys Gly Arg Met Met Ile Thr Thr Trp Arg Gln Arg Gly Met Ala
20 25 30

Ile Val Ala Met Leu Thr Gly Leu Ile Ile Met Val Gly Val Val Phe
35 40 45

Gly Ser Ala Asn Thr Ala Tyr Ala Ala Thr Leu Thr Pro Ala Asp Glu
50 55 60

Arg Tyr His Val Ala Phe Pro Tyr Asn Asp Met Glu Tyr Tyr Val Gly
65 70 75 80

Val Ala Gly Leu Asp Ala
85

<210> 108
<211>
<212> PRT
<213> Bifidobacterium spp.

<220>
<223> SP52L30

<400> 108

Met Ser Phe His Val Ser Ala Gln Ser Val Arg Ala Val Ala Gly Gly
1 5 10 15

Leu Val Ala Ala Ala Thr Leu Leu Ser Gly Leu Ala Leu Ala Pro Thr
20 25 30

Ala Met Ala Ala Asp Ser Ala Thr Ala Asp Asn Ala Pro Ser Val Ala
35 40 45

Gly His Ala Tyr Asn Glu Leu Pro Tyr Asn Asn Pro Asp Val Thr Val
50 55 60

Thr
65

<210> 109
<211> 78
<212> PRT
<213> Bifidobacterium spp.

<220>
<223> SP55L30

<400> 109

Met Val Arg Arg Ala Gln Ala Ala Leu Arg Ser Val Glu His Thr Ser
1 5 10 15

Val Arg Gln Leu Arg Val Leu Ala Ala Ile Val Phe Leu Phe Ala Met
20 25 30

Val Ile Ala Thr Val Ala Pro Ala Val Thr Ala Pro Met Ala Phe Ala
35 40 45

Asp Ser Ser Thr Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Val Asp
50 55 60

Tyr Ala Thr Trp Ala Glu Val Ser Lys Ala Met Asp Lys Gln
65 70 75

<210> 110
<211> 62
<212> PRT
<213> Bifidobacterium spp.

<220>
<223> SP58L30

<400> 110

Met Ala Met Ala Arg Arg Trp Thr Pro Gln Arg Phe Val Thr Leu Arg
1 5 10 15

Arg Ile Arg Val Ile Ala Cys Ile Ala Ala Thr Ser Ile Ala Leu Ala
20 25 30

Gly Ser Phe Ala Phe Thr Ala Arg Lys Ser Val Ala Leu Asn Ile Asn
35 40 45

Gly Gln Thr Thr Gln Val Thr Thr Tyr Ala Met Thr Ala Thr
50 55 60

<210> 111
<211> 61
<212> PRT
<213> Bifidobacterium spp.

<220>
<223> SP64L30

<400> 111

Met Lys Ser Leu Ile Arg Asn Val Ala Ala Gly Val Leu Ala Ala Ala
1 5 10 15

Thr Met Leu Gly Ile Ala Gly Leu Gly Ala Thr Thr Ala Ser Ala Glu
20 25 30

Asp Ala Thr Gly Thr Leu Thr Val Thr Ser Ser Asp Ala Ala Phe Asn
35 40 45

Gly Lys Lys Val Asn Ala Tyr Gln Met Phe Ser Ala Ser
50 55 60

<210> 112
<211> 56
<212> PRT
<213> Bifidobacterium spp.

<220>
<223> SP66L30

<400> 112

Met Lys His Trp Lys Lys Met Ala Ala Ser Leu Ile Ala Ile Ser Thr
1 5 10 15

Met Val Ala Val Val Pro Thr Thr Tyr Ala Met Glu Ser Glu Asp Ser
20 25 30

Gln Pro Gln Thr Thr Asp Thr Ala Thr Val Gln Thr Thr Lys Ala Ala
35 40 45

Glu Pro Thr Leu Leu Ala Ser Trp
50 55

<210> 113
<211> 63
<212> PRT
<213> Bifidobacterium spp.

<220>
<223> SP67L30

<400> 113

Met Lys Ile Asn Asn Lys Gly Lys Gly Ala Leu Ile Ala Ala Ile Thr
1 5 10 15

Ala Ala Ala Thr Leu Leu Ser Cys Gly Leu Ala Ala Ala Ser Ala Ser
20 25 30

Ala Ala Gly Val Asp Tyr Leu Pro Thr Ile Gly Gln Val Pro Thr Tyr
35 40 45

Thr Lys Phe Gln Pro Thr Ala Asp Pro Gly Lys Asn Ala Ser Asp
50 55 60

<210> 114
<211> 73
<212> PRT
<213> Bifidobacterium spp.

<220>
<223> SP68L30

<400> 114

Met Val Tyr Asn Ile His Ile Leu Gln Thr Arg Lys Thr Gly Arg Val
1 5 10 15

Val Ala Ala Ala Ala Ala Ser Val Leu Cys Cys Met Gly Ala Val Phe
20 25 30

Pro Ala Thr Ile Gly Val Thr Ala Ala Ser Ala Asp Glu Pro Val Glu
35 40 @@ 45

Leu Val Val Asn Gly Gly Phe Glu Asp Asp Leu Asn Gly Trp Lys Ser
50 55 60

Gly Thr Val Trp Asn Ser Ser Ala Ser
65 70

<210> 115
<211> 60
<212> PRT
<213> Bifidobacterium spp.

<220>
<223> SP69L30

<400> 115

Met Asn Tyr Leu Arg Gln Lys Ile Ser Ala Ser Ala Ile Ala Val Leu
1 5 10 15

Ser Thr Cys Gly Leu Ile Leu Ala Pro Met Pro Val Phe Ala Asp Asp
20 25 30

Ser Thr Pro Ser Ser Thr Pro Ser Asp Gly Ser Tyr Thr Thr Thr Asp
35 40 45

Ser Gly Asp Gly Thr Tyr Ser Ile Pro Met Leu Asn

50 55 60

<210> 116

<211> 99
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(99)
<223> SP7

<400> 116
atg gcg ttg atg atg agc gtt aag act att att tcc aca tca gtg gcg 48
Met Ala Leu Met Met Ser Val Lys Thr Ile Ile Ser Thr Ser Val Ala
1 5 10 15

att atc gcc acg ggt gcc atg ttt gcg tgc gta gcc ccg ttt gcc tct 96
Ile Ile Ala Thr Gly Ala Met Phe Ala Cys Val Ala Pro Phe Ala Ser
20 25 30

gcc 99
Ala

<210> 117
<211> 102
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(102)
<223> SP45

<400> 117
atg aag cac ctc tcc cac cgc acc atc gcc atc atc gtc gcg ttg ctc 48
Met Lys His Leu Ser His Arg Thr Ile Ala Ile Ile Val Ala Leu Leu
1 5 10 15

tcc acg ctg tca ctg gca ctt gcc gtc atc tcc ctt ccg cac cag gcg 96
Ser Thr Leu Ser Leu Ala Leu Ala Val Ile Ser Leu Pro His Gln Ala
20 25 30

tac gca 102
Tyr Ala

<210> 118
<211> 168
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(168)
<223> SP50

<400> 118
atg atc gtg gcc tac ccg cac aca gtg cag tat gcg ggg aaa cgt acc 48
Met Ile Val Ala Tyr Pro His Thr Val Gln Tyr Ala Gly Lys Arg Thr
1 5 10 15

agg aaa gga cga atg atg ata acg aca tgg cgg caa cgg ggc atg gcc 96
Arg Lys Gly Arg Met Met Ile Thr Thr Trp Arg Gln Arg Gly Met Ala
20 25 30

atc gta gcg atg ctg acc ggt ctg ata ata atg gtg gga gtg gtg ttc 144
Ile Val Ala Met Leu Thr Gly Leu Ile Ile Met Val Gly Val Val Phe
35 40 45

ggc tcg gcg aat acg gcg tat gcc 168
Gly Ser Ala Asn Thr Ala Tyr Ala
50 55

<210> 119
<211> 105
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(105)
<223> SP52

<400> 119
atg agt ttc cat gta tcc gcg caa tcg gtt cgc gcg gtg gcc ggt gga 48
Met Ser Phe His Val Ser Ala Gln Ser Val Arg Ala Val Ala Gly Gly
1 5 10 15

ctc gtc gcc gca gcg aca ttg ctg tca ggc ctt gcc ctt gcg ccg acc 96
Leu Val Ala Ala Ala Thr Leu Leu Ser Gly Leu Ala Leu Ala Pro Thr
20 25 30

gca atg gcc 105
Ala Met Ala
35

<210> 120
<211> 144
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(144)
<223> SP55

<400> 120
atg gtt cgt cgc gcc cag gct gct ctt cgg tcc gtt gaa cac acc agc 48
Met Val Arg Arg Ala Gln Ala Ala Leu Arg Ser Val Glu His Thr Ser
1 5 10 15

gtg agg cag ctg cga gtc ctg gct gcg att gta ttt ctg ttc gcc atg 96
Val Arg Gln Leu Arg Val Leu Ala Ala Ile Val Phe Leu Phe Ala Met
20 25 30

gtc atc gcc act gtg gct cct gca gtc acg gct ccg atg gcc ttc gcc 144
Val Ile Ala Thr Val Ala Pro Ala Val Thr Ala Pro Met Ala Phe Ala
35 40 45

<210> 121
<211> 96
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(96)
<223> SP58

<400> 121
atg gca atg gca cgg cgt tgg acc ccg cag cgg ttt gtt aca ctg cgt 48
Met Ala Met Ala Arg Arg Trp Thr Pro Gln Arg Phe Val Thr Leu Arg
1 5 10 15

cgc atc cgc gta atc gcc tgt atc gcg gcg acc agt atc gcg ctg gct 96
Arg Ile Arg Val Ile Ala Cys Ile Ala Ala Thr Ser Ile Ala Leu Ala
20 25 30

<210> 122
<211> 93
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(93)
<223> SP64

<400> 122
atg aag tca cta atc agg aat gta gcg gcg ggc gtc ctc gca gcc gcc 48
Met Lys Ser Leu Ile Arg Asn Val Ala Ala Gly Val Leu Ala Ala Ala
1 5 10 15

acg atg ctc ggc atc gcc ggc ctc ggc gcc acc acc gcc tcc gcg 93
Thr Met Leu Gly Ile Ala Gly Leu Gly Ala Thr Thr Ala Ser Ala
20 25 30

<210> 123
<211> 78
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(78)
<223> SP66

<400> 123
gtg aag cat tgg aag aag atg gca gca tcg ttg att gca ata tca acg 48
Met Lys His Trp Lys Lys Met Ala Ala Ser Leu Ile Ala Ile Ser Thr
1 5 10 15

atg gtg gca gta gtt ccg acg acg tat gcc 78
Met Val Ala Val Val Pro Thr Thr Tyr Ala
20 25

<210> 124
<211> 99
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(99)
<223> SP67

<400> 124
atg aag ata aac aat aag ggc aag ggc gct ctt atc gcg gca att acc 48
Met Lys Ile Asn Asn Lys Gly Lys Gly Ala Leu Ile Ala Ala Ile Thr
1 5 10 15

gcc gcg gca acg cta ttg tca tgc ggg ctg gcc gct gca agt gcc agt 96
Ala Ala Ala Thr Leu Leu Ser Cys Gly Leu Ala Ala Ala Ser Ala Ser
20 25 30

gcg 99
Ala

<210> 125
<211> 129
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(129)
<223> SP68

<400> 125
atg gtt tat aac att cac ata ttg caa aca agg aaa acc ggt cgt gtg 48
Met Val Tyr Asn Ile His Ile Leu Gln Thr Arg Lys Thr Gly Arg Val
1 5 10 15

gtt gct gct gcg gct gca tcc gtg ctg tgt tgc atg ggg gct gta ttt 96
Val Ala Ala Ala Ala Ala Ser Val Leu Cys Cys Met Gly Ala Val Phe
20 25 30

cca gcg act atc gga gtg act gcg gcg tcg gcc 129
Pro Ala Thr Ile Gly Val Thr Ala Ala Ser Ala
35 40

<210> 126
<211> 90
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(90)
<223> SP69

<400> 126
atg aat tat tta cga caa aaa att tcg gct agt gct atc gcg gtg ttg 48
Met Asn Tyr Leu Arg Gln Lys Ile Ser Ala Ser Ala Ile Ala Val Leu
1 5 10 15

tcg act tgt ggg ttg att ttg gcg cca atg ccg gtc ttt gcg 90
Ser Thr Cys Gly Leu Ile Leu Ala Pro Met Pro Val Phe Ala
20 25 30

<210> 127
<211> 90
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(90)
<223> Linker peptide (SP7)

<400> 127
gat tcc gcg cag acg agt gct gtg gtg tcc tca cgt tct ttc ccg aag 48
Asp Ser Ala Gln Thr Ser Ala Val Val Ser Ser Arg Ser Phe Pro Lys
1 5 10 15

gcg agt tcg gtg aag aag aat ttg ttc gcc gaa tcc acc tcc 90
Ala Ser Ser Val Lys Lys Asn Leu Phe Ala Glu Ser Thr Ser
20 25 30

<210> 128
<211> 90
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(90)
<223> Linker peptide (SP45)

<400> 128
gtc gac ggc aca gac ggc aca agc ggc acg aac agt acg tct cag gga 48
Val Asp Gly Thr Asp Gly Thr Ser Gly Thr Asn Ser Thr Ser Gln Gly
1 5 10 15

agc gac ggc gat tcc gcg cca atc gcc ggc ccg gtg ccg aac 90
Ser Asp Gly Asp Ser Ala Pro Ile Ala Gly Pro Val Pro Asn
20 25 30

<210> 129
<211> 90
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(90)
<223> Linker peptide (SP50)

<400> 129
gcg acg ttg acg ccc gcc gac gaa aga tat cac gtg gcg ttt cca tac 48
Ala Thr Leu Thr Pro Ala Asp Glu Arg Tyr His Val Ala Phe Pro Tyr
1 5 10 15

aac gat atg gaa tat tac gtc ggt gtc gcg ggg ctg gac gct 90
Asn Asp Met Glu Tyr Tyr Val Gly Val Ala Gly Leu Asp Ala
20 25 30

<210> 130
<211> 90
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(90)
<223> Linker peptide (SP52)

<400> 130
gcc gat tca gcc acc gct gac aac gcg ccc agc gtt gcc ggt cac gcg 48
Ala Asp Ser Ala Thr Ala Asp Asn Ala Pro Ser Val Ala Gly His Ala
1 5 10 15

tat aac gaa ctg ccg tat aac aat cct gat gtc acc gtc acc 90
Tyr Asn Glu Leu Pro Tyr Asn Asn Pro Asp Val Thr Val Thr
20 25 30

<210> 131
<211> 90
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(90)
<223> Linker peptide (SP55)

<400> 131
gac agc agc acc agc tcg tcc agt tca tca agc tcg tcc agc gtt gac 48
Asp Ser Ser Thr Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Val Asp

1 5 10 15

tac gcc acc tgg gcc gaa gtc tcc aag gcg atg gac aag cag 90
Tyr Ala Thr Trp Ala Glu Val Ser Lys Ala Met Asp Lys Gln
20 25 30

<210> 132
<211> 90
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(90)
<223> Linker peptide (SP58)

<400> 132
gga tca ttc gcg ttc acc gcg cgt aaa tcc gtg gcc ctg aac atc aac 48
Gly Ser Phe Ala Phe Thr Ala Arg Lys Ser Val Ala Leu Asn Ile Asn
1 5 10 15

ggg caa acc acc caa gtc acc aca tat gcg atg acg gcc acc 90
Gly Gln Thr Thr Gln Val Thr Thr Tyr Ala Met Thr Ala Thr
20 25 30

<210> 133
<211> 90
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(90)
<223> Linker peptide (SP64)

<400> 133
gaa gat gct acc ggc acg ctg acc gtc acc agc tcg gat gcc gcg ttc 48
Glu Asp Ala Thr Gly Thr Leu Thr Val Thr Ser Ser Asp Ala Ala Phe
1 5 10 15

aac ggc aag aag gtg aac gcc tac cag atg ttc tcc gct tcc 90
Asn Gly Lys Lys Val Asn Ala Tyr Gln Met Phe Ser Ala Ser
20 25 30

<210> 134
<211> 90
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(90)
<223> Linker peptide (SP66)

<400> 134
atg gaa tcg gaa gat tcc caa cca cag aca acc gat acc gcg aca gtg 48
Met Glu Ser Glu Asp Ser Gln Pro Gln Thr Thr Asp Thr Ala Thr Val
1 5 10 15

cag act act aag gct gct gaa ccg acg ctg ctc gcc agc tgg 90
Gln Thr Thr Lys Ala Ala Glu Pro Thr Leu Leu Ala Ser Trp
20 25 30

<210> 135

<211> 90
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(90)
<223> Linker peptide (SP67)

<400> 135
gca ggt gtg gat tac ctg cct acc atc ggc caa gtg ccg aca tac acc 48
Ala Gly Val Asp Tyr Leu Pro Thr Ile Gly Gln Val Pro Thr Tyr Thr
1 5 10 15

aag ttc cag ccc aca gcc gat ccg ggc aag aac gct agc gat 90
Lys Phe Gln Pro Thr Ala Asp Pro Gly Lys Asn Ala Ser Asp
20 25 30

<210> 136
<211> 90
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(90)
<223> Linker peptide (SP68)

<400> 136
gat gaa ccc gtc gaa ttg gtc gtc aac ggc gga ttc gaa gac gat ctt 48
Asp Glu Pro Val Glu Leu Val Val Asn Gly Gly Phe Glu Asp Asp Leu
1 5 10 15

aat ggt tgg aag tct gga acg gtg tgg aat agc tcc gca tcg 90
Asn Gly Trp Lys Ser Gly Thr Val Trp Asn Ser Ser Ala Ser
20 25 30

<210> 137
<211> 90
<212> DNA
<213> Bifidobacterium spp.

<220>
<221> CDS
<222> (1)..(90)
<223> Linker peptide (SP69)

<400> 137
gat gat tca acg cca tct tca acg cca tcg gat ggc agt tac acc acg 48
Asp Asp Ser Thr Pro Ser Ser Thr Pro Ser Asp Gly Ser Tyr Thr Thr
1 5 10 15

act gat agc ggt gat ggc acg tat tcc att ccc atg ttg aac 90
Thr Asp Ser Gly Asp Gly Thr Tyr Ser Ile Pro Met Leu Asn
20 25 30

<210> 138
<211> 245
<212> PRT
<213> Homo sapiens

<220>
<223> scFv-PD-1-1

<400> 138

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Met Gln Val Gln Leu Val Gln Ser Gly Ser Glu Leu Lys Lys Pro Gly
1               5                   10                  15

Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn
            20                  25                  30

Tyr Gly Met Asn Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Gln Trp
        35                  40                  45

Met Gly Trp Ile Asn Thr Asp Ser Gly Glu Ser Thr Tyr Ala Glu Glu
    50                  55                  60

Phe Lys Gly Arg Phe Val Phe Ser Leu Asp Thr Ser Val Asn Thr Ala
65                  70                  75                  80

Tyr Leu Gln Ile Thr Ser Leu Thr Ala Glu Asp Thr Gly Met Tyr Phe
                85                  90                  95

Cys Val Arg Val Gly Tyr Asp Ala Leu Asp Tyr Trp Gly Gln Gly Thr
            100                 105                 110

Leu Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
        115                 120                 125

Gly Gly Gly Gly Ser Glu Ile Val Leu Thr Gln Ser Pro Ser Ser Leu
    130                 135                 140

Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Arg Ser
145                 150                 155                 160

Ser Val Ser Tyr Met His Trp Phe Gln Gln Lys Pro Gly Lys Ala Pro
                165                 170                 175

Lys Leu Trp Ile Tyr Arg Thr Ser Asn Leu Ala Ser Gly Val Pro Ser
            180                 185                 190

Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Cys Leu Thr Ile Asn
        195                 200                 205

Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Arg Ser
    210                 215                 220

Ser Phe Pro Leu Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys His
225                 230                 235                 240

His His His His His
                245
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<210> 139
<211> 249
<212> PRT
<213> homo sapiens

<220>
<223> scFv-PD-1-2

<400> 139

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Met Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser
1               5                  10                  15

Gln Ser Leu Ser Leu Thr Cys Thr Val Thr Gly His Ser Ile Thr Ser
            20                  25                  30

Asp Tyr Ala Trp Asn Trp Ile Arg Gln Phe Pro Gly Asp Lys Leu Glu
        35                  40                  45

Trp Met Gly Tyr Ile Ser Tyr Ser Gly Tyr Thr Thr Tyr Asn Pro Ser
    50                  55                  60

Leu Lys Ser Arg Val Ser Ile Thr Arg Asp Thr Ser Lys Asn Gln Phe
65                  70                  75                  80

Phe Leu Gln Leu Asn Ser Val Thr Thr Glu Asp Thr Ala Thr Tyr Phe
                85                  90                  95

Cys Ala Arg Asp Leu Asp Tyr Gly Pro Trp Phe Ala Tyr Trp Gly Gln
            100                 105                 110

Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly
        115                 120                 125

Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Met Thr Gln Ser Pro Ala
    130                 135                 140

Ser Leu Ser Ala Ser Val Gly Glu Thr Val Thr Leu Thr Cys Arg Ala
145                 150                 155                 160

Ser Glu Asn Ile His Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Gln Gly
                165                 170                 175

Lys Ser Pro Gln Leu Leu Val Tyr Asn Val Lys Thr Leu Ala Asp Gly
            180                 185                 190

Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Gln Tyr Ser Leu
        195                 200                 205

Lys Ile Asn Ser Leu Gln Pro Glu Asp Phe Gly Ser Tyr Tyr Cys Gln
    210                 215                 220

His Phe Trp Ser Ser Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu
225                 230                 235                 240

Ile Lys Arg His His His His His His
                245
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<210> 140
<211> 253
<212> PRT
<213> Mus musculus

<220>
<223> scFv-PD-1-3

<400> 140

Met Glu Val Arg Leu Leu Glu Ser Gly Gly Gly Leu Val Lys Pro Glu
1 5 10 15

Gly Ser Leu Lys Leu Ser Cys Val Ala Ser Gly Phe Thr Phe Ser Asp
20 25 30

Tyr Phe Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp
35 40 45

Val Ala His Ile Tyr Thr Lys Ser Tyr Asn Tyr Ala Thr Tyr Tyr Ser
50 55 60

Gly Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Arg Ser
65 70 75 80

Met Val Tyr Leu Gln Met Asn Asn Leu Arg Thr Glu Asp Thr Ala Thr
85 90 95

Tyr Tyr Cys Thr Arg Asp Gly Ser Gly Tyr Pro Ser Leu Asp Phe Trp
100 105 110

Gly Gln Gly Thr Gln Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly
115 120 125

Gly Gly Gly Ser Gly Gly Gly Gly Ser Tyr Glu Leu Thr Gln Pro Pro
130 135 140

Ser Ala Ser Val Asn Val Gly Glu Thr Val Lys Ile Thr Cys Ser Gly
145 150 155 160

Asp Gln Leu Pro Lys Tyr Phe Ala Asp Trp Phe His Gln Arg Ser Asp
165 170 175

Gln Thr Ile Leu Gln Val Ile Tyr Asp Asp Asn Lys Arg Pro Ser Gly
180 185 190

Ile Pro Glu Arg Ile Ser Gly Ser Ser Ser Gly Thr Thr Ala Thr Leu
195 200 205

Thr Ile Arg Asp Val Arg Ala Glu Asp Glu Gly Asp Tyr Tyr Cys Phe
210 215 220

Ser Gly Tyr Val Asp Ser Asp Ser Lys Leu Tyr Val Phe Gly Ser Gly
225 230 235 240

Thr Gln Leu Thr Val Leu Gly His His His His His His
245 250

<210> 141
<211> 254
<212> PRT
<213> Homo sapiens

<220>
<223> scFv-CTLA-4-1

<400> 141

Met Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly
1 5 10 15

Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser
20 25 30

Tyr Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp
35 40 45

Val Ala Val Ile Trp Tyr Asp Gly Ser Asn Lys Tyr Tyr Ala Asp Ser
50 55 60

Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu
65 70 75 80

Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr
85 90 95

Cys Ala Arg Asp Pro Arg Gly Ala Thr Leu Tyr Tyr Tyr Tyr Tyr Gly
100 105 110

Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly
115 120 125

Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln
130 135 140

Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val
145 150 155 160

Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Asn Ser Tyr Leu Asp Trp
165 170 175

Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Ala Ala
180 185 190

Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser
195 200 205

Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe
210 215 220

Ala Thr Tyr Tyr Cys Gln Gln Tyr Tyr Ser Thr Pro Phe Thr Phe Gly
225 230 235 240

Pro Gly Thr Lys Val Glu Ile Lys His His His His His His
245 250

<210> 142
<211> 248
<212> PRT
<213> Homo sapiens

<220>
<223> scFv-CTLA-4-2

<400> 142

Met Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly
1 5 10 15

Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser
20 25 30

Tyr Thr Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp
35 40 45

Val Thr Phe Ile Ser Tyr Asp Gly Asn Asn Lys Tyr Tyr Ala Asp Ser
50 55 60

Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu
65 70 75 80

Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Ile Tyr Tyr
85 90 95

Cys Ala Arg Thr Gly Trp Leu Gly Pro Phe Asp Tyr Trp Gly Gln Gly
100 105 110

Thr Leu Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly
115 120 125

Ser Gly Gly Gly Gly Ser Glu Ile Val Leu Thr Gln Ser Pro Gly Thr
130 135 140

Leu Ser Leu Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser
145 150 155 160

Gln Ser Val Gly Ser Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly
165 170 175

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Gln Ala Pro Arg Leu Leu Ile Tyr Gly Ala Phe Ser Arg Ala Thr Gly
            180                 185                 190

Ile Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu
        195                 200                 205

Thr Ile Ser Arg Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln
    210                 215                 220

Gln Tyr Gly Ser Ser Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
225                 230                 235                 240

Ile Lys His His His His His His
                245
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