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(72) **Inventeurs/Inventors:**
KANO, YASUNOBU, JP;
HAMAJI, YOSHINORI, JP;
FUJIMORI, MINORU, JP;
SASAKI, TAKAYUKI, JP;
KOHNO, KYOKO, JP;
AMANO, JUN, JP;
TANIGUCHI, SHUN'ICHIRO, JP

(73) **Propriétaire/Owner:**

(54) **Titre : VECTEUR NAVETTE**
(54) **Title: NOVEL SHUTTLE VECTOR**

(57) **Abrégé/Abstract:**
The present invention provides a shuttle vector for a microorganism of the genus Bifidobacterium (BM) and Escherichia coli having a wide host range and a large copy number in BM and capable of highly expressing a desired protein when used as an expression vector; an expression vector capable of expressing a desired gene in BM by use of the shuttle vector; BM transformed with the expression vector; and an antitumor agent comprising the BM as an active ingredient. It comprises a pTB6-derived region portion comprising a replication origin (oriV)-repB region of pTB6 but not comprising MembB, MobA, Orfl, and oriT regions of pTB6 and an Escherichia coli-derived plasmid portion comprising a replication origin (oriC) region of Escherichia coli but having deleted DNA encoding an N-terminal region of an ampicillin resistance gene (ampR) expression product β-lactamase, and constitute a shuttle vector for Escherichia coli with a BM having an average copy number of 6 to 30.

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(73) **Propriétaires(suite)/Owners(continued):**ANAEROPHARMA SCIENCE INC., JP

(74) **Agent:** SMART & BIGGAR

ABSTRACT

The present invention provides a shuttle vector for a microorganism of the genus Bifidobacterium (BM) and Escherichia coli having a wide host range and a large copy number in BM and capable of highly expressing a desired protein when used as an expression vector; an expression vector capable of expressing a desired gene in BM by use of the shuttle vector; BM transformed with the expression vector; and an antitumor agent comprising the BM as an active ingredient. It comprises a pTB6-derived region portion comprising a replication origin (oriV)-repB region of pTB6 but not comprising MembB, MobA, OrfI, and oriT regions of pTB6 and an Escherichia coli-derived plasmid portion comprising a replication origin (oriC) region of Escherichia coli but having deleted DNA encoding an N-terminal region of an ampicillin resistance gene (ampR) expression product β-lactamase, and constitute a shuttle vector for Escherichia coli with a BM having an average copy number of 6 to 30.

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DESCRIPTION

TITLE OF THE INVENTION

NOVEL SHUTTLE VECTOR

Technical Field

[0001]

The present invention relates to a shuttle vector for a microorganism of the genus Bifidobacterium and Escherichia coli; an expression vector capable of expressing a desired gene in a microorganism of the genus Bifidobacterium by use of the shuttle vector; a microorganism of the genus Bifidobacterium transformed with the expression vector; and an antitumor agent comprising the microorganism of the genus Bifidobacterium as an active ingredient.

Background Art

[0002]

Bifidobacterium longum is a Gram-positive anaerobic bacterium and which has a genome with a high GC content (see e.g., Non-Patent Document 1). This Bifidobacterium longum is nonpathogenic and constitutes the most part of normal microflora in the large intestines of humans and other animals (see e.g., Non-Patent Document 2). This microorganism is said to have properties of promoting host's health such as enhancement of immunoreaction (see e.g., Non-Patent Document 3), inhibitory effect on the onset of cancer (see e.g., Non-Patent Document 4), protection of hosts against viral infection (see e.g., Non-Patent Documents 5 and 6), and possibility of producing antibacterial substance (see e.g., Non-Patent Document 7).

Some microorganisms of the genus Bifidobacterium are widely used in the world in the preparation of fermented dairy products.

[0003]

Furthermore, plasmids of Bifidobacterium are expected to be applied to probiotics vectors and oral vaccine vectors against infectious disease. Recent reports have revealed that Bifidobacterium longum is accumulated in hypoxic solid tumor after systemic administration (see e.g., Non-Patent Documents 8 and 9), and that a recombinant plasmid pBLES100-S-eCD that bears Escherichia coli codA fused with a Bifidobacterium longum hup promoter expresses cytosine deaminase in microorganisms (see e.g., Patent Document 1 and Non-Patent Documents 10 and 11). This confirmed the theory that recombinant Bifidobacterium longum is effective for enzyme-prodrug therapy. However, while these plasmids are getting attention in the fields of foods, pharmaceutical drugs, and industry, their genetic properties are little known due to the lack of an efficient replicable gene transfer system.

[0004]

pBLES100, which was used in the construction of the recombinant plasmid pBLES100-S-eCD, is a shuttle vector constructed from the plasmid pTB6 of a Bifidobacterium longum BK51 and the plasmid pBR322 of Escherichia coli (see e.g., Non-Patent Document 12). This shuttle vector pBLES100 transformed Bifidobacterium longum at an efficiency of 2.2×10^4 transformants/μg DNA and was stable in the cells in terms of structure and segregation of phenotypes (see e.g., Non-Patent Document 13). However, as the plasmid having unmodified DNA can be cleaved with a restriction enzyme in the microorganism

during transfection, cloning of a foreign gene requires higher transformation efficiency.

[0005]

Patent Document 1: Japanese Laid-Open Patent Application No. 2002-97144

Non-Patent Document 1: Scardovi, Bergey's Manual of Systematic Bacteriology vol 2, eds. Sneath et al., pp. 1418-1434 (1986)

Non-Patent Document 2: Mitsuoka, Elsevier Applied Science, pp 69-114 (1992)

Non-Patent Document 3: Yasui et al., J. Dairy Sci., 74, 1187-1195 (1991)

Non-Patent Document 4: Reddy et al., Cancer Res., 53, 3914-3918 (1993)

Non-Patent Document 5: Duffy et al., Pediatr. Res., 35, 690-695 (1994)

Non-Patent Document 6: Saaverdra et al., Lancet., 344, 1046-1049 (1994)

Non-Patent Document 7: Ibrahim et al., J. Food Prot., 56, 713-715 (1993)

Non-Patent Document 8: Yazawa et al., Cancer Gene Ther., 7, 269-274 (2000)

Non-Patent Document 9: Yazawa et al., Breast Cancer Res. Treat., 66, 165-170 (2001)

Non-Patent Document 10: Nakamura et al., Biosci. Biotechnol. Biochem., 66, 2362-2366 (2002)

Non-Patent Document 11: Fujimori et al., Curr. Opin. Drug Discov. Devel., 5, 200-203 (2002)

Non-Patent Document 12: Matsumura et al., Biosci. Biotechnol. Biochem., 61, 1211-1212 (1997)

Non-Patent Document 13: Matsumura et al., Biosci. Biotechnol. Biochem., 61, 1211-1212 (1997)

Disclosure of the Invention

[Problem to be solved by the Invention]

[0006]

As described above, a shuttle vector pBLES100 for a microorganism of the genus Bifidobacterium and Escherichia coli transformed Bifidobacterium longum at an efficiency of 2.2×10^4 transformants/μg DNA and was stable in the cells in terms of structure and segregation of phenotypes. However, as the plasmid having unmodified DNA can be cleaved with a restriction enzyme in the microorganism during transfection, cloning of a foreign gene requires higher transformation efficiency. The object of the present invention is to provide a shuttle vector for a microorganism of the genus Bifidobacterium and Escherichia coli having a wide host range and a large copy number in microorganisms of the genus Bifidobacterium and capable of highly expressing a desired protein in a microorganism of the genus Bifidobacterium when used as an expression vector; an expression vector capable of expressing a desired gene in a microorganism of the genus Bifidobacterium by use of the shuttle vector; a microorganism of the genus Bifidobacterium transformed with the expression vector; and an antitumor agent comprising the microorganism of the genus Bifidobacterium as an active ingredient.

[Means to solve the problems]

[0007]

The present inventors determined the complete sequence (3624 bp; SEQ ID NO: 1) of a plasmid pTB6 derived from

Bifidobacterium longum BK51 and identified some gene sequences of this microorganism of the genus Bifidobacterium including the plasmid replication origin OriV. The present inventors found out that OriV is a 358-bp sequence containing dso and sso, and that a pTB6-derived fragment of approximately 1900 bp in full length containing a replication protein (RepB, 693 bp) is available as a "replication unit of a microorganism of the genus Bifidobacterium." Replication ability was not confirmed for a unit containing OriV and a portion of RepB. The present inventors completed the present invention by finding out that the expression of an expression product of an ampicillin resistance gene serving as a selective marker, particularly the expression of the N-terminal region thereof, is unfavorable in terms of expansion of a host range in microorganisms of the genus Bifidobacterium.

[0008]

Namely, the present invention relates to (1) a shuttle vector for a microorganism of the genus Bifidobacterium and Escherichia coli comprising a pTB6-derived region portion comprising a replication origin (oriV)-repB region of pTB6 but not comprising MembB, MobA, OrfI and oriT regions of pTB6, and an Escherichia coli-derived plasmid portion comprising a replication region from an Escherichia coli-derived plasmid comprising pUC plasmid replication origin; (2) the shuttle vector for a microorganism of the genus Bifidobacterium and Escherichia coli according to (1), wherein the pTB6-derived region portion consists of a pTB6-derived region portion comprising a 1260-bp nucleotide sequence consisting of a nucleotide sequence represented by nucleotides 305 to 1564 in SEQ ID NO: 1; (3) the shuttle vector for a microorganism of the genus Bifidobacterium and Escherichia coli according to (1) or (2), wherein the

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Escherichia coli-derived plasmid portion comprising a replication origin (ori) region of Escherichia coli is a plasmid portion not comprising an ampicillin resistance gene (ampR) or having deleted DNA encoding an N-terminal region of an ampicillin resistance gene (ampR) expression product β-lactamase; (4) the shuttle vector for a microorganism of the genus Bifidobacterium and Escherichia coli according to any one of (1) to (3), wherein the microorganism of the genus Bifidobacterium is Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium animalis, Bifidobacterium bifidum, or Bifidobacterium adolescentis; (5) the shuttle vector for a microorganism of the genus Bifidobacterium and Escherichia coli according to any one of (1) to (4), wherein the shuttle vector has an average copy number of 6 to 30; (6) the shuttle vector for a microorganism of the genus Bifidobacterium and Escherichia coli according to any one of (1) to (5), wherein the shuttle vector is pAV001; (7) an expression vector having an ability to express a desired gene in a microorganism of the genus Bifidobacterium, comprising the shuttle vector for a microorganism of the genus Bifidobacterium and Escherichia coli according to any one of (1) to (6) into which an expression unit of the desired gene ligated in-frame between a promoter and a terminator involved in the expression of a Bifidobacterium longum-derived gene encoding a histone-like DNA-binding protein (HU protein) is inserted; (8) the expression vector according to (7), wherein the desired gene is a cytosine deaminase gene; (9) a microorganism of the genus Bifidobacterium transformed with the expression vector according to (7) or (8); (10) an antitumor agent comprising the microorganism of the genus Bifidobacterium according to

(9) as an active ingredient; (11) a pTB6-derived plasmid replication unit of a microorganism of the genus Bifidobacterium comprising a replication origin (oriV)-repB region of pTB6 but not comprising MembB, MobA, OrfI, and oriT regions of pTB6; and (12) the pTB6-derived plasmid replication unit of a microorganism of the genus Bifidobacterium according to claim 11, comprising a 1260-bp nucleotide sequence consisting of a nucleotide sequence represented by nucleotides 305 to 1564 in SEQ ID NO: 1.

Brief Description of Drawings

[0009]

[Fig. 1]

Figure 1 is a diagram showing a linear map of a plasmid pTB6 (3624 bp) and the structure of oriV. Figure 1(a) represents putative RepB, MembB, MobA, and a putative protein OrfI. The arrows denote the direction of translation, and the numerals denote nucleotide position. Figure 1(b) schematically represents 2 sets of IR (305 to 417) in sso and 1 set of IR (481 to 504) and 4 repeat units (indicated by the triangles) (577 to 664) in dso located in oriV. The diagram is indicated without reference to scale.

[Fig. 2]

Figure 2 is a diagram showing sequences in RepB and oriT. The amino acid sequences (a) of region 1 and region 2 in RepB and the nucleotide sequences of DR (b) and IR (c) in oriV are arranged in the diagram. The numerals above the sequence denote amino acid position in predicted RepB of pTB6, and the numerals above the dotted line denote the number of amino acids in the regions. The nucleotide (nuc) position in Figure 2(b) denotes

the gene locus of DR, and the nucleotide sequences of the repeat units are arranged. The nucleotide (nuc) position in Figure 2(c) denotes the gene locus of IR, and their sequences are arranged. The rectangles overlaid with dots represent the conserved regions of the amino acids or the nucleotides.

[Fig. 3]

Figure 3 is a diagram showing an amino acid sequence in MobA and a nucleotide sequence in oriV. In Figure 3(a), the partial amino acid sequences (amino acids 1 to 27 and 121 to 145) of MobA of pTB6 and the partial amino acid sequences (amino acids 1 to 27 and 108 to 131) of a MOBσ-line plasmid RSF1010 (Deposition No. M28829) are arranged. The symbol * denotes an active Tyr associated with DNA nicking-closing activity in Motif I and 3H motif in Motif III. In Figure 3(b), the nucleotide sequence of nucleotides 3454 to 3510 of putative oriV of pTB6 and the nucleotide sequence at position 3169 to 3132 of oriT of RSF1010 are arranged. The arrows denote characteristic IR, and the arrowhead denotes a nicking (nic) site by MobA of RSF1010. The numerals above and below the sequences denote the amino acid position of MobA or the nucleotide position of pTB6. The regions indicated by the rectangles overlaid with dots denote nucleotide sequences homologuous with matched amino acids.

[Fig. 4]

Figure 4 is a diagram showing linear maps of reconstituted plasmids. The PstI-FspI fragment of pTB6 (nucleotides 1 to 1872) was inserted into the PstI-FspII site of pUC18 to obtain a composite plasmid. The 1.6 kbp-HincII-HindIII fragment containing spc of pBLES100 was ligated with the HindIII-SspI fragment (4.0 kbp) and the HindIII-FspI fragment (3.4 kbp) of the composite plasmid to obtain pBRASTA100 (5.6 kbp) and

pBRASTA101 (5.0 kbp), respectively. The 0.6 kbp-SspI-FspI fragment (indicated by the rectangle with a short underline and a shading line) of pUC18 was inserted into the BamHI site and the BamHI and HindIII sites of pBRASTA101 to obtain pBRASTA102 (5.6 kbp) and pBRASTA103 (6.2 kbp), respectively. A plasmid pSS030Sp (5.4 kbp) was constructed by the same procedure as in the construction of pBRASTA100 except that the PstI-FspI DNA fragment (nucleotides 1873 to 3624) of pTB6 instead of the PstI-FspI DNA fragment (nucleotides 1 to 1872) was inserted into the PstI-HincII site of pUC18. The AatII-EcoRV fragment containing spc (1.3 kbp) of pBLES100 was ligated to a recombinant molecule AatII-FspI fragment (1.4 kbp) constructed by inserting the DNA fragment (nucleotides 472 to 1872) of pTB6 into the AatII-HincII site of pUC18, to construct a plasmid pDSO44Sp (4.3 kbp). The rectangles with a shading line denote sso and dso. The diagram is indicated without reference to scale.

[Fig. 5]

Figure 5 is a diagram showing the production processes of Bifidobacterium longum::pAV001-HU-eCD and Bifidobacterium longum::pBLES100-S-eCD.

[Fig. 6]

Figure 6 is a diagram showing the comparison result of the expression level of a cytosine deaminase protein in Bifidobacterium longum. The diagram shows that the cytosine deaminase protein is expressed 2 to 8-fold by Bifidobacterium longum::pAV001-HU-eCD compared to that expressed by Bifidobacterium longum::pBLES100-S-eCD.

[Fig. 7]

Figure 7 is a diagram showing a temporal shift in bacterial counts obtained by a result of comparison of enzyme activity of the cytosine deaminase protein (comparison of activity of converting 5FC to 5FU) in Bifidobacterium longum.

[Fig. 8]

Figure 8 is a diagram showing a 5-FU concentration obtained by a result of comparison of enzyme activity of the cytosine deaminase protein (comparison of activity of converting 5FC to 5FU) in Bifidobacterium longum.

[Fig. 9]

Figure 9A is a diagram showing a result of Southern analysis, after culturing Bifidobacterium longum::pAV001-HU-eCD and Bifidobacterium longum::pBLES100-S-eCD, digesting DNAs extracted from the respective bacterial cells with a restriction enzyme, and separating nucleic acids by agarose gel electrophoresis to subject to Southern analysis using AlkPhos Direct Labelling Reagents (Amersham Bioscience). Figure 9B is a diagram showing the comparison result of plasmid copy numbers of Bifidobacterium longum::pAV001-HU-eCD and Bifidobacterium longum::pBLES100-S-eCD on the basis of signal intensity.

Best Mode of Carrying Out the Invention

[0010]

A shuttle vector for a microorganism of the genus Bifidobacterium and Escherichia coli of the present invention is not particularly limited as long as it is a shuttle vector comprising a pTB6-derived region portion comprising a replication origin (oriV; nucleotides 305 to 664 in a nucleotide sequence represented by SEQ ID NO: 1)-repB (nucleotides 872

to 1564 in the nucleotide sequence represented by SEQ ID NO: 1) region of pTB6 but not comprising MembB (nucleotides 1543 to 2355 in the nucleotide sequence represented by SEQ ID NO: 1), MobA (nucleotides 2212 to 3300 in the nucleotide sequence represented by SEQ ID NO: 1), OrfI (nucleotides 3587 to 517 in the nucleotide sequence represented by SEQ ID NO: 1), and oriT (nucleotides 3454 to 3510 in the nucleotide sequence represented by SEQ ID NO: 1) regions of pTB6 and an Escherichia coli-derived plasmid portion comprising a replication origin (ori) region of Escherichia coli. However, a replication unit comprising a 1260-bp nucleotide sequence consisting of a nucleotide sequence represented by nucleotides 305 to 1564 in SEQ ID NO: 1, for example, a 1260-bp minimum replication unit consisting of a nucleotide sequence represented by nucleotides 305 to 1564 in SEQ ID NO: 1, a 1900-bp replication unit consisting of a nucleotide sequence represented by nucleotides 1 to 1900 in SEQ ID NO: 1, and a 2355-bp maximum replication unit consisting of a nucleotide sequence represented by nucleotides 1 to 2355 in SEQ ID NO: 1, are preferable. Thus, by using a smaller replication unit without the MembB, MobA, OrfI, and OriT regions, the copy number in host cells can be increased. It is preferred that the shuttle vector has a copy number larger than that of a shuttle vector pBLES100 for Bifidobacterium longum and Escherichia coli that can be constructed by the method described in Biosci. Biotech. Biochem., 61, 1211 (1997), and has an average copy number increased of, for example, 3 to 30, particularly preferably 6 to 30.

[0011]

In the shuttle vector of the present invention, it is preferred that the Escherichia coli-derived plasmid portion

comprising a replication origin (ori) region of Escherichia coli does not comprise an ampicillin resistance gene (ampR) or, if any, a portion wherein DNA encoding the N-terminal region of its expression product β-lactamase is deleted is preferable. The expression lacking the N-terminal region of β-lactamase allows for the expansion of a host range in microorganisms of the genus Bifidobacterium.

[0012]

In the shuttle vector of the present invention, the microorganism of the genus Bifidobacterium can concretely be exemplified by Bifidobacterium longum, Bifidobacterium adolescentis, Bifidobacterium breve, Bifidobacterium animalis, and Bifidobacterium bifidum. In contrast, the replication of the shuttle vector pBLES100 in microorganisms of the genus Bifidobacterium other than Bifidobacterium longum is not confirmed.

[0013]

The shuttle vector of the present invention can preferably be exemplified by the shuttle vector pAV001 described in Example 2.

[0014]

A pTB6-derived plasmid replication unit of a microorganism of the genus Bifidobacterium of the present invention is not particularly limited as long as it is a pTB6-derived region portion comprising a replication origin (oriV; nucleotides 305 to 664 in the nucleotide sequence represented by SEQ ID NO: 1)-repB (nucleotides 872 to 1564 in the nucleotide sequence represented by SEQ ID NO: 1) region of pTB6 but not comprising MembB (nucleotides 1543 to 2355 in the nucleotide sequence represented by SEQ ID NO: 1), MobA

(nucleotides 2212 to 3300 in the nucleotide sequence represented by SEQ ID NO: 1), OrfI (nucleotides 3587 to 517 in the nucleotide sequence represented by SEQ ID NO: 1), and oriT (nucleotides 3454 to 3510 in the nucleotide sequence represented by SEQ ID NO: 1) regions of pTB6. The plasmid replication unit can preferably be exemplified by a replication unit comprising a 1260-bp nucleotide sequence consisting of a nucleotide sequence represented by nucleotides 305 to 1564 in SEQ ID NO: 1, for example, a 1260-bp minimum replication unit consisting of a nucleotide sequence represented by nucleotides 305 to 1564 in SEQ ID NO: 1, a 1900-bp replication unit consisting of a nucleotide sequence represented by nucleotides 1 to 1900 in SEQ ID NO: 1, and a 2355-bp maximum replication unit consisting of a nucleotide sequence represented by nucleotides 1 to 2355 in SEQ ID NO: 1.

[0015]

An expression vector of the present invention capable of expressing a desired gene in a microorganism of the genus Bifidobacterium is not particularly limited as long as it is a vector comprising the shuttle vector for a microorganism of the genus Bifidobacterium and Escherichia coli of the present invention into which an expression unit of the desired gene ligated in-frame between a promoter and a terminator involved in the expression of Bifidobacterium longum-derived gene encoding a histone-like DNA-binding protein (HU protein) is inserted. The desired gene is not particularly limited and can preferably be exemplified by a gene encoding a cytokine or angiogenic inhibitor having antitumor activity and a gene encoding an enzyme capable of converting a low toxic antitumor

substance precursor to an antitumor substance (hereinafter, abbreviated to as a converting enzyme).

[0016]

The promoter and the terminator in the expression unit can preferably be exemplified by a DNA represented by nucleotides 1 to 192 and DNA represented by nucleotides 472 to 600, respectively, in the nucleotide sequence that can be represented by SEQ ID NO: 2. The expression vector having the promoter and the terminator involved in the expression of a HU gene can be prepared by cleaving a HU gene with a restriction enzyme from the DNA of Bifidobacterium longum, then incorporating the HU gene into a cloning vector, and further incorporating the desired gene to the downstream of the promoter involved in the expression of the HU gene. The desired gene can be expressed efficiently by using the promoter and the terminator involved in the expression of the HU gene. Examples of a method for isolating the HU gene include a method digesting the chromosomal DNA of Bifidobacterium longum with the restriction enzyme HindIII. To be more specific, the chromosomal DNA of Bifidobacterium longum is first digested with a restriction enzyme HindIII and then purified by phenol treatment and ethanol precipitation. On the other hand, pBR322 (TAKARA SHUZO) is also digested with HindIII and subjected to dephosphorylation treatment, followed by purification in the same way. The respective DNAs are ligated to obtain recombinant DNA. Next, the recombinant DNA is used to transform Escherichia coli mH3 (Gene, 45, 37 (1986)) according to a conventional method to obtain a transformant exhibiting ampicillin resistance and tetracycline sensitivity. Plasmid DNA is extracted from the obtained transformant according to a conventional method and

introduced into an Escherichia coli YK2741 strain (Gene, 89, 133 (1990)) according to a conventional method to transform the bacterial strain. The YK2741 strain, which has HU and IHF (integration host factor) genes deleted, exhibits cold sensitivity, and with this property the transformant is selected by plating onto an ampicillin-containing agar medium and culturing at 27°C. Subsequently, the transformant of the YK2741 strain thus obtained is further cultured, and plasmid retained in the strain is extracted according to a conventional method and introduced into Escherichia coli YK1340 (J. Mol. Biol., 204, 581 (1988)) according to a conventional method to transform the bacterial strain. The obtained transformant is subjected to an infection test with Mu phage according to a conventional method. The YK1340 strain is a HU genes-deleted strain, and as the proliferation of the Mu phage requires HU proteins, a transformant that is lysed by Mu phage infection and proliferation can be a potent candidate of a strain bearing the Bifidobacterium longum-derived HU gene. Thus, the plasmid pBLHU15 having the promoter and the terminator involved in the expression of the Bifidobacterium longum-derived HU gene can be obtained by selecting a plasmid retained in the strain exhibiting ampicillin resistance and is lysed by Mu phage infection and proliferation. Moreover, a site developed by using NsvV and HpaI is preferable as an introduction site of the desired gene.

[0017]

For the gene encoding a cytokine having antitumor activity, examples include interferon (IFN)-α, IFN-β, IFN-γ, granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin (IL)-1α, Il-1β, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10,

IL-12, IL-13, IL-15, IL-18, 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), leukemia inhibitory factor (LIF), co-stimulatory factors B7 (CD80) and B7-2 (CD86) for T-cell activation, Kit ligand, and oncostatin M. Particularly, IL-2 is preferable. Alternatively, these cytokines may be used in combination of two or more of them, and, for example, the combination of IL-6 with TNF-α, IFN-α, IFN-β, or IFN-γ, the combination of TNF-α with IFN-γ, and the combination of anti-Fas with IFN-γ are preferable. Angiogenic inhibitors such as endostatin, angiostatin, kringle 1, kringle 2, kringle 3, kringle 4, kringle 5, and NK4 can be used advantageously as substances having antitumor activity other than the cytokine.

[0018]

Examples of the gene encoding a converting enzyme include: a combination of 5-fluorocytosine (5-FC) as an antitumor substance precursor, 5-fluorouracil (5-FU) as an antitumor substance, and cytosine deaminase as a converting enzyme; a combination of 5-aziridino-2,4-dinitrobenzamide (CB1954) as an antitumor substance precursor; an alkylating agent known to cause bridge bond in double-stranded DNA as an antitumor substance, and nitroreductase as a converting enzyme; and a combination of ganciclovir as an antitumor substance precursor, a metabolite thereof as an antitumor substance, and herpes simplex virus type 1 thymidine kinase (HSV1-TK) as a converting enzyme. In addition, by converting an antitumor substance into a precursor (including an inactivated product) low toxic to human bodies by modification such as glucuronide conjugation, glycine

conjugation, or lysine conjugation, and the enzyme that demodifies the precursor can be used as a converting enzyme. Any of known per se in the art may be used as enzyme that demodifies the precursor. For example, the combination of a glucuronide-conjugated antitumor substance precursor with β-glucuronidase as a converting enzyme can be exemplified.

[0019]

The microorganism of the genus Bifidobacterium of the present invention is not particularly limited as long as it is a microorganism of the genus Bifidobacterium transformed with the expression vector of the present invention. Concrete examples thereof include Bifidobacterium longum, Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium pseudolongum, Bifidobacterium thermophirum, Bifidobacterium breve, Bifidobacterium infantis, and Bifidobacterium animalis. Among these, Bifidobacterium longum, Bifidobacterium adolescentis, Bifidobacterium bifidum, or Bifidobacterium infantis known to inhabit in the intestines of humans regardless of age, are preferable, and Bifidobacterium longum, is more preferable. All of these bacteria are commercially available or can be obtained easily from depository institutions. For example, Bifidobacterium longum ATCC-15707, Bifidobacterium bifidum ATCC-11863, and Bifidobacterium infantis ATCC-15697 can be used.

[0020]

The plasmid replication unit, the shuttle vector, the expression vector, and the transformed microorganism of the genus Bifidobacterium of the present invention can be prepared according to the methods described in commercially available experiment manuals, for example, Gene Manual (Kodansha),

Methods for Experiments in Gene Manipulation (ed., Yasutaka Takagi, Kodansha), Molecular Cloning (Cold Spring Harbor Laboratory (1982)), Molecular Cloning, 2nd ed. (Cold Spring Harbor Laboratory (1989)), Methods in Enzymology, 194 (1991), and Gene Experiments Using Yeasts, Experimental Medicine Suppl. (Yodosha (1994)).

[0021]

The transformed microorganism of the genus Bifidobacterium can proliferate only in tumor tissues under anaerobic environment and express substances having antitumor activity, converting enzymes and the like, in the tumor tissues. Thus, the transformed microorganism of the genus Bifidobacterium is used as a medicine effective for treating tumor having anaerobic environment, preferably solid tumor. The antitumor agent of the present invention is not particularly limited as long as it comprises the microorganism of the genus Bifidobacterium of the present invention as an active ingredient. The administration of the antitumor agent of the present invention is not particularly limited and include oral administration and parenteral administration, while parenteral administration is particularly preferable. Examples of parenteral administration can include airway, intrarectal, subcutaneous, intramuscular, and intravenous administration. Examples of preparations suitable for oral administration include tablets, granules, fine granules, powders, syrups, solutions, capsules, and suspensions. Examples of preparations suitable for parenteral administration include injections, infusions, inhalants, propellants, suppositories, transdermal absorbents and transmucosal absorbents. In the

present invention, it is preferred that the preparations are used as injections, particularly intravenous injections.

[0022]

The transformed microorganism of the genus Bifidobacterium may be supplied to aftertreatment known per se in the art. For example, partial purification can be performed by centrifugation. Moreover, after partial purification,the microorganism may then be dissolved or suspended, in a solvent conventionally used in the art such as saline, PBS (phosphate-buffered saline), or lactated Ringer's solution, if desired. Further, lypholization or spray drying can be performed to make powdered or granulated product, if desired.

[0023]

The solution or suspension of the transformed microorganism of the genus Bifidobacterium or the dried powdered or granulated product of the transformed microorganism may be administered directly as the antitumor agent of the present invention. However, it is generally desirable that the antitumor agent is administered in the form of a pharmaceutical composition comprising the above substances serving as an active ingredient and one or more pharmaceutical additives. Such pharmaceutical compositions can be produced according to a method well known or commonly used per se in the pharmaceutical field.

[0024]

Examples of pharmaceutical additives that can be used in the production of the liquid preparations suitable for oral administration include: water; saccharides such as cane sugar, sorbit, and fruit sugar; glycols such as polyethylene glycol

and propylene glycol; oils such as sesame oil, olive oil, and soybean oil; and preservatives such as p-hydroxybenzoic acid esters. For the production of the solid preparations such as capsules, tablets, powders, and granules include: excipients such as lactose, glucose, cane sugar, and mannite; disintegrants such as starch and sodium alginate; lubricants such as magnesium stearate and talc; binders such as polyvinyl alcohol, hydroxypropylcellulose, and gelatin; surfactants such as fatty acid ester; and plasticizers such as glycerin, can be used.

[0025]

Of the preparations suitable for parenteral administration, preparations for intravascular administration such as injections and infusions can preferably be prepared by using an aqueous medium isotonic with human blood. For example, injections can be prepared as a solution, suspension, or dispersion together with an appropriate auxiliary according to a conventional method by using an aqueous medium selected from salt solution, glucose solution, or a mixture of salt solution and glucose solution. Suppositories for intestinal administration can be prepared by using a carrier, for example, cocoa butter, hydrogenated fat, or hydrogenated carboxylic acid. Propellants can be prepared by using a carrier that gives no stimulation to human oral cavity and airway mucosa and that can promote absorption of the microorganism of the genus Bifidobacterium of the present invention serving as an active ingredient by dispersing the microorganism as fine particles. For example, lactose or glycerin can be used as a carrier. In addition, it can be prepared as preparations in forms of aerosols and dry powders. For example, one or two or more pharmaceutical additives selected from diluents, flavors, preservatives,

excipients, disintegrants, lubricants, binders, surfactants, and plasticizers can be used in the production of preparations for parenteral administration. The form and production method of the antitumor agent of the present invention are not limited to the specific examples mentioned above.

[0026]

The dosage and administration frequency of the antitumor agent of the present invention are not particularly limited and can appropriately be selected according to various conditions such as the type of gene retained in the microorganism of the genus Bifidobacterium, the type of a morbid condition to be treated, administration routes, age and body weight of the patient, symptoms, and the severity of disease. For example, when the antitumor agent is systemically administered by intravenous injection, the daily dosage in adult is preferably approximately 2×10^6 to 2×10^7 microorganisms/body. When the antitumor agent is locally administered into tumor, the dosage is preferably approximately 5×10^8 microorganisms/tumor. However, the dosage is not limited to this specific example.

[0027]

The antitumor agent according to the present invention can be applied to tumor having anaerobic environment, preferably a variety of solid cancers. Examples of solid cancers include large bowel cancer, brain tumor, head and neck cancer, breast cancer, lung cancer, esophagus cancer, gastric cancer, hepatic cancer, gallbladder cancer, bile duct cancer, pancreatic cancer, islet cell cancer, choriocarcinoma, colonic cancer, renal cell carcinoma, adrenal cortical cancer, bladder cancer, testicle cancer, prostate cancer, testicular tumor, ovarian cancer, uterine cancer, choriocarcinoma, thyroid cancer, malignant

carcinoid tumor, skin cancer, malignant melanoma, osteosarcoma, soft tissue sarcoma, neuroblastoma, Wilms tumor, retinoblastoma, melanoma, and squamous cell carcinoma.

[0028]

The medicine of the present invention may be used with other medicines and the like. Particularly, when the microorganism of the genus Bifidobacterium incorporating therein the gene encoding a converting enzyme is administered, the administration of an antitumor substance precursor is essential. However, the antitumor substance precursor may form a single preparation together with the microorganism of the genus Bifidobacterium incorporating therein the gene encoding a converting enzyme; or otherwise, it may be separate preparations to be administered simultaneously or at time intervals. Moreover, it is preferred to be used with Lactulose. Lactulose is a nutrition source for the microorganism of the genus Bifidobacterium and is not metabolized by humans, mice, and pigs. Therefore, by administrating Lactulose, the number of the microorganism of the genus Bifidobacterium in tumor tissue increases in a specific manner. The daily dosage thereof in adult is preferably approximately 24 to 48 g/body, and the administration frequency is not limited. Moreover, the medicine of the present invention can be used in combination with other antitumor agents and the like.

[0029]

The microorganism of the genus Bifidobacterium of the present invention administered to a patient can be killed easily by an antibiotic. This is important for more enhancing the safety of the gene transfer system according to the present invention.

[0030]

Hereinafter, the present invention will be described more fully with reference to Examples. However, the technical scope of the present invention is limited to these illustrations.

Example 1

[0031]

(Structural analysis of plasmid pTB6)

[Materials and Method]

1. Bacterial strains, plasmids, and media

Bacterial strains and plasmids used in the present invention are listed in Table 1. Escherichia coli was aerobically cultured at 37°C in LB broth (10 g of Bacto-tryptone, 5 g of yeast extract, 5 g of NaCl, and 0.1% glucose per litter), and colonies were allowed to form in LB broth containing 1.5% agar. Bifidobacterium longum 105-A (see Non-Patent Document 12) was anaerobically cultured at 37°C in MRS broth (Difco Laboratories, USA) supplemented with 50 mM sucrose, 0.34% cysteine, and 0.02% sodium ascorbate. An antibiotic (50 μg/ml ampicillin (Ap) and/or 75 μg/ml spectinomycin (Sp)) was added according to need. Colonies were allowed to form on broth containing 1.5% agar by using Gas-Pak anaerobic system (BL, USA) according to the method described in Non-Patent Document 12.

[0032]

[Table 1]

Bacterial strains and plasmids used in this study

Strain or Plasmids	Relevant characteristics	Source or Reference
Bacterial strains		
E. coli HMS174	*recA1 hsdR rif*Sp^s	Sambrook *et al.* *
B. longum 105-A	Sp^s	Matsumura *et al.* **
Plasmids		
pUC18	2.7kbp;ColE1 *ori, amp*	Sambrook *et al.* *
pBLES100	9.1kbp;ColE1 *ori, whole* pTB6, *spc*	Matsumura *et al.* **
pBRASTA100	5.6kbp;ColE1 *ori*, pTB6 *oriV-repB, spc, amp*	This study
pBRASTA101	5.0kbp;ColE1 *ori*, pTB6 *oriV-repB, spc,*	This study
pBRASTA102	5.6kbp;ColE1 *ori*, pTB6 *oriV-repB, spc,*	This study
pBRASTA103	6.2kbp;ColE1 *ori*, pTB6 *oriV-repB, spc,*	This study
pDS044Sp	4.3kbp;ColE1 *ori*, pTB6 *oriV-repB, Δsso, spc*	This study
pSS030Sp	5.4kbp;ColE1 *ori*, pTB6 *mobA, spc, amp*	This study

*)Sambrook, J.E., Fritsch, F., and Maniatis, T., In" Molecular cloning: a laboratory manual" 2nd. eds. Cold Spring Harbor Laboratory, New York, (1989).

**)Matsumura, H., Takeuchi, A., ando Kano, Y., Construction of *Escherichia coli-Bifidobacterium longum* shuttle vector transforming *B. longum* 105-A and 108-A. *Biosci. Biotechnol. Biochem.*, 61, 1211-1212 (1997).

[0033]

2. Plasmid DNA isolation and molecular manipulation

Qiagen Plasmid Kit (QIAGEN, USA) was used according to procedures described in the accompanying manual to extract plasmid DNA by a modified alkali lysis method. The Bifidobacterium longum was washed with 0.9% NaCl and treated at 37°C for 30 minutes with lysozyme (1 mg/ml) before alkali lysis and at 37°C for 15 minutes with proteinase K (0.1 mg/ml) before phenol treatment.

[0034]

Competent cells of Bifidobacterium longum were prepared according to the method described in Non-Patent Document 12. The cells harvested at late log phase were washed three times with 50 mM buffered sucrose solution and were resuspended in

ice-cold glycerin (10% v/v) of approximately one hundredth of the volume of the original cultured product. Moreover, competent cells of the Escherichia coli were prepared according to the method described previously (Takeuchi et al., Biosci. Biotechnol. Biochem., 66, 598-603 (2002)). GenePulser apparatus (Bio-Rad Labs., USA) was used under the conditions of 200 Ω parallel resistance, 2.5 kV/cm, and 25 μF according to the method described in Non-Patent Document 12 to perform electroporation using 100 ng of the plasmid DNA and 50 μl of the bacterial solutions.

[0035]

DNA was amplified using GeneAmp PCR System 9600 and LA Taq Polymerase (TAKARA). Two primers [5'-GGCCGGAATTCTGAGCAAAAGGCCAGCAAAAGGCC-3' (SEQ ID NO: 3) and 5'-GGCCGGAATTCAGTACTCATATATACTTTAGATTGATTTA-3' (SEQ ID NO: 4)] were used to amplify ColE1 ori from pUC18 by PCR. Next, by using two primers [5'-GCGGCGGATCCATTGAAAAAGGAAGAGTAT-3' (SEQ ID NO: 5) and 5'-CGGCCGGATCCTGCGCAACGTTGTTGCCAT-3' (SEQ ID NO: 6)] for constructing pBRASTA102 and two primers [5'-GCGGCAAGCTTATTGAAAAAGGAAGAGTAT-3' (SEQ ID NO: 7) and 5'-CGGCCAAGCTTTGCGCAACGTTGTTGCCAT-3' (SEQ ID NO: 8)] for constructing pBRASTA103, a half amp region proximal to the promoter was amplified from pUC18. Other DNA manipulations were all performed according to the method described previously (Sambrook et al., Molecular cloning: a laboratory manual 2nd eds. Cold Spring Harbor Laboratory (1989)).

[0036]

3. DNA sequencing and sequence analysis

pBLES100 was cleaved with PstI to prepare the full-length DNA (3.6 kbp) of pTB6. After digestion with Sau3AI or AluI,

the DNA was subcloned in the multi-cloning site of pUC18. ALF express II DNA sequencer and Thermo Sequenase Cycle Sequencing Kit or Thermo Sequenase CyTM 5 Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) were used to perform sequencing. DNASIS-Mac v2.2 and GENETYX-MAC software were used to practice sequence assembly and sequence analysis using computer. FASTA and BLAST servers were used for homology search.

[0037]

[Results]

1. Plasmid structure

The complete nucleotide sequence (3,624 bp) of pTB6 of Bifidobacterium longum BK51 serving as a component of the shuttle vector pBLES100 was determined. pTB6 (Figure 1(a)) was predicted from data base search to contain 4 open reading frames (Orf), that is, RepB (872 to 1564), MembB (1543 to 2355), MobA (3300 to 2212), and a hypothetical protein OrfI (3587 to 1 to 517). GC content of pTB6 was 65.1 mol% and had a level usually observed in the genomic DNA and plasmid DNA of Bifidobacterium longum.

[0038]

The complete nucleotide sequence of pTB6 exhibited 95% homology to Bifidobacterium longum plasmids pKJ36 (3625 bp) and pB44 (3624 bp) belonging to the rolling circle replication-type (RCR) plasmid family group 1, 92% homology to pNAC2 (3684 bp), and 89% homology to pBLO1 (3626 bp). Homology as high as 92% was also detected to a plasmid pDOJH10L (5308 to 8999 in 10073 bp) of the group 1 (Table 2).

[0039]

[Table 2]

Nucleotide homology and aa identity

Plasmid	nuc homology (%)*	aa identity (%)			
		RepB**	MobA	MembB	Orf I***
pKJ36	95	92	85	88	-
pB44	95	91	85	-	31
pNAC2	92	89	58	-	31
pDOJH10L	92	90	46	57	30
pBLO1	89	81	57	-	31

Numeral shows percent of nuc homology and aa identity to pTB6.

*; Complete nuc sequences were compared to that of pTB6 (3,624 bp) except pDOJH10L where nuc position 5308 to 8999 was compared to complete nuc sequence of pTB6. **; Amino acid sequence of RepB from aa position 1 to 230 (pKJ36), from 54 to 283 (pB44), and from 54 to 281 (pNAC2, pDOJH10L and pBLO1) was compared with the complete RepB aa sequence (230 aa) of pTB6. Number of aa in complete RepB was 230, 299, 297, 297, 297 for pKJ36, pB44, pNAC2, pDOJH10L and pBLO1, respectively. ***; Orf of pB44, Orf III of pNAC2, Orf II of pDOJH10L, and Orf I of pBLO1 were compared with Orf I. -; Orf homologous to Orf I have not been reported. The GenBank accession numbers for plasmid DNA sequences and plasmid nuc size are AF139129 for pKJ36 (3,625 bp), AY066026 for pB44 (3,624 bp), AY112723 for pNAC2 (3,684 bp), AF538868 for pDOJH10L (10,073 bp) and AF540971 for pBLO1 (3,626 bp).

[0040]

2. RepB and oriV

The amino acid identity of predicted Orf of pTB6 is shown in Table 2. RepB (230 amino acids (aa)) exhibited 92%, 91%, 90%, 89%, and 81% identity with the complete RepB amino acid sequence (230 aa) of pKJ36, the Rep B aa region of amino acids 54 to 283 of pB44, and the RepB aa region of amino acids 54 to 281 of pDOJH10L, pNAC2, and pBLO1, respectively. Two local sites, region 1 (aa 117 to 138) and region 2 (aa 183 to 188), were not conserved in these plasmids (Figure 2(a)).

[0041]

The replication origin oriV consists of dso (double strand origin) and sso (single strand origin) and is necessary for the initiation of replication of reading and lagging strands (Del Solar et al., Microbiol. Mol. Biol. Rev., 62, 434-494 (1998)). This replication origin was predicted in a region between nucleotides 305 and 664 of pTB6 (Figure 1(b)). Two sets of IR (305 to 417) were detected in sso, while IR (481 to 504) and DR (577 to 644) were detected in dso (Figure 1(b)). DR consisted of four identical 22-mer sequences (5'-AACCTACACCAAAAGGGGAGCG-3'; SEQ ID NO: 9) except that the last one sequence had T at the 3' terminus. Their 11 nucleotides (5'-CAAAA-3' and 5'-GGAGCG-3') were conserved in the DR of pKJ36, pB44, pNAC2, pDOJH10L, and pBLO1 (Figure 2(b)). The nucleotide sequence of IR in dso was also conserved in these plasmids except for 2 nucleotides in the loop region (22 of 24 nucleotides) (Figure 2(c)).

[0042]

3. MobA and oriT

MobA is a primary DNA processing protein for the conjugative mobilization of the plasmid (Becker et al., J. Bacteriol., 185, 3538-3546 (2003)). The amino acid identity of putative MobA of pTB6 was 85%, 85%, 58%, 57%, and 46% to putative MobA of pKJ36, pB44, pNAC2, pBLO1, and pDOJH10L, respectively (Table 2). MobA of pTB6 also exhibited remarkable similarity to MobA of a plasmid RSF1010 of MOBΩ family (Francia et al., FEMS Microbiol. Rev., 28, 79-100 (2004)). The tyrosine residue of motif I (Francia et al., FEMS Microbiol. Rev., 28, 79-100 (2004)) involved in DNA nicking-closing action and the 3H motif of motif III helping the initiation of transfer by motif I are conserved in MobA of pTB6 (Figure 3(a)). In oriT of RSF1010, the 13-mer nucleotide sequence 5'-GTAAGTGCGCCCT-3' (SEQ ID NO: 10) adjacent to the inverted repeat is cleaved by MobA (Figure 3(b)). The region of nucleotides 3454 to 3510 in oriT of pTB6 exhibited structural similarity to the nucleotide sequence seen in oriT of RSF1010 (Francia et al., FEMS Microbiol. Rev., 28, 79-100 (2004)). From these results, the oriT of pTB6 was estimated to be cleaved at the nic site of the 13-mer sequence by MobA expressed from pTB6.

[0043]

4. MembB and Orf1

MembB of pKJ36 and pDOJH10L exhibited 88% and 57% aa identity, respectively, to putative MembB of pTB6. Putative OrfI of pTB6 consists of 184 aa and exhibited considerably low aa identity (33% to 31%) to Orf of pB44, OrfIII of pNAC2, OrfII of pDOJH10L, and OrfI of pBLO1 (Table 2). The functions of these proteins remain unexplained.

[0044]

5. Essential region to plasmid replication

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For understanding the control of plasmid replication, it is important to know the DNA region essential to plasmid replication. The present inventors constructed recombinant plasmids consisting of pUC18, the oriV-repB region (nucleotides 1 to 1872) of pTB6, and spc (1.1 kbp) of pBLES100 (Table 3, Figure 4). The resulting plasmids pBRASTA100 and pBRASTA101 were extracted from Escherichia coli HMS174 and transferred to Bifidobacterium longum 105-A. As shown in Table 3, both of the plasmids efficiently transformed Bifidobacterium longum. The plasmid pBRSTA100 was transformed to Bifidobacterium longum 105-A efficiently (5.9×10^4 transformants/μg DNA). This efficiency was as high as that obtained by pBLES100 (2.2×10^4 transformants/μg DNA) previously reported. Transformation efficiency 40 times better than that of pBRASTA100 was observed in pBRASTA101 (2.5×10^6 transformants/μg DNA). These plasmids were stable in the transformants in terms of the structure and the segregation of phenotypes. An sso-deleted plasmid pDSO44Sp (Table 3, Figure 4) consisting of pUC18, pTB6 dso-repB, and spc transformed Bifidobacterium longum at an efficiency of 1.3×10^6 transformants/μg DNA (Table 3). On the other hand, the recombinant plasmid pSSO30Sp bearing mobA and a portion of membB (1873 b to 3624 b) but not bearing orfI, oriV, and repB could not transform Bifidobacterium longum at all (Table 3). These results concluded that only the presence of the dso-repB region is sufficient for the replication of pTB6 in Bifidobacterium longum, and the sso region is not essential.

[0045]

[Table 3]

Transformation of *B. longum* with plasmid

Plasmid	Transformants obtained / μg DNA	Ratio
pBRASTA100	5.9×10^4	1.0
pBRASTA101	2.5×10^6	42.3
pDSO44Sp	1.3×10^6	22.0
pSSO30Sp	N.D.	-
pBRASTA102	7.5×10^4	1.3
pBRASTA103	7.3×10^2	0.012

Plasmids were prepared from *E. coli* HMS174, and transferred in *B. longum* 105-A. Transformants were selected on agar plate in the presence of Sp. N.D.; Transformants were not detected.

[0046]

6. High-efficiency transformation of Bifidobacterium longum

As described above, the plasmid pBRASTA101 was shown to transform Bifidobacterium longum at high efficiency exceeding 40 times higher than that of pBRASTA100 and 100 times higher than that of pBLES100. pBRASTA100 and pBLES100 retained a normal amp site, and pBRASTA101 had mutation with half of amp proximal to the promoter deleted (deletion of 0.6 kbp-SspI-FspI DNA segment). For confirming that the deletion in amp actually influences transformation, the 0.6-kbp fragment was inserted into the BamHI site of pBRASTA101, and the obtained pBRASTA102 (Figure 4) was in turn extracted from Escherichia coli HMS174 and transferred into Bifidobacterium longum 105-A. As shown in Table 3, almost the same transformation efficiency as that of pBRASTA100 was obtained in pBRASTA102 (7.5×10^4

transformants/μg DNA). pBRASTA103 was constructed by inserting the 0.6-kbp fragment into the HindIII site of pBRASTA102. It was shown that this plasmid had 2 copies of this fragment (Figure 4), and transformation ability was further decreased in proportion to the copy number of the fragment. When these plasmids were transferred to Escherichia coli HMS174, such decrease in transformation efficiency was not seen. The accurate mechanism underlying the restriction of the plasmid induced by the 0.6 kbp-DNA remains unexplained. However, these results suggested that plasmid replication is restricted by the cleavage of the 0.6 kbp-DNA transfected into Bifidobacterium longum 105-A.

[0047]

[Discussion]

In the present invention, the nucleotide sequence of the plasmid pTB6 of Bifidobacterium longum was determined, and the aa sequence of putative Orf was predicted. The results of sequence analysis revealed that pTB6 is a plasmid associated with the RCR plasmid family group 1 reported by Corneau et al (Plasmid, 51, 87-100 (2004)). The high aa identity and the high homology of oriV nucleotide sequences between plasmids of the group 1, particularly pKJ36 and pTB6, clearly suggested that pTB6 replicates by the rolling circle mechanism.

[0048]

The nucleotide sequence conserved in oriT of the MOBΩ-line plasmid was identified in putative oriT of pTB6. Moreover, the remarkable similarity of motif I and motif III of putative MobA to motif I and motif III of the MOBΩ-line plasmid was found in pTB6. This result suggested that pTB6 is mobilized when the factor is further supplied from host cells or other plasmids.

From this point of view, the present inventors asuume that the plasmids pBRASTA101 and pDSO44Sp having membB and orfI as well as oriT and mobA deleted are useful as safe vehicles of gene transfer.

[0049]

Recently, Nakamura et al. and Fujimori et al. succeeded in the expression of the cytosine deaminase gene (codA) of Escherichia coli in Bifidobacterium longum by using the composite plasmid pBLES100 between pBR322 and pTB6. This showed that pTB6 is an useful vector for the expression of a foreign gene in Bifidobacterium longum. However, Bifidobacterium microorganisms are known to generate site-specific endonuclease. Thus, unmodified DNA may be damaged when introduced into the cells of Bifidobacterium microorganisms, (Khosaka et al., Gene. 17, 117-122 (1982); Khosaka et al., FRBS Lett., 14, 63, 170-174 (1983); Khosaka et al., Gene 31, 251-255 (1984); and Skrypina et al., Mol. Gen. Mikrobiol. Virusol., 5, 15-16 (1988)). Because the small plasmids pBRASTA101 and pDSO44Sp lack oriT and mobA and have high transformation ability, the present inventors assume that these plasmids are practical for the cloning and expression of a foreign gene in Bifidobacterium longum.

Example 2

[0050]

(Construction of Shuttle vector pAV001)

[Construction of plasmid]

Enterococcus faecalis-derived sequence containing spectinomycin adenyltransferase (AAD cassette) was amplified from pBLES100 by PCR and subcloned into pCR-Blunt II-TOPO vector (Invitrogen) to prepare pCRTOPO-ScaI-AAD-Eam1105I.

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Restriction enzymes sites ScaI and Eam1105I were added to forward and reverse primers, respectively.

[0051]

As shown in Figure 5, a cloning vector pGFPuv (DEFINITION: Cloning vector pGFPuv. ACCESSION: U62636 VERSION: U62636.1 GI: 1490528) purchased from Invitrogen is composed of a GFPuv gene, multi-cloning sites (MCS) located at both ends thereof, an ampicillin resistance gene, and a plasmid replication origin Ori (pUC Ori) of Escherichia coli.

[0052]

The ampicillin resistance gene site was cleaved with restriction enzymes Eam1105I and ScaI to prepare a long fragment without the site. Similarly, pCRTOPO-ScaI-AAD-Eam1105I was cleaved with restriction enzymes Eam1105I and ScaI to prepare a fragment (approximately 1100 bp) containing the AAD cassette. These two fragments were ligated by use of T4DNA ligase to prepare pGFPuv-SpR. The addition of a spectinomycin resistance property to the prepared plasmid pGFPuv-SpR and the deficiency of an ampicillin resistance property of the plasmid were respectively confirmed in Escherichia coli.

[0053]

pGFPuv-SpR was digested with restriction enzymes SalI (present in the multi-cloning site upstream of the GFPuv gene) and SpeI (present in the multi-cloning site downstream of the GFPuv gene) to prepare a plasmid pAVN in which the GFPuv gene was deleted.

[0054]

A sequence of approximately 1900 bp containing RepB, SDO, DDO, AT-rich repeats, and DnaA-binding motifs was identified as a plasmid replication unit of Bifidobacterium longum from

the information on the complete nucleotide sequence of the Bifidobacterium longum-derived plasmid pTB6 obtained in Example 1.

[0055]

The sequence of approximately 1900 bp containing the plasmid replication unit of Bifidobacterium longum was amplified from pTB6 by PCR and subcloned into pCR-Blunt II-TOPO vector to prepare pCRTOPO-ApaI-1900-ScaI. Restriction enzyme sites ApaI and ScaI were added to forward and reverse primers, respectively.

[0056]

A long fragment (approximately 2400 bp) obtained from pAVN digested with restriction enzymes ApaI and ScaI and a short fragment (approximately 1900 bp) obtained from pCRTOPO-ApaI-1900-ScaI digested with restriction enzymes ApaI and ScaI were ligated by use of T4 DNA ligase to prepare a Bifidobacterium longum-Escherichia coli shuttle vector pAV001 (approximately 4300 bp).

Example 3

[0057]

(Cytosine deaminase gene expression vector pAV001-HU-eCD)

[Construction of expression vector]

Next, pBLES100-S-eCD was cleaved with restriction enzymes HindIII and SpeI to extract a sequence of approximately 2900 bp containing a HU gene promoter, an Escherichia coli-derived cytosine deaminase gene, and a HU gene terminator. A long fragment obtained from the shuttle vector pAV001 cleaved at the restriction enzyme sites in the multi-cloning site with HindIII and SpeI and the above fragment of approximately 2900

bp were ligated by use of T4 DNA ligase to prepare pAV001-HU-eCD (approximately 7100 bp).

[0058]

[Comparison of plasmid copy number in Bifidobacterium longum]

The pAV001-HU-eCD and pBLES100-S-eCD constructed this time were respectively introduced into Bifidobacterium longum to prepare two recombinant microorganisms, Bifidobacterium longum::pAV001-HU-eCD and Bifidobacterium longum::pBLES100-S-eCD. The presence of the respective plasmids was confirmed by PCR. Bifidobacterium longum::pAV001-HU-eCD and Bifidobacterium longum::pBLES100-S-eCD were respectively subcultured at 37°C for 2 or more days in MRS media (OXID) under anaerobic conditions. The respective bacterial cells (1 x 10^9 CFU) were separated by centrifugation from the resulting culture media, and total DNAs were extracted by using Puregene DNA Isolation Kit (Gentra Systems). The collected total DNAs were digested with restriction enzymes HindIII and Eco81I, and nucleic acids were separated by agarose gel electrophoresis and subjected to Southern analysis using AlkPhos Direct Labelling Reagents (Amersham Bioscience) to compare plasmid copy numbers (see Figure 9A). For a sensitization method of signals, the nucleic acids allowed to develop chemiluminescence with alkaline phosphatase were exposed to X-ray films. In the comparison of signal intensity, the X-ray films after exposure were scanned using a gel scanning system (ATTO), and the obtained scan images were analyzed with CS analyzer (software; ATTO) to determine plasmid copy numbers. As a result, the plasmid copy number of pBLES100-S-eCD was approximately 3.26/cell, whereas the plasmid copy number of pAV001-HU-eCD was approximately

11.68/cell. Therefore, the plasmid copy number of pAV001-HU-eCD was confirmed to be 3.6 times larger than that of pBLES100-S-eCD, and significant difference was observed in the number of the pAV001-HU-eCD plasmid replicating, when compared to that of pBLES100-S-eCD (see Figure 9B).

[0059]

[Comparison of expression level of cytosine deaminase protein in Bifidobacterium longum]

Bifidobacterium longum::pAV001-HU-eCD and Bifidobacterium longum::pBLES100-S-eCD were respectively subcultured at 37°C for 2 or more days in MRS media under anaerobic conditions. The respective bacterial cells (1×10^9 CFU) were separated by centrifugation from the resulting culture media and disrupted by ultrasonication to extract the respective proteins in the bacterial cells. The above extracted proteins were separated by SDS-polyacrylamide gel electrophoresis, and the signal intensity of cytosine deaminase proteins was compared by Western analysis. A rabbit anti-cytosine deaminase monoclonal antibody (Sawaday Technology) and a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G complex (Santa Cruz Biotechnology, Inc) were used as primary and secondary antibodies, respectively. For a sensitization method of signals, the proteins allowed to develop light by an ECL method were exposed to X-ray films. In the comparison of signal intensity, the X-ray films after the exposure were scanned using a gel scanning system (ATTO), and the obtained scan images were analyzed with CS analyzer (software; ATTO) to compare the expression levels. The result is shown in Figure 6. The result of comparison of signal intensity revealed that Bifidobacterium longum::pAV001-HU-eCD expresses the cytosine

deaminase protein 2 to 8 times compared to Bifidobacterium longum::pBLES100-S-eCD.

[0060]

[Comparison of enzyme activity of cytosine deaminase protein (comparison of activity of converting 5FC to 5FU) in Bifidobacterium longum]

In addition to Bifidobacterium longum::pAV001-HU-eCD(D) and Bifidobacterium longum::pBLES100-S-eCD (B), a wild-type strain of Bifidobacterium longum (A) and Bifidobacterium longum::pAVN without the introduced cytosine deaminase gene(C) used as controls were respectively subcultured at 37°C for 2 or more days in MRS media under anaerobic conditions. The respective bacterial cells (2×10^9 CFU) were separated by centrifugation from the resulting culture media and resuspended in 4.5 ml of MRS media. Subsequently, the cells were supplemented with 0.5 ml of 5FC (20 mg/ml) at the final concentration of 2 mg/ml and cultured at 37°C under anaerobic conditions. The respective supernatants from which the bacterial cells were removed by centrifugation were collected from the culture media on 0, 4, 8, 18 and 24 hours to measure a converted 5FU concentration by gas chromatography (5FU GC-MS methods, BML). A temporal shift in bacterial counts is shown in Figure 7, and the 5-FU concentration is shown in Figure 8. The results of analysis revealed that the amount of 5FU converted in Bifidobacterium longum::pAV001-HU-eCD(D)was approximately 1.83 times (on 4 hours), approximately 1.52 times (on 8 hours), approximately 1.34 times (on 18 hours), approximately 1.57 times (on 24 hours), and approximately 1.57 times (on average) larger than those in Bifidobacterium longum::pBLES100-S-eCD (B).

[0061]

(Gene transfer of cytosine deaminase gene expression vector pAV001-HU-eCD to microorganisms of genus Bifidobacterium)

The cytosine deaminase gene expression vector pAV001-HU-eCD was introduced into bacterial strains by electroporation shown in Table 4 to prepare recombinant strains of Bifidobacterium.

[0062]

[Table 4]

	Bacterial strains
1	Bifidobacterium longum (standard strain of common name: Bifidobacterium infantis) JCM1222
2	Bifidobacterium longum(standard strain of common name: Bifidobacterium lactenis)JCM1210
3	Bifidobacterium breve(standard strain)JCM1190
4	Bifidobacterium longum aE-194b strain
5	Bifidobacterium longum bs-601 strain

At present, infantis and lactenis are included as subspecies of longum in longum from the standpoint of taxonomy whileas they were previously known as distinct bacterial species belonging to the genus Bifidobacterium.

[0063]

(Measurement of cytosine deaminase activity of recombinant strains of Bifidobacterium)

After the culture of the recombinant strains of Bifidobacterium, the respective bacterial cells were centrifuged from the resulting culture media, then suspended in buffer solutions containing 50 mM HEPES, and disrupted in a ultrasonicator. These disruption solutions were centrifuged, and the supernatants from which unnecessary fractions were removed were used as extracted protein samples. Aliquots corresponding to the total protein amount of 0.05 mg were taken

from the extracted protein samples and supplemented with a buffer solution to exactly adjust the total amount to 250 μL. Further, 250 μL of 20 mM 5FS solution was exactly added to the resulting solutions, which were then incubated at 37°C for 60 minutes in water bath. After the addition of trichloroacetic acid thereto and extraction, the samples neutralized with sodium hydroxide were measured by HPLC for the amount of 5FU produced and the amount of 5FC remaining. HPLC measurement was conducted twice to confirm that there were only a few measurement error.

[0064]

The cytosine deaminase gene expression vector pAV001-HU-eCD using the novel shuttle vector pAV001 was capable to introduce five bacterial strains shown in Table 4. Moreover, the transgene cytosine deaminase was actually highly expressed as a protein having activity, and the activity was high (see Table 5).

[0065]

[Table 5]

Sample	5FU (mM)	**average 5FU**	5FC (mM)	Amount of 5FC converted (%)
Bifidobacterium infantis / pAV001-HU-eCD	1.767	**1.767**	8.024	**18.0**
	1.768		8.119	
Bifidobacterium lactenis / pAV001-HU-eCD	0.999	**1.006**	9.148	**9.8**
	1.013		9.293	
Bifidobacterium breve / pAV001-HU-eCD	0.848	**0.844**	9.029	**8.6**
	0.840		8.967	
Bifidobacterium longum aE-194b strain / pAV001-HU-eCD	0.595	**0.596**	9.233	**6.1**
	0.597		9.230	
Bifidobacterium longum bs-601 strain / pAV001-HU-eCD	0.637	**0.637**	9.543	**6.3**

Industrial Applicability

[0066]

According to the present invention, a shuttle vector for a microorganism of the genus Bifidobacterium and Escherichia coli having a wide host range and a large copy number in microorganisms of the genus Bifidobacterium and capable of highly expressing of a desired protein in a microorganism of the genus Bifidobacterium when used as an expression vector; an expression vector capable of expressing a desired gene in a microorganism of the genus Bifidobacterium by use of the shuttle vector; a microorganism of the genus Bifidobacterium transformed with the expression vector; and an antitumor agent comprising the microorganism of the genus Bifidobacterium as an active ingredient can be obtained.

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 41

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JUMBO APPLICATIONS/PATENTS

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CONTAINING PAGES 1 TO 41

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NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

77513-56

CLAIMS:

1. A shuttle vector for a microorganism of the genus Bifidobacterium and Escherichia coli comprising a pTB6-derived region portion comprising a replication origin (oriV)-repB region of pTB6 but not comprising MembB, MobA, OrfI and oriT regions of pTB6, and an Escherichia coli-derived plasmid portion comprising a replication region from an Escherichia coli-derived plasmid comprising pUC plasmid replication origin.

2. The shuttle vector according to claim 1, wherein the pTB6-derived region portion consists of a pTB6-derived region portion comprising a 1260-bp nucleotide sequence consisting of a nucleotide sequence represented by nucleotides 305 to 1564 in SEQ ID NO: 1.

3. The shuttle vector according to claim 1 or 2, wherein the Escherichia coli-derived plasmid portion comprising a replication origin (ori) region of Escherichia coli is a plasmid portion not comprising an ampicillin resistance gene (ampR) or having deleted DNA encoding an N-terminal region of an ampicillin resistance gene (ampR) expression product β-lactamase.

4. The shuttle vector according to any one of claims 1 to 3, wherein the microorganism of the genus Bifidobacterium is Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium animalis, Bifidobacterium bifidum, or Bifidobacterium adolescentis.

5. The shuttle vector according to any one of claims 1 to 4, wherein the shuttle vector has an average copy number of 6 to 30.

6. An expression vector having an ability to express a desired gene in a microorganism of the genus Bifidobacterium, comprising the shuttle vector for a microorganism of the genus Bifidobacterium and Escherichia coli according to any one of claims 1 to 5 into which an expression unit of the desired gene ligated in-frame between a promoter and a terminator involved in the expression of a Bifidobacterium longum-derived gene encoding a histone-like DNA-binding protein (HU protein) is inserted.

7. The expression vector according to claim 6, wherein the desired gene is a gene encoding a cytokine having an antitumor activity, a gene encoding an angiogenic inhibitor, or a gene encoding an enzyme capable of converting an antitumor substance precursor to an antitumor substance.

8. The expression vector according to claim 7, wherein the gene encoding an enzyme capable of converting an antitumor substance precursor to an antitumor substance is a cytosine deaminase gene.

9. A microorganism of the genus Bifidobacterium transformed with the shuttle vector for a microorganism of the genus Bifidobacterium and Escherichia coli according to any one of claims 1 to 5.

10. The microorganism of the genus Bifidobacterium according to claim 9, which is Bifidobacterium longum, Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium pseudolongum, Bifidobacterium thermophirum, Bifidobacterium breve, Bifidobacterium infantis, or Bifidobacterium animalis.

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11. A microorganism of the genus Bifidobacterium transformed with the expression vector according to claim 7 or 8.

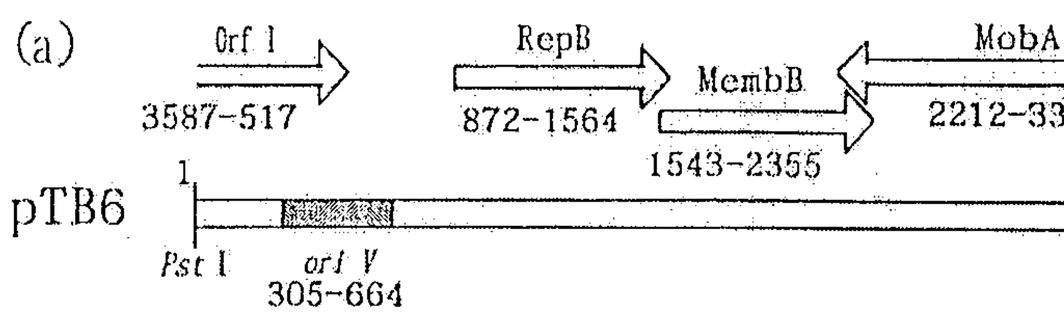
12. The microorganism of the genus Bifidobacterium according to claim 11, which is Bifidobacterium longum, Bifidobacterium adolescentis, Bifidobacterium bifidum, Bifidobacterium pseudolongum, Bifidobacterium thermophirum, Bifidobacterium breve, Bifidobacterium infantis, or Bifidobacterium animalis.

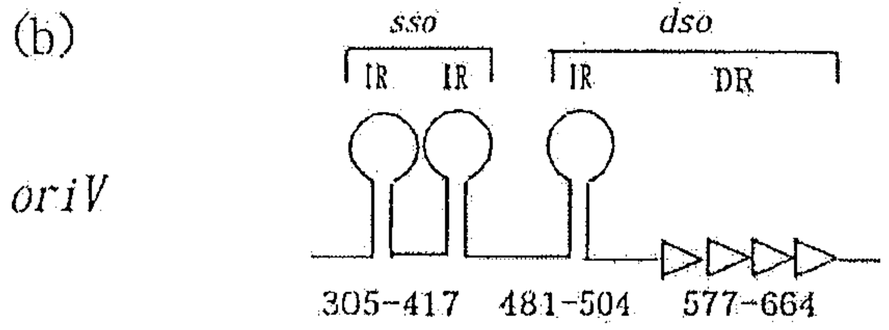
13. An antitumor agent comprising the microorganism of the genus Bifidobacterium according to claim 11 or 12 as the active ingredient.

14. A pTB6-derived plasmid replication unit of a microorganism of the genus Bifidobacterium comprising a replication origin (oriV)-repB region of pTB6 but not comprising MembB, MobA, OrfI, and oriT regions of pTB6.

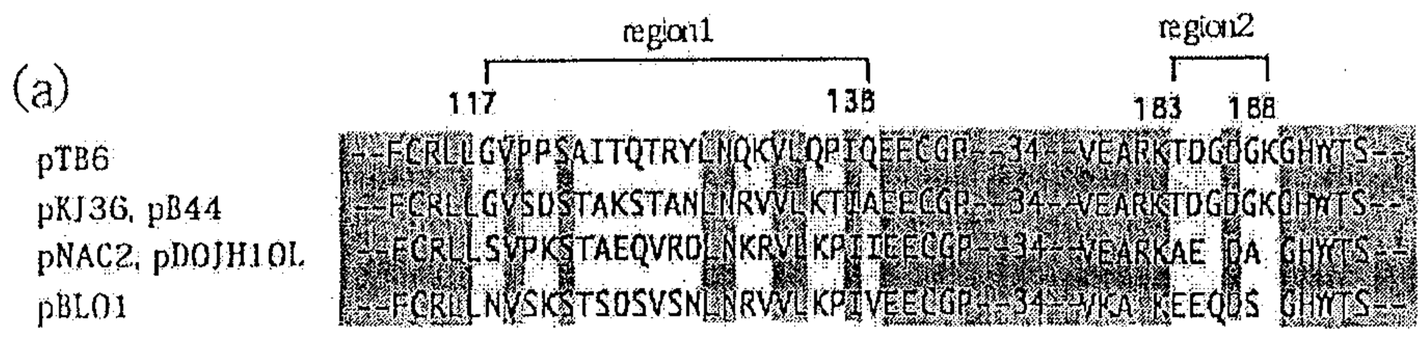
15. The pTB6-derived plasmid replication unit of the microorganism of the genus Bifidobacterium according to claim 14, comprising a 1260-bp nucleotide sequence consisting of a nucleotide sequence represented by nucleotides 305 to 1564 in SEQ ID NO: 1.

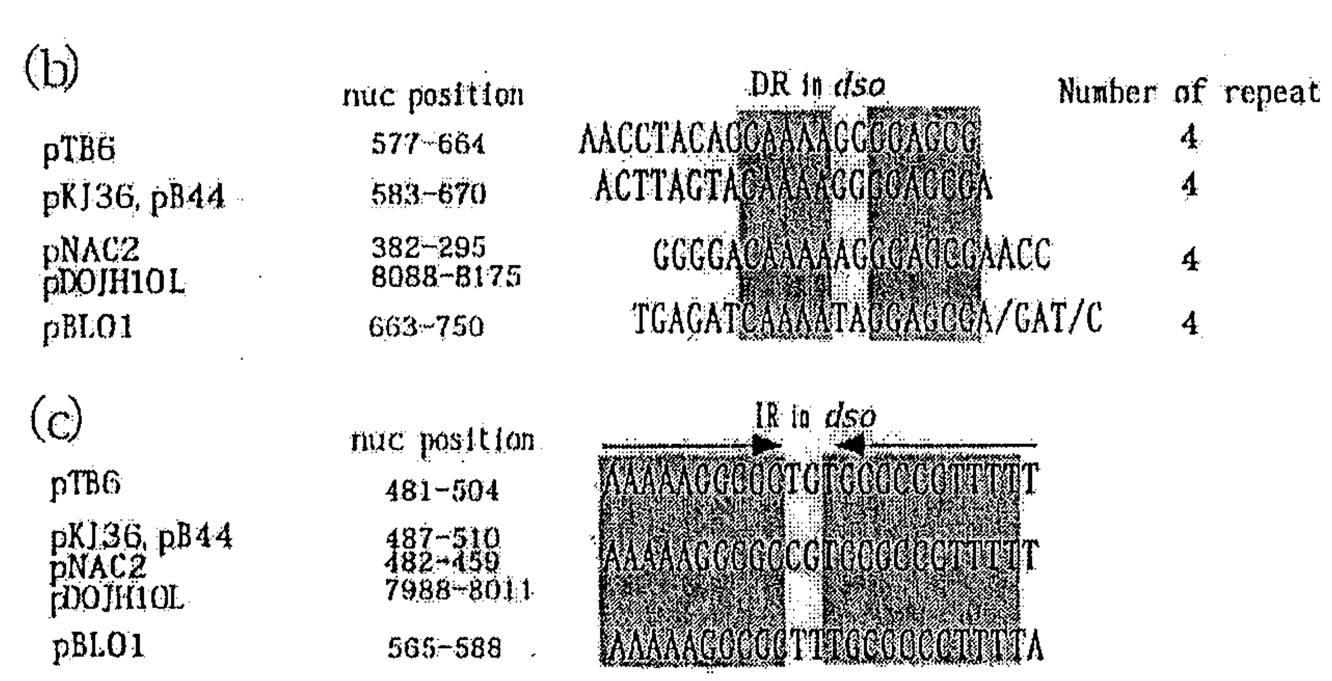
[Fig. 1]



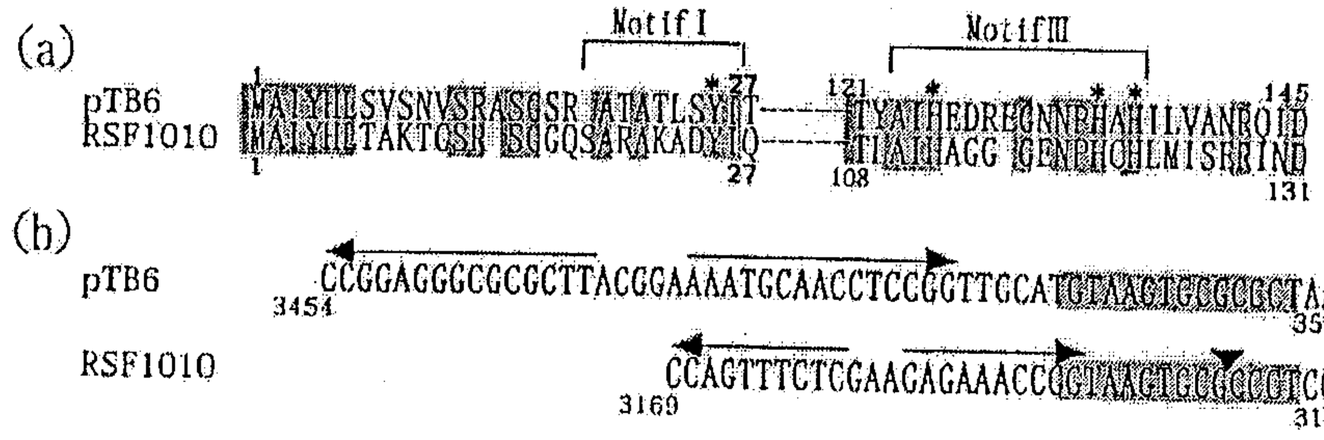


[Fig. 2]

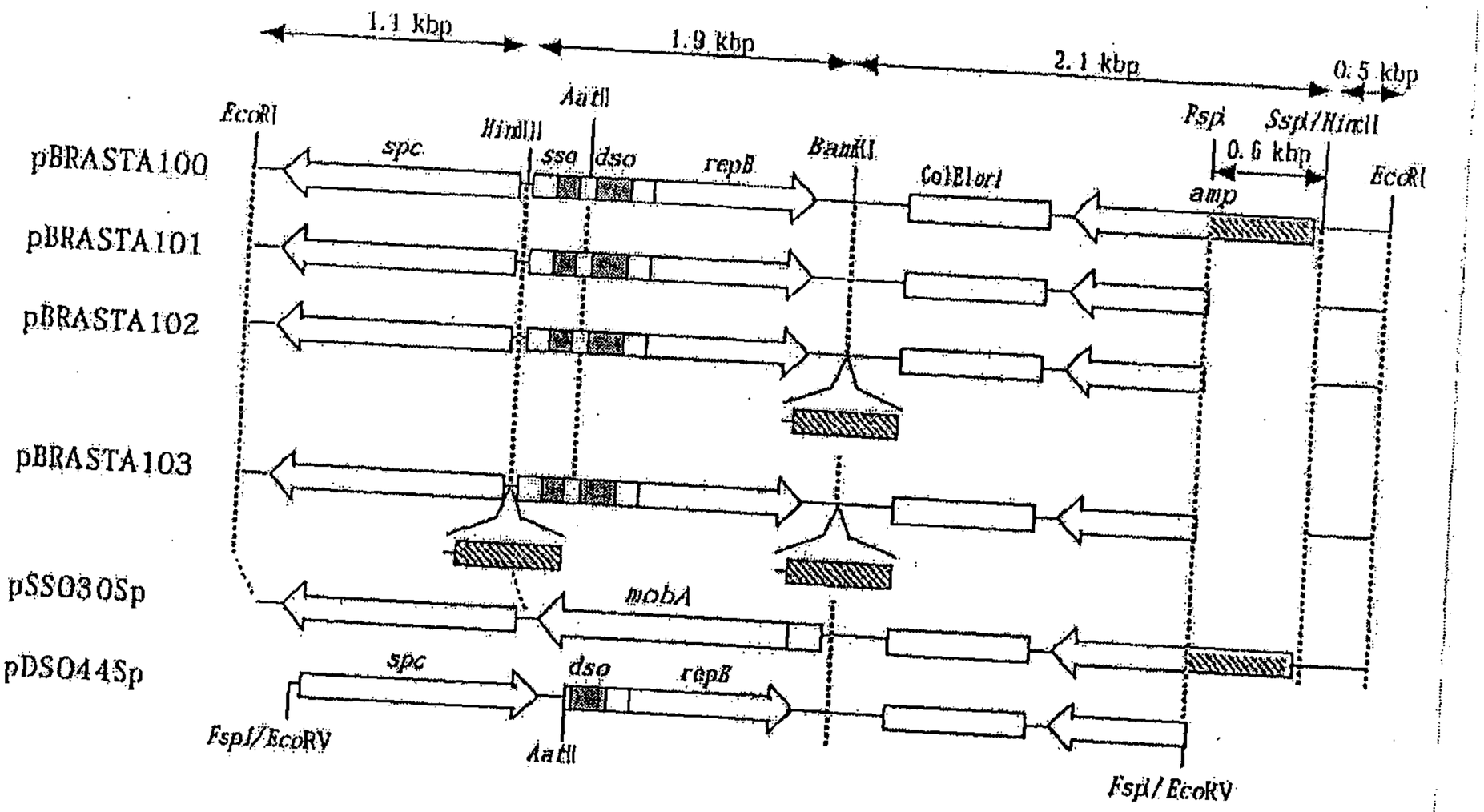




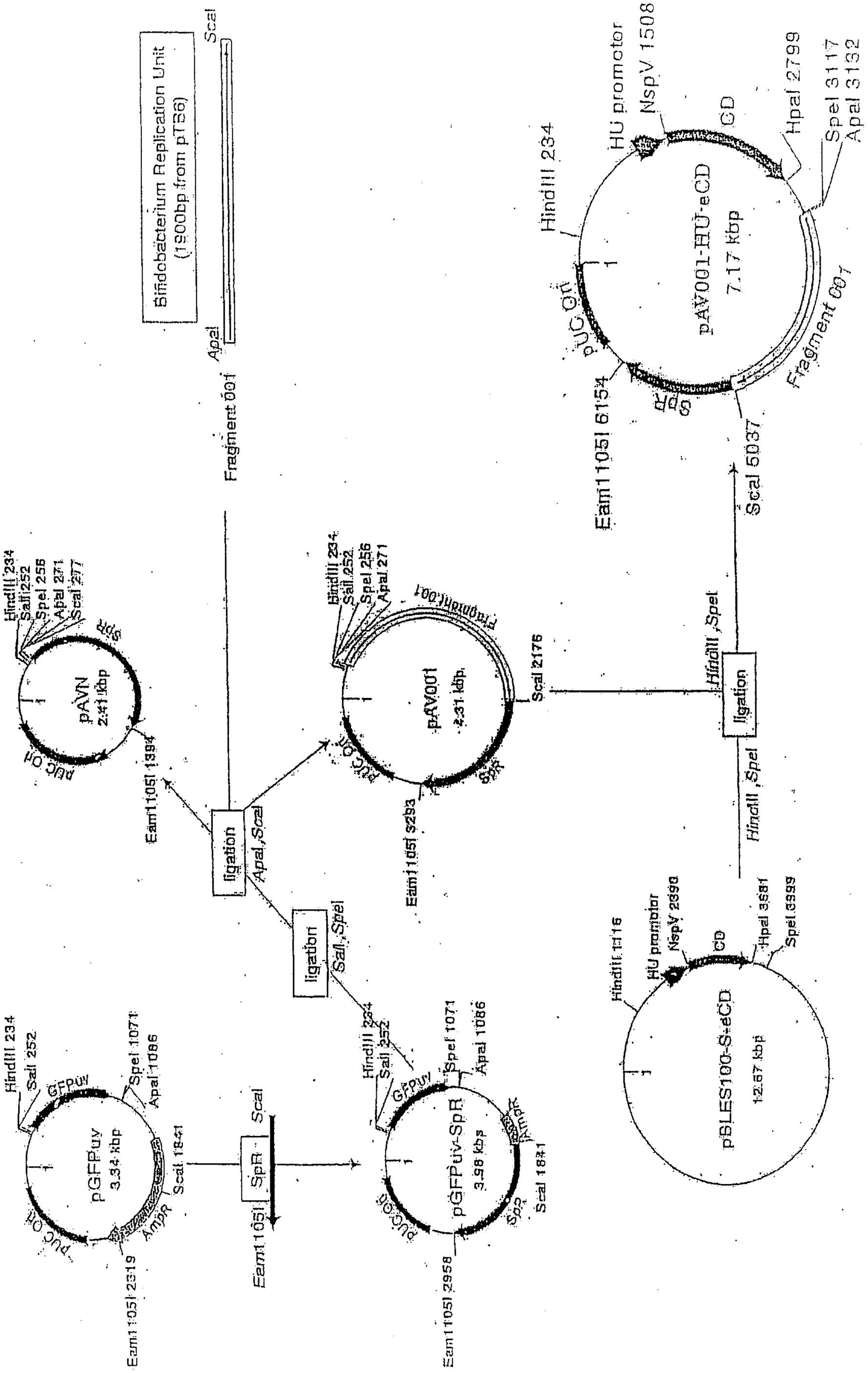
[Fig. 3]



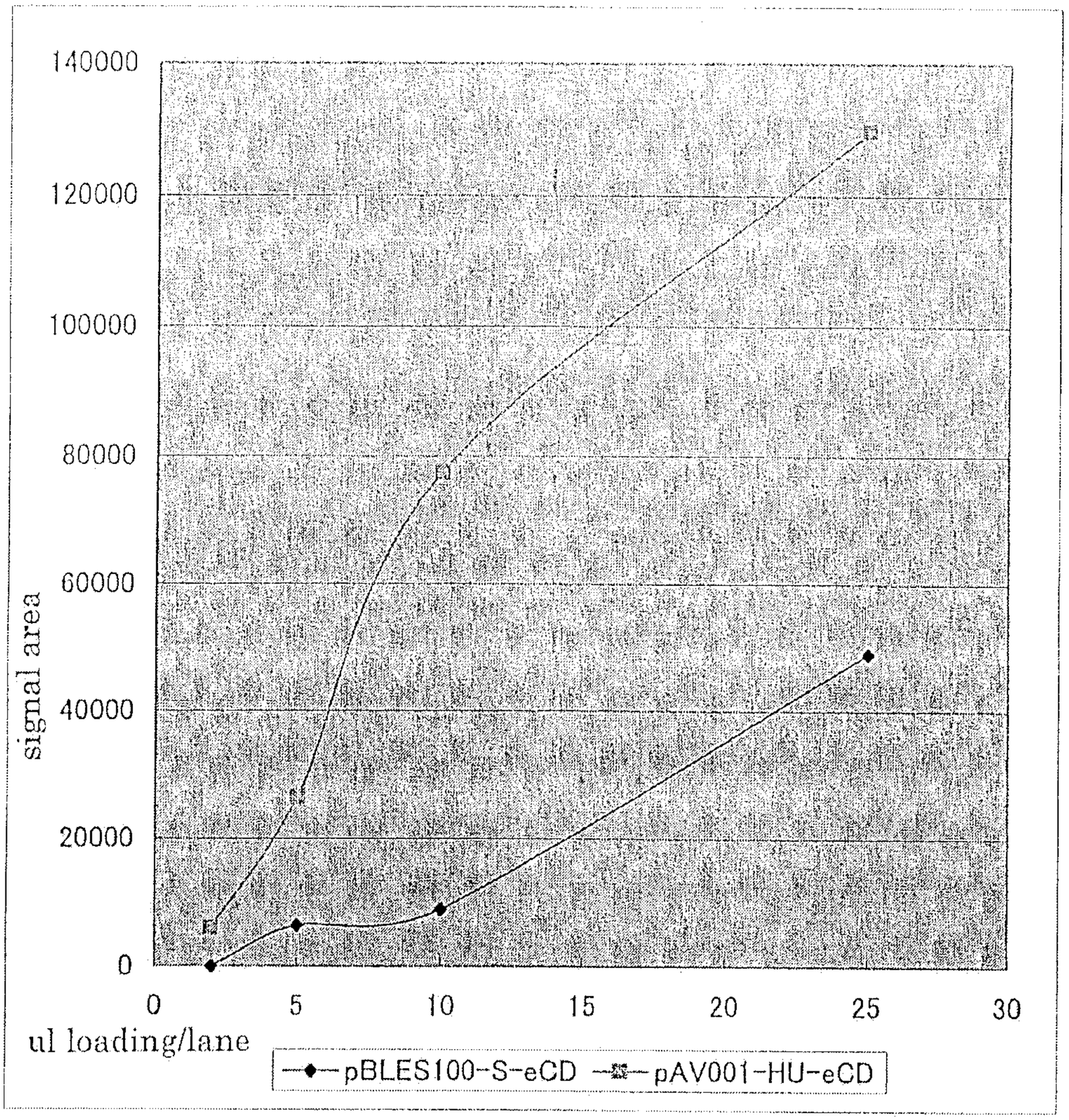
[Fig. 4]


1.1 kbp
1.9 kbp
2.1 kbp
0.5 kbp
EcoRI
HindIII
AatII
PspI
SspI/HincII
0.6 kbp
EcoRI
pBRASTA100
spc
ssa
dsa
repB
BanHI
ColE1ori
amp
pBRASTA101
pBRASTA102
pBRASTA103
pSSO30Sp
mobA
pDSO44Sp
spc
dso
repB
FspI/EcoRV
AatII
FspI/EcoRV

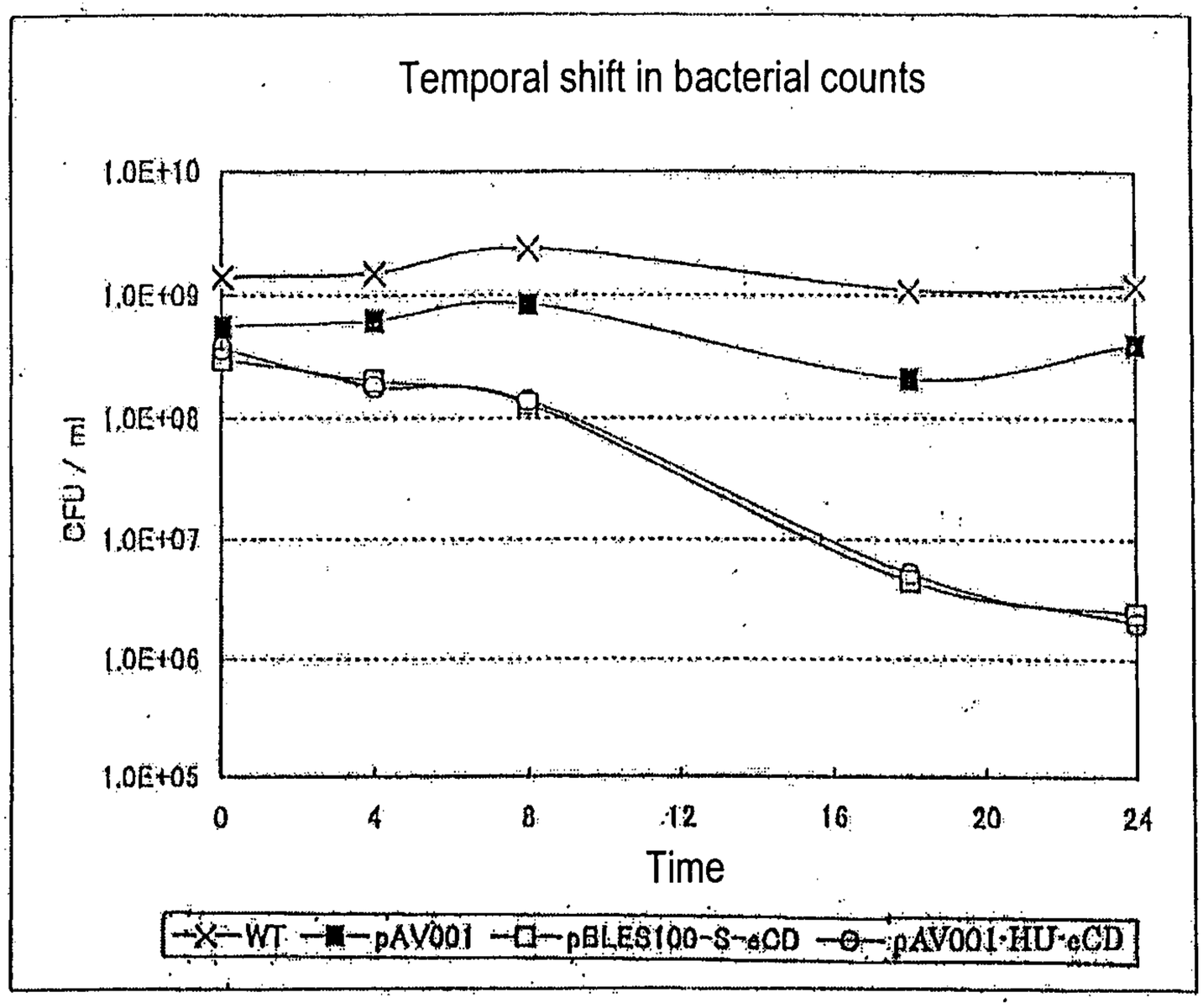
[Fig. 5]


Bifidobacterium Replication Unit
(1900bp from pTB6)
ScaI
ApaI
Fragment 001
pAVN
2.41 kbp
HindIII 234
SalI 252
SpeI 256
ApaI 271
ScaI 277
pUC Ori
Ampr
EamI105I 1394
ligation
ApaI,ScaI
pAV001
2.31 kbp
ScaI 2175
EamI105I 2293
ligation
SalI,SpeI
HindIII 234
SalI 252
SpeI 256
ApaI 271
Fragment 001
SpeI 1071
ApaI 1086
pGFPuv
3.34 kbp
GFPuv
SpeI 1071
ApaI 1086
ScaI 1841
AmpR
EamI105I 2319
EamI105I
SpR
ScaI
pGFPuv-SpR
3.98 kbp
SpR
EamI105I 2958
HindIII 234
SalI 252
SpeI 256
ApaI 271
ScaI 1841
HindIII,SpeI
ligation
EamI105I 6154
ScaI 5037
pAV001-HU-eCD
7.17 kbp
HU promotor
NspV 1508
eCD
HpaI 2799
SpeI 3117
ApaI 3132
Fragment 001
HindIII,SpeI
ligation
HindIII 1116
HU promotor
NspV 2390
eCD
HpaI 3581
SpeI 3599
pBLES100-S-eCD
12.67 kbp

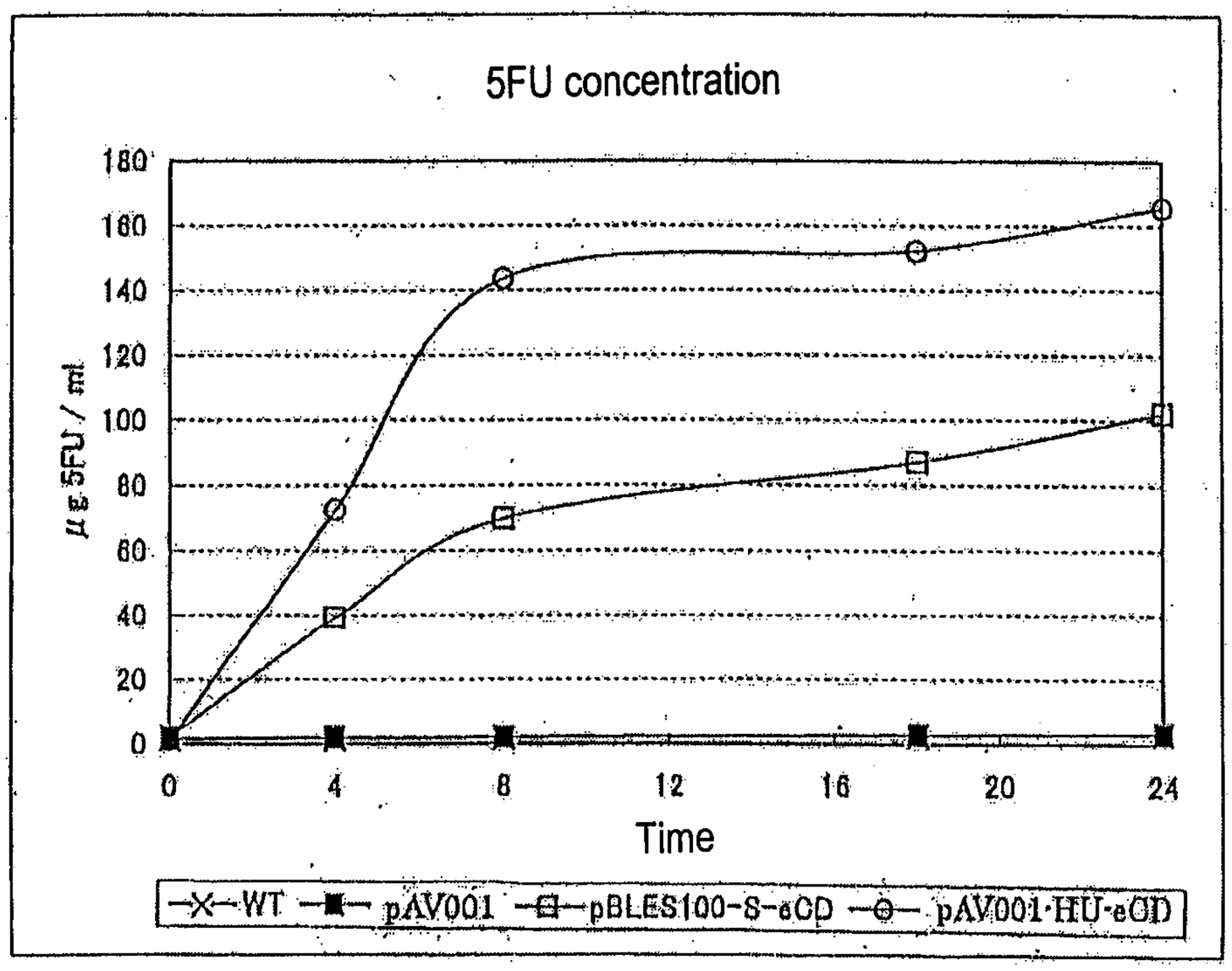
[Fig. 6]


140000
120000
100000
80000
60000
40000
20000
0
signal area
0
5
10
15
20
25
30
ul loading/lane
pBLES100-S-eCD
pAV001-HU-eCD

[Fig. 7]

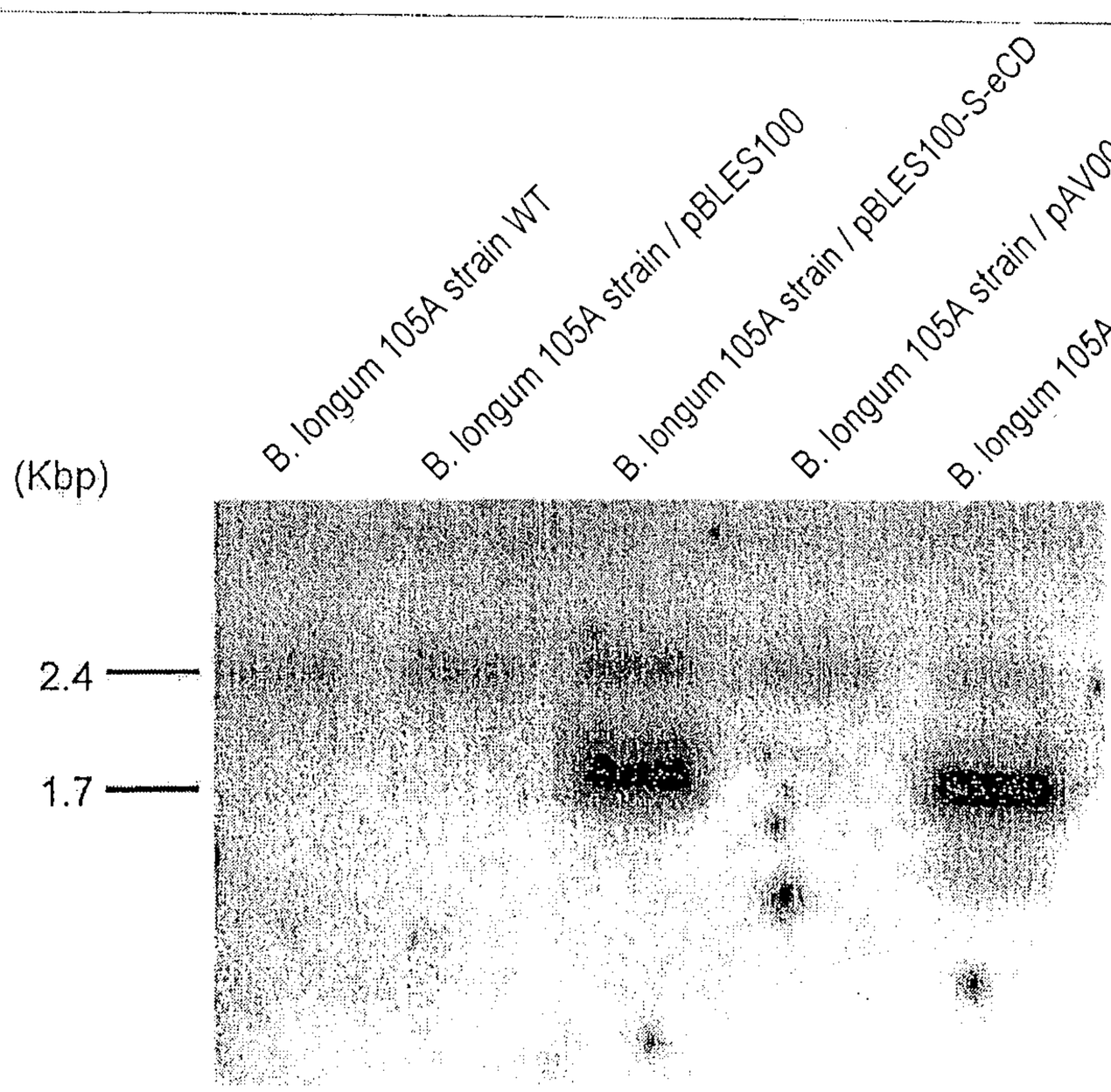

Temporal shift in bacterial counts
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
CFU / ml
0
4
8
12
16
20
24
Time
WT
pAV001
pBLES100-S-sCD
pAV001-HU-sCD

[Fig.8]


5FU concentration
180
160
140
120
100
80
60
40
20
0
μg 5FU / ml
0
4
8
12
16
20
24
Time
WT
pAV001
pBLES100-S-sCD
pAV001-HU-sCD

[Fig. 9]

A



B

hup gene (Genome)	14212	15164	29784	15708	8228
hup promoter (Plasmid)			97104		96084
Plasmid Copy Number			3.26		11.68