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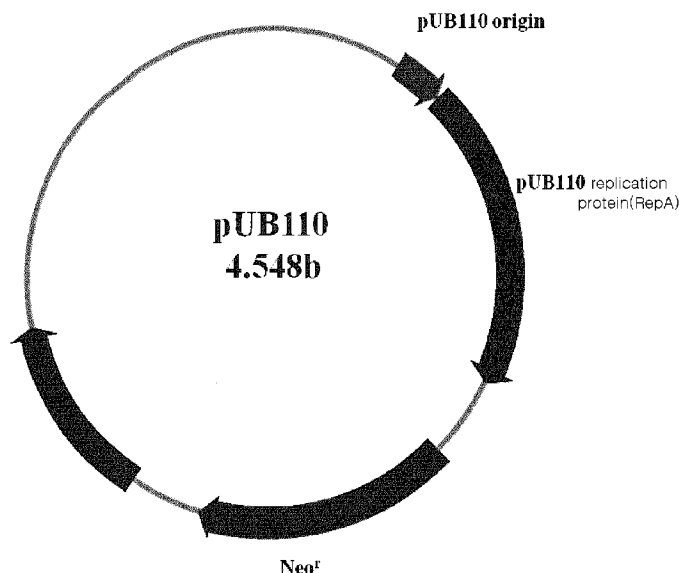
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(54) Titre : UN PLASMIDE NAVETTE REPLICABLE DANS LES CLOSTRIDIA ET L'ESCHERICHIA COLI

(54) Title: A SHUTTLE PLASMID REPLICABLE IN BOTH CLOSTRIDIA AND ESCHERICHIA COLI



(57) Abrégé/Abstract:

Disclosed is a shuttle plasmid replicable in both Clostridium and Escherichia coli comprising: a base sequence of a first replication origin replicable in Escherichia coli; and a base sequence of a second replication origin derived from pUB110 plasmid.

ABSTRACT OF THE DISCLOSURE

Disclosed is a shuttle plasmid replicable in both *Clostridium* and *Escherichia coli* comprising: a base sequence of a first replication origin replicable in *Escherichia coli*; and
5 a base sequence of a second replication origin derived from pUB110 plasmid.

A SHUTTLE PLASMID REPLICABLE IN BOTH CLOSTRIDIA AND ESCHERICHIA COLI

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BACKGROUND

1. Technical Field

The present invention relates to a shuttle plasmid replicable in both *Clostridium* and *Escherichia coli*.

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2. Description of the Related Art

Microorganisms of the genus *Clostridium* are Gram-positive bacteria. They are obligate anaerobes capable of producing spores and have a variety of activity as biocatalysts. Recently, as the full genomes of some microorganisms of the genus *Clostridium* have recently been sequenced, it is expected that studies into *Clostridium* will contribute to the production of biofuels from renewable biomass and development of methods for interpreting and preventing pathogenic mechanisms of some microorganisms of the genus *Clostridium*. Although *Clostridium* is important industrially and medicinally, research of *Clostridium* has been extremely limited due to absence of useful genetic engineering techniques. Accordingly, there is a need for development of effective host/plasmid systems useful for developing *Clostridium* strains having an effectively high metabolic activity.

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On the other hand, in order to replicate a plasmid in *acetobutylicum* and/or *Escherichia coli*, shuttle plasmids such as pMTL500E (Minton NP, Oultram JD. 1988. Host: vector systems for gene cloning in *Clostridium*. Microbiological Sciences 5: 310-315), pIM1 (Mermelstein LD, Papoutsakis ET. 1993. In vivo methylation in *Escherichia coli* by the *Bacillus subtilis* phage phi 3TI methyltransferase to protect plasmids from restriction upon transformation of *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 59:1077-1081.), pJIR418 (Sloan J, Warner TA, Scott PT, Bannam TL, Berryman DI, Rood JJ. 1992. Construction of a sequenced *Clostridium perfringens*-

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Escherichia coli shuttle plasmid. Plasmid 27:207-219.), and pCB102 (Fox ME, Lemmon MJ, Mauchline ML, Davis TO, Giaccia AJ, Minton NP, Brown JM. 1996. Anaerobic bacteria as a delivery system for cancer gene therapy: in vitro activation of 5-fluorocytosine by genetically engineered clostridia. Gene therapy 3: 173-178.), and the like have been developed. The *Clostridial* replication origins (origins of replication) are derived from pAM β 1, pIMP13, pIP404, and pCB102, respectively. However, no plasmid is used industrially since their segregational stability is very low under culture conditions with no antibiotic present. Namely, since use of antibiotics may deteriorate economic feasibility of microorganisms in terms of industrial application or can cause problems related to environmental stability, in order for plasmids to be used industrially, segregational stability of the plasmids in a medium containing no antibiotics should be guaranteed. The shuttle plasmids such as pMTL500E, pIM1, pJIR418, pCB102, and the like do not possess the requisite segregational stability.

The present inventors have endeavored to find a novel plasmid having excellent segregational stability in a culture medium containing no antibiotics and capable of replication in *Clostridium acetobutylicum*, and constructed plasmids including specific replication origins and regions encoding replication proteins, and the like which are found to be replicable in both *Clostridium* and *Escherichia coli* and have high segregational stability. The present invention is based on this finding.

BRIEF SUMMARY

It is an aspect of the present invention to provide a shuttle plasmid replicable in both *Clostridium* and *Escherichia coli*.

In accordance with one aspect of the present invention, there is provided a shuttle plasmid replicable in both *Clostridium* and *Escherichia coli*, including: a base sequence of a first replication origin replicable in *Escherichia coli*; and a base sequence of a second replication origin derived from pUB110 plasmid.

The shuttle plasmid may be replicable in both *Clostridium* and *Escherichia coli*.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other aspects, features, and advantages of the present invention will become apparent from the detailed description of the following embodiments in conjunction with the accompanying drawings, in which:

Fig. 1 shows a genetic map of cryptic plasmid pUB110 derived from *Staphylococcus aureus*;

Fig. 2 shows a process for constructing a shuttle plasmid pLK1-MCS from pUB110 cryptic plasmid and pMTL500E plasmid including a base sequence of a replication origin of pUC19 plasmid;

Fig. 3 shows segregational stability of a shuttle plasmid pLK1-MCS of the present invention; and

Fig. 4 shows a process for constructing a recombinant plasmid in which a recombinant gene is cloned so that acetone is converted into isopropanol by using the shuttle plasmid of the present invention.

Explanation of SEQ ID NO.

SEQ ID NO: 1 and SEQ ID NO: 2 are base sequences of two primers used in the amplification of a region encoding a replication protein and a replication origin of pUB110 plasmid.

SEQ ID NO: 3 is a base sequence of a replication origin of pUB110 plasmid.

SEQ ID NO: 4 is a base sequence of a region encoding a replication protein of pUB110 plasmid.

SEQ ID NO: 5 is an amino acid sequence of a replication protein (RepA) of pUB110 plasmid.

SEQ ID NO: 6 is a base sequence of a DNA fragment including a multiple cloning site used in the construction of a shuttle plasmid pLK1-MCS of the present invention.

SEQ ID NO: 7 is a base sequence of an erythromycin resistance gene.

SEQ ID NO: 8 is a base sequence of an ampicillin resistance gene.

SEQ ID NO: 9 is a base sequence of a replication origin of pUC19 plasmid.

SEQ ID NO: 10 is a base sequence of a shuttle plasmid pLK1-MCS of the present invention.

SEQ ID NO: 11 and SEQ ID NO: 12 are base sequences of primers used in the amplification of a region encoding a secondary alcohol dehydrogenase through PCR reaction.

DETAILED DESCRIPTION

The present invention relates to a shuttle plasmid replicable in both *Clostridium* and *Escherichia coli* including:

- a base sequence of a first replication origin replicable in *Escherichia coli*; and
- a base sequence of a second replication origin derived from pUB110 plasmid.

In addition, the present invention relates to a shuttle plasmid replicable in both *Clostridium* and *Escherichia coli* including:

- a base sequence of pMTL500E plasmid including a replication origin of pUC19 plasmid (pUC origin) and ampicillin and erythromycin antibiotic resistance genes; and
- a base sequence of a base sequence of a replication protein and a replication origin derived from pUB110 plasmid (pUB110 origin).

Further, the present invention relates to a method for constructing a transformed microorganism including:

- preparing a shuttle plasmid of the present invention; and
- introducing the shuttle plasmid into a microorganism.

Furthermore, the present invention relates to a transformed microorganism including a shuttle plasmid of the present invention.

Furthermore, the present invention relates to a method for producing a culture including:

- culturing a transformed microorganism including a shuttle plasmid of the present

invention; and

harvesting the culture.

Hereinafter, embodiments of the present invention will be described in detail.

5 Base sequence of a first replication origin replicable in *Escherichia coli*

The shuttle plasmid of the present invention includes a base sequence of a first replication origin replicable in *Escherichia coli*. The base sequence of the first replication origin may be preferably a base sequence of a replication origin derived from pUC19 plasmid. Namely, the base sequence of the first replication origin may be derived from
10 pUC (pUC origin). In addition, the base sequence of a replication origin derived from pUC19 plasmid may be derived from pMTL500E plasmid.

Base sequence of a second replication origin derived from pUB110 plasmid

The shuttle plasmid of the present invention includes a base sequence of a second
15 replication origin derived from pUB110 plasmid. pUB110 plasmid is a cryptic plasmid derived from *Staphylococcus aureus*, and the genetic map thereof is depicted in Fig. 1. The base sequence of a replication origin derived from pUB110 plasmid may be a base sequence of SEQ ID NO: 3. In addition, the base sequence may have 70% or more, preferably 80% or more, more preferably 90% or more homology with SEQ ID NO: 3 and
20 maintain functionality of the replication origin.

Base sequence encoding a replication protein derived from pUB110 plasmid

The shuttle plasmid of the present invention may include a base sequence encoding a replication protein and derived from pUB110 plasmid. The base sequence
25 encoding the replication protein may be a base sequence of SEQ ID NO: 4. The base sequence may have 70% or more, preferably 80% or more, more preferably 90% or more homology with SEQ ID NO: 4 and encode a protein maintaining the functionality of the replication protein. On the other hand, the amino acid sequence of the replication protein may be an amino acid sequence of SEQ ID NO: 5. The amino acid sequence may have 70%
30 or more, preferably 80% or more, more preferably 90% or more homology with SEQ ID

NO: 5 and maintain the functionality of the replication protein.

First antibiotic resistance gene

5 The shuttle plasmid of the present invention may include a first antibiotic resistance gene capable of being expressed in *Escherichia coli* and serving as a selective marker in *Escherichia coli*. The first antibiotic resistance gene is expressed in *Escherichia coli* and serves as a selective marker. Preferably, the first antibiotic resistance gene is an ampicillin antibiotic resistance gene.

Second antibiotic resistance gene

10 The shuttle plasmid of the present invention may include a second antibiotic resistance gene capable of being expressed in *Clostridium* and serving as a selective marker in *Clostridium*. The second antibiotic resistance gene is expressed in *Clostridium*, preferably *Clostridium acetobutylicum* and serves as a selective marker. Preferably, the
15 second antibiotic resistance gene is an erythromycin antibiotic resistance gene.

However, it is not intended that the first antibiotic resistance gene is limited to the ampicillin antibiotic resistance gene, or the second antibiotic resistance gene is limited to erythromycin antibiotic resistance gene. For example, the first antibiotic resistance gene
20 and the second antibiotic resistance gene may be identical. The first antibiotic resistance gene may be any antibiotic resistance gene so long as such gene can serve as a selective marker in *Escherichia coli*. The second antibiotic resistance gene may be any antibiotic resistance gene so long as such gene can serve as a selective marker in *Clostridium*.

Shuttle plasmid of the present invention

25 The shuttle plasmid of the present invention is a shuttle plasmid replicable in both *Clostridium* and *Escherichia coli* including:

- a base sequence of a first replication origin replicable in *Escherichia coli*; and
- a base sequence of a second replication origin derived from pUB110 plasmid.

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Further, the shuttle plasmid of the present invention may be a shuttle plasmid replicable in both *Clostridium* and *Escherichia coli* including:

- a base sequence of a first replication origin replicable in *Escherichia coli*;
- a base sequence of a second replication origin derived from pUB110 plasmid;
- 5 a base sequence encoding a replication protein derived from pUB110 plasmid;
- a first antibiotic resistance gene expressed in *Escherichia coli*; and
- a second antibiotic resistance gene expressed in *Clostridium*.

The shuttle plasmid of the present invention may include a base sequence of SEQ ID NO: 7, or a base sequence having 80% or more, preferably 90% or more, more preferably 95% or more homology with SEQ ID NO: 7 so long as the base sequence maintains replication capability in both *Escherichia coli* and *Clostridium*, and expression and selection marker capability of antibiotic resistance genes in both *Escherichia coli* and *Clostridium*. Further, the shuttle plasmid of the present invention may be the pLK1-MCS.

15 In addition, the shuttle plasmid of the present invention can be easily constructed using pUB110 cryptic plasmid and pMTL500E plasmid, wherein pMTL500E plasmid includes a replication origin of pUC19 plasmid, the first antibiotic resistance gene and the second antibiotic resistance gene (Fig. 2).

20 The shuttle plasmid of the present invention is a shuttle vector replicable in both *Clostridium* and *Escherichia coli*. Particularly, the shuttle plasmid has high segregational stability in *Clostridium*. Therefore, use of the shuttle plasmid of the present invention may allow the shuttle plasmid and a target gene to be recombined, which functions as an operably linked recombinant shuttle plasmid. Further, the shuttle plasmid of the present invention is capable of replication in both *Clostridium* and *Escherichia coli* under an environment with no antibiotics and under an environment with antibiotics. Specifically,

25 the shuttle plasmid of the present invention is capable of replication in both *Clostridium* and *Escherichia coli* under the environment with no antibiotics, and has high segregational stability, thereby being industrially applicable.

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Further, the shuttle plasmid of the present invention has high segregational stability in *Clostridium* strains, and is sufficiently replicable in a culture medium containing no antibiotics, thereby ensuring stability in a fermentation process or a biotransformation process.

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Clostridium may include *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharobutylicum*, *Clostridium saccharoperbutylacetonicum*, *Clostridium perfringens*, *Clostridium tetani*, *Clostridium difficile*, *Clostridium butyricum*, *Clostridium butylicum*, *Clostridium kluyveri*, *Clostridium tyrobutylicum* or *Clostridium tyrobutyricum*. Preferably, *Clostridium* is *Clostridium acetobutylicum*.

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Method for constructing transformed microorganisms

The present invention provides a method for constructing a transformed microorganism including: preparing a shuttle plasmid of the present invention; and introducing the shuttle plasmid into a microorganism.

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After preparing the shuttle plasmid, a foreign gene is cloned to the shuttle plasmid, which is introduced into a microorganism.

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Furthermore, the present invention provides a method for producing a transformed microorganism including: preparing a shuttle plasmid of the present invention; cloning a foreign gene into the shuttle plasmid; and introducing the shuttle plasmid into which the foreign gene is cloned into a microorganism.

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In addition, the present invention provides a transformed microorganism including the shuttle plasmid of the present invention. The microorganism may be *Escherichia coli* or *Clostridium*.

Method for producing culture products

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The present invention provides a method for producing a culture including:

culturing a transformed microorganism including the shuttle plasmid of the present invention; and harvesting the culture.

5 Further, the present invention provides a method for producing a fermented product including: culturing a transformed microorganism including the shuttle plasmid of the present invention; collecting the culture; and harvesting a fermented product produced by the transformed microorganism from the culture.

10 Further, the present invention provides a method for producing a fermented product including: preparing a shuttle plasmid of the present invention; cloning a foreign gene into the shuttle plasmid; and introducing the shuttle plasmid into a microorganism to construct a transformed microorganism; culturing the transformed microorganism; collecting the culture; and harvesting a fermented product from the culture.

15 The microorganism may be *Escherichia coli* or *Clostridium*. The culture contains fermented products produced by the transformed microorganism. The fermented products may be fermented products originally produced by the microorganism or fermented products produced by the foreign gene. For example, the fermented products may be alcohols, organic acids, ketones, and the like, preferably, alcohols having carbon number of 20 7 or less, polyhydric alcohols, and the like. For example, the fermented products may be butanol, isopropanol, ethanol, 1,3-propanol, 2,3-butandiol, propionic acid, acetone, and the like, without being limited thereto.

25 The above and other aspects, features, and advantages of the present invention will become apparent from the detailed description of the following embodiments in conjunction with the accompanying drawings. However, it should be understood that the present invention is not limited to the following embodiments and may be embodied in different ways, and that the embodiments are provided for complete disclosure and thorough understanding of the invention by those skilled in the art. The scope of the 30 invention should be defined only by the accompanying claims and equivalents thereof.

Materials and methods

pUB110 plasmid and *Clostridium acetobutylicum* ATCC 824 were purchased from the US strain depository authority, American Type Culture Collection (ATCC).

5

<Experimental Example 1>

Construction of pLK1-MCS shuttle plasmid

A region encoding a replication protein and a replication origin of pUB110 plasmid were amplified and obtained using a primer having a base sequence of SEQ ID NO: 1 and a primer having a base sequence of SEQ ID NO: 2 and using pUB110 cryptic plasmid as a template. The base sequence of the replication origin of pUB110 plasmid is the base sequence of SEQ ID NO: 3; the base sequence encoding a replication protein (RepA) is the base sequence of SEQ ID NO: 4; and the amino acid sequence of a replication protein is the amino acid of SEQ ID NO: 5.

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100 µl of PCR reaction mixture was prepared by combining 250 µM dNTP, 20 pmol of each primer, 1.5mM MgCl₂, 10 µl of 10 x buffer, 100 ng of DNA template, and 1 unit of pfu polymerase. In PCR reaction, the reaction repeated 30 cycles consisting of initial denaturing at 95°C for 5 minutes, followed by denaturing at 95°C for one minute, annealing at 58°C for one minute and then polymerizing at 72°C for two minutes. PCR reaction in the following Examples was performed in the same manner as above. The amplified DNA fragment was purified on a 1% agarose gel, and then digested with *SacI/BglII* restriction enzymes to isolate a DNA fragment.

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TABLE I

SEQ ID NO: 1	ATAGAGCTCACGAAGTCGAGATCAGGGAATGAG
SEQ ID NO: 2	GCGAGATCTCTCGTCTTCCTAAGCATCCTTCAATCC
SEQ ID NO: 3	CTTGTTCTTTCTTATCTTGATACATATAGAAATAACGTCATTTTATTTTAGTTGCTGAAAGGTGC GTTGAAGTGTTGGTATGTATGTGTTTTAAAGTATTGAAAACCTTAAATTTGGTTGCACAGAAAA ACCCCATCTGTTAAAGTTATAAGTGAATAACAAATAACTAAATAGA
SEQ ID NO: 4	ATGGGGGTTTCTTTAATATTATGTGTCCTAATAGTAGCATTTATTCAGATGAAAAATCAAGGGT TTTAGTGGACAAGACAAAAAGTGAAAAAGTGAGACCATGGAGAGAAAAGAAAATCGTAATG TTGATTACTTTGAACTTCTGCATATCTTGAATTTAAAAAGGCTGAAAGAGTAAAGATTGTGCT GAAATATTAGAGTATAAACAAAATCGTGAACAGGCGAAAGAAAGTTGATCGAGTGTGGTTT TGTAATCCAGGCTTTGTCCAATGTGCAACTGGAGGAGAGCAATGAAACATGGCATTCACTCA CAAAAGGTTGTGCTGAAGTTATTAACAAAAGCCAACAGTTCGTTGGTTGTTTCTCACATTAAC AGTTAAAAATGTTTATGATGGCGAAGAATTAATAAGAGTTTGTGATATGCTCAAGGATTT CGCCGAATGATGCAATATAAAAAAATTAATAAAAAATCTTGTGGTTTATGCGTGCAACGGAAG TGACAATAAATAATAAGATAATCTTATAATCAGCACATGCATGTATTGGTATGTGTGGAACC AACTTATTTAAGAATACAGAAAACACGTGAATCAAAAACAATGGATTCAATTTTGAAAAAG GCAATGAAATTAGACTATGATCCAAATGTAAAAGTTCAAATGATTGACCGAAAAATAATATA AATCGGATATACAATCGCAATTGACGAAACTGCAAAATATCCTGTAAAGGATACGGATTTTAT GACCGATGATGAAGAAAAGAATTTGAAACGTTTGTCTGATTGGAGGAAGGTTTACACCGTAA AAGGTTAATCTCTATGGTGGTTTGTAAAAGAAATACATAAAAAATTAACCTTGATGACACA GAAGAAGGCGATTGATTCATACAGATGATGACGAAAAAGCCGATGAAGATGGATTTTCTATTA TTGCAATGTGGAATTGGGAACGGAATAATTTTATTAAAGAGTAG
SEQ ID NO: 5	MGVSFNIMCPNSSIYSDEKSRVLVDKTKSGKVRPWREKKIANVDYFELLHILEFKKAERVKDCAEILEY KQNRETGERKLYRVWFCKSRLCPMCNWRRAMKHGIQSQKVVAEVIKQKPTVRWFLTLTVKNVYD GEELNKSLSDMAQGFRRMMQYKKINKNLVGFMRATEVTINNKNDSYNQHMHLVLCVEPTYFKNT ENYVNQKQWQFWKKAMKLDYDPNVKVQMRPKNKYKSDIQSAIDETAKYPVKDTFMTDDEEK NLKRLSDLEGLHRKRLISYGGLLKEIHKKLNLDTEEGDLIHTDDDEKADEDEGFSIIMWNWERKNY FIKE

On the other hand, as depicted in Fig. 2, pMTL500E plasmid was cleaved with *SacI/BamHI* restriction enzymes and the resulting DNA fragment containing an ampicillin resistance gene, an erythromycin resistance gene and a replication origin region of pUC19 plasmid (pUC origin) were purified in a 1% agarose gel, and then, the digested fragment

5 ligated with DNA fragments (SEQ ID NOs: 3 and 4) comprising a replication origin of pUB110 and a replication protein, which was amplified by PCR reaction as mentioned above, digested with *SacI/BglIII* restriction enzymes to construct pLK1-temp. The constructed pLK1-temp was digested with *PvuII/SacI* restriction enzymes, cloned together

10 with a DNA fragment excised from DNA (SEQ ID NO: 6) by digestion of *SmaI/SacI*, which include a promoter and a multiple cloning site synthesized by Bioneer Corp. to

construct a final pLK1-MCS (SEQ ID NO: 10, Fig. 2). The pLK1-MCS includes a base sequence of the erythromycin resistance gene (SEQ ID NO: 7), a base sequence of the ampicillin resistance gene (SEQ ID NO: 8) and a base sequence of the replication origin of pUC19 plasmid (SEQ ID NO: 9).

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The pLK1-MCS (DNA plasmid) was deposited with accession number of KCTC 12755BP at the Korea Research Institute of Bioscience and Biotechnology (BRIBB) on February 4, 2015.

10 TABLE 2

SEQ ID NO: 6	ATACCCGGGCATGATTTTAAGGGGGTTAGCAGATGCATAAGTTTAATTTTTTTGTTAAAAAATAT TAAACTTTGTGTTTTTTTAAACAAATATATTGATAAAAAATAATAAGTGGGTATAATTAAGTTG TTAGAGAAAACGTATAAATTAGGGATAAACTATGGAACCTATGAAATAGATTGAAATGGTTAT CTGTTACCCCGTATCAAATTTAGGAGGTTAGTTTAAACCTGCAGAGATCTCTCGAGGCGGCCG CGTCGACTCTAGACCCGGGAATTCAGTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCT GGCGTTACCCAACTTAATCGCCTTGACGACATCCCCCTTCGCCAGCTGGCGTAATAGCGAAG AGGCCCGCACCGATCGCCCTTCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGC GGTATTTCTCCTACGCATCTGTGCGGTATTTACACCGAGCTCATA
SEQ ID NO: 7	ATGAACAAAAATATAAAATATTCTCAAACTTTTTAACGAGTGAAAAAGTACTCAACCAATAA TAAACAATTGAATTTAAAGAAACCGATACCGTTTACGAAATTGGAACAGGTAAAGGGCATT AACGACGAAACTGGCTAAATAAGTAAACAGGTAACGTCTATTGAATTAGACAGTCATCTATTC AACTTATCGTCAGAAAAATAAACTGAATACTCGTGTCACITTAATTCACCAAGATATTCTACA GTTTCAATTCCTAACAAACAGAGGTATAAAATTGTTGGGAGTATTCCTTACCATTTAAGCACAC AAATTATTAATAAAGTGGTTTTTAAAGCCATGCGTCTGACATCTATCTGATTGTTGAAGAAGG ATTCTACAAGCGTACCTTGGATATTCACCGAACACTAGGGTTGCTCTTGACACTCAAGTCTCG ATTCAGCAATTGCTTAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAAAGTAAACAGTGTCT TAATAAACTTACCCGCCATACCACAGATGTTCCAGATAAATATTGGAAGCTATATACGTACTTT GTTTCAAATGGGTCAATCGAGAATATCGTCAACTGTTTACTAAAAATCAGTTTCATCAAGCAAT GAAACACGCCAAAGTAAACAATTTAAGTACCGTTACTTATGAGCAAGTATTGTCTATTTTAATA GTTATCTATTATTAACGGGAGGAAATAA
SEQ ID NO: 8	ATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTGCGGCATTTTGCCTTCCTGTTTTG CTACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGT ACATCGAACTGGATCTCAACAGCGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCC AATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAA GAGCAACTCGGTGCGCCGATACACTATTCTCAGAATGACTTGGTTGAGTACTACCAGTCACAG AAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTG ATAACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTT GCACAACATGGGGGATCATGTAACCTGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCAT ACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACCTATT AACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAA AGTTGCAGGACCACTTCTGCGCTCGGCCCTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGA GCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGT ATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCT GAGATAGGTGCCTCACTGATTAAGCATTGGTAA

SEQ ID NO: 9	TCAAAGGATCTTCTTGAGATCCTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAACCA CCGCTACCAGCGGTGGTTTGTGGCCGGATCAAGAGCTACCAACTCTTTTCCGAAGGTAAGT GCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTT CAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAAGTGGCTGCTGCC AGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAG CGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGA ACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGG ACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGG AAACGCCTGGTATCTTTATAGTCTGTCTGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGT GATGCTCGTCAGGGGGGCGGAGCCTATGAAAAACGCCAGCAACG
SEQ ID NO: 10	CATGATTTTAAGGGGTTAGCAGATGCATAAGTTTAATTTTTTTGTTAAAAATATTAACTTTGT GTTTTTTTAAACAAATATATTGATAAAAAATAATAAGTGGGTATAATTAAGTTGTTAGAGAAA ACGTATAAATTAGGGATAAATATGGAACCTTATGAAATAGATTGAAATGGTTTATCTGTTACCCC GTATCAAAATTTAGGAGGTTAGTTTAAACCTGCAGAGATCTCTCGAGGCGGCCGCGTCGACTCT AGACCCGGGAATTCAGTGGCGTCGTTTTACAACGTCGTACTGGGAAAACCTGGCGTTACC CAACTTAATCGCCTTGACGACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGC ACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTC TCCTTACGCATCTGTGCGGTATTTACACCGAGCTCACGAAGTCGAGATCAGGGAATGAGTTTA TAAATAAAAAAGCACCTGAAAAGGTGTCTTTTTTGTATGGTTTTGAACCTGTTCTTTCTTATCT TGATACATATAGAAATAACGTCATTTTTATTTTAGTTGCTGAAAGGTGCGTTGAAGTGTGGTAT GTATGTGTTTTAAAGTATTGAAAACCTTAAATTTGGTTGCACAGAAAAACCCATCTGTTAAAG TTATAAGTGACTAAACAAATAACTAAATAGATGGGGGTTCTTTTAATATTATGTGTCCTAATAG TAGCATTTATTCAGATGAAAAATCAAGGGTTTTAGTGGACAAGACAAAAAGTGGAAGTGAG ACCATGGAGAGAAAAAGAAATCGCTAATGTTGATTACTTTGAACCTCTGCATATTCTTGAATTTA AAAAGGCTGAAAGAGTAAAGATTGTGCTGAAATATTAGAGTATAACAAAATCGTGAAACAG GCGAAAGAAAGTTGTATCGAGTGTGGTTTTGTAAATCCAGGCTTTGTCCAATGTGCAACTGGAG GAGAGCAATGAAACATGGCATTAGTCACAAAAGGTTGTGCTGAAGTTATTAACAAAAGCC AACAGTTCGTTGGTTGTTTCTCACATTAACAGTTAAAAATGTTTATGATGGCGAAGAATTAATA AGAGTTTGTAGATATGGCTCAAGGATTTGCCGAATGATGCAATATAAAAAATTAATAAAAA TCTTGTGTTTTATGCGTGCAACGGAAGTGACAATAAATAATAAGATAATTCTTATAATCAGC ACATGCATGTATTGGTATGTGTGGAACCAACTTATTTTAAGAATACAGAAAACACTACGTGAATCA AAAACAATGGATTCAATTTTGGAAAAAGGCAATGAAATTAGACTATGATCCAAATGTAAAAGTT CAAATGATTGACCGAAAAATAAATAAATCGGATATACAATCGGCAATTGACGAAACTGCA AAATATCCTGTAAAGGATACGGATTTTATGACCGATGATGAAGAAAAGAAATTGAAACGTTTGT CTGATTTGGAGGAAGGTTTACACCGTAAAAGGTTAATCTCCTATGGTGGTTTGTAAAAGAAAT ACATAAAAAATTAACCTTGATGACACAGAAGAAGGCGATTTGATTCATACAGATGATGACGA

AAAAGCCGATGAAGATGGATTTCTATTATTGCAATGTGGAATTGGGAACGGAAAAATTATTTT ATTAAAGAGTAGTTCAACAAACGGGCCAGTTTGTGAAGATTAGATGCTATAATTGTTATTTAA AGGATTGAAGGATGCTTAGGAAGACGAGAGATCCTAGCAGCACGCCATAGTGACTGGCGATG CTGTCGGAATGGACGATCAAAATCCCCGTAGGCGCTAGGGACCTCTTAGCTCCTTGAAGCTG TCAGTAGTATACCTAATAATTTATCTACATCCCTTTAGTAACGTGTAACCTTCCAAATTTACAAA AGCGACTCATAGAATTATTTCTCCGTTAAATAATAGATAACTATTAATAATAGACAATACTTG CTCATAAGTAACGGTACTTAAATTGTTTACTTTGGCGTGTTCATTGCTTGATGAAACTGATTTTT AGTAAACAGTTGACGATATTCTCGATTGACCCATTTTGAACAAAGTACGTATATAGCTTCCAAT ATTTATCTGGAACATCTGTGGTATGGCGGTAAGTTTATTAAGACACTGTTTACTTTTGGTTTGA GATGAAAGCATTCCGCTGGCAGCTTAAGCAATTGCTGAATCGAGACTTGAGTGTGCAAGAGCA ACCCTAGTGTTCCGTGAATATCCAAGGTACGCTTGTAATCCTTCTCAACAATCAGATAGAT GTCAGACGCATGGCTTTCAAAAACCACTTTTTAATAATTTGTGTGCTTAAATGGTAAGGAATAC TCCAACAATTTTATACCTCTGTTTGTAGGGAATTGAAACTGTAGAATATCTTGGTGAATTTAA GTGACACGAGTATTCAGTTTAAATTTTCTGACGATAAGTTGAATAGATGACTGTCTAATCAAT AGACGTTACCTGTTTACTTATTTTAGCCAGTTTCGTCGTTAAATGCCCTTACCCTGTTCCAATTC GTAAACGGTATCGGTTTCTTTAAATCAATTGTTTTATTATTTGGTTGAGTACTTTTCACTCGTT AAAAAGTTTTGAGAATATTTTATATTTTGTTCATGTAATCACTCCTTCTTAATTACAAATTTTGA CATCTAATTTAACTTCAATTCCTATTATACAAAATTTAAGATACTGCACTATCAACACACTCTTA AGTTTGCTTCTAAGCTTATTTCCATACTCTTTTACGTTTCCGCCATTCTTTGCTGTTTCGATTT TATGATATGGTGCAAGTCAGCACGAACACGAACCGTCTTATCTCCATTATATCTTTTTTGCAC TGATTGGTGTATCATTTCTGTTTTCTTTTTATCCCGCAAGAGGCCCGGCAGTCAGGTGGCACTTT CGGGGAAATGTGCGCGGAACCCCTATTTGTTATTTTCTAAATACATTCAAATATGTATCCGCT CATGAGACAATAACCCGTGATAAATGCTTCAATAATTGAAAAAGGAAGAGTATGAGTATTTCAA CATTTCCGTGTCGCCCTTATCCCTTTTTTGCGGCATTTTGCCTTCTGTTTTGCTCACCCAGAAA CGCTGGTGAAAGTAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTACATCGAACTGG ATCTAACAGCGGTAAGATCCTTGAGAGTTTTGCCCGAAGAAGCTTTTCCAATGATGAGCAC TTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGT CGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTA CGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGG CCAACCTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGACAACATGG GGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACG AGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAATTAATACTGGCGAAC TACTTACTAGCTTCCCGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGAC CACTTCTGCGCTCGGCCCTCCGGCTGGCTGGTTATTGCTGATAAATCTGGAGCCGGTGAGCG TGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATC TACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGC

	CTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTAA AACTTCATTTTAAATTTAAAGGATCTAGGTGAAGATCCTTTTGTATAATCTCATGACCAAAATCC CTTAACGTGAGTTTTCTGTTCCACTGAGCGTCAGACCCGTAGAAAAGATCAAAGGATCTTCTTG AGATCCTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAACACCGCTACCAGCGGTG GTTTGTGCGCGATCAAGAGCTACCAACTCTTTTCCGAAGGTAAGTGGCTTCAGCAGAGCGC AGATACCAAACTGTTCTTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGC ACCGCTACATACCTCGCTCTGCTAATCCTGTTACCAAGTGGCTGCTGCCAGTGGCGATAAGTCG TGTCTTACCGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTGCGGCTGAACG GGGGGTCGTGCACACAGCCAGCTTGAGCGAACGACCTACCCGAAGTGGAGATACCTACA GCGTGAGCTATGAGAAAGCGCCACGCTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAA GCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTCCAGGGGAAACGCTGGTATCT TTATAGTCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTGTGATGCTCGTCAGGGG GGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCTTTTACGGTTCCTGGCCTTTTGCTGGC CTTTGCTCACATGTTCTTCTGCGTTATCCCTGATTCTGTGGATAACCGTATTACCGCCTTG AGTGAGCTGATACCGCTCGCCGAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGA AGCGGAAGAGCGCCCAATACGCAAACCGCTCTCCCGCGCGTTGGCCGATTCATTATGCAG GGG
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<Experimental Example 2> Evaluation of segregational stability of shuttle plasmid (pLK1-MCS)

5

<2-1> Construction of transformants

The shuttle plasmid prepared in Experimental Example 1 was introduced into *Clostridium acetobutylicum* to prepare a transformed recombinant microorganism.

10 Detailed methods are as follows. *Clostridium acetobutylicum* was cultured in 100ml of liquid CGM (Clostridium Growth Medium) (0.75 g/L K_2HPO_4 , 0.75 g/L KH_2PO_4 , 0.7 g/L, $MgSO_4 \cdot 7H_2O$, 0.017 g/L $MnSO_4 \cdot 5H_2O$, 0.01 g/L, $FeSO_4 \cdot 7H_2O$, 2 g/L $(NH_4)_2SO_4$, 1 g/L NaCl, 2 g/L asparagine, 0.004 g/L p-aminobenzoic acid, 5 g/L, yeast extract, and 10 g/L glucose) under anaerobic conditions until OD600 reached 1.0. The culture solution was
15 left on ice for 10 minutes, followed by subjecting to centrifugation at 7000 g for 10 minutes at 4°C, thereby obtaining cell pellets. The obtained cell pellets were washed with a buffer solution three times and suspended in 20 ml of the same buffer solution to prepare cells for transformation. To 500 μ l of the prepared cells for transformation, 5.0 μ g of shuttle plasmids were added, followed by performing electroporation (4mm cuvette, 2.5kV,

$\infty\Omega$, 25 μ F) using a Gene PulserTM II prepared by Bio-Rad Corp. Transformed strains were identified in a medium to which erythromycin was added (Table 3).

The plasmids used in the transformation were all methylated in *Escherichia coli* TOP10 strain transformed with pAN1 plasmid (having genes for methylating inner cytosine in case that GCNGC sequence is present) before electroporation so that the plasmids were not affected by the restriction system of *Clostridium acetobutylicum* strain.

<2-2> Evaluation of segregational stability of pLK1-MCS in *Clostridium*

The segregational stability of the shuttle plasmid pLK1-MCS was evaluated in *Clostridium acetobutylicum* strain containing the pLK1-MCS shuttle plasmid constructed in Experimental Example 1. The evaluation was performed by adapting the existing evaluation method (Shin MH, Jung MW, Lee J-H, Kim MD, Kim KH. 2008. Strategies for producing recombinant sucrose phosphorylase originating from *Bifidobacterium longum* in *Escherichia coli* JM109. Process Biochemistry 43:822-828).

The shuttle plasmid was introduced into *Clostridium acetobutylicum* ATCC 824 strain by electroporation, and then cultured in a solid medium containing erythromycin under anaerobic culture conditions at 37°C for two days. One colony taken from the culture solution was cultured in a culture tube with 40ml liquid CGM with no antibiotic present at 37°C until cell concentration (OD600 nm) reached 1.0. The cell concentration was measured using a spectrophotometer (Hach, USA).

The cultured cells were diluted, streaked on solid CGM with no antibiotic present, and cultured at 37°C for 36 hours. The number of colonies formed was identified. Thereafter, 50 colonies formed were replica plated onto solid CGM containing erythromycin, and the number of cells in which the shuttle plasmid was lost was identified. In case that the shuttle plasmid was lost, colonies could not be formed upon replica plating since there was no erythromycin antibiotic resistance.

Further, 40 ml of liquid CGM with no antibiotic present (diluted to 1/1000 of concentration of the initial culture solution) was inoculated with 40 μ L of initial liquid culture solution, and then the aforementioned procedures were repeated 10 times to identify the number of cells in which the shuttle plasmid was lost. The stability from shuttle plasmid loss was evaluated over 100 generations. Since cells were inoculated in a concentration of 1: 1000 dilution every time, each generation was assumed to have been divided 10 times ($2^{10} \approx 1024$).

Results are shown in Fig. 3. It can be seen that the novel shuttle plasmid pLK1-MCS showed remarkably improved stability from plasmid loss as compared with the existing pMTL500E (including a replication origin of pAM β 1 and a base sequence encoding a replication protein) used as *Escherichia coli*-*Clostridium* shuttle plasmid. Further, the novel shuttle plasmid pLK1-MCS was also found to have better segregational stability as compared with pGS1-MCS shuttle plasmid including a replication origin of pIM13 plasmid and a base sequence encoding a replication protein. As a result, the novel shuttle plasmid pLK1-MCS was found to have better segregational stability as compared with the existing shuttle plasmid (Table 3).

TABLE 3

Plasmid	Number of colony	Temperature ($^{\circ}$ C)
pLK1-MCS	4.3×10^2	37
pMTL500E	2.6×10^2	37
pGS1-MCS	4.6×10^2	37

<2-3> Evaluation of segregational stability of pLK1-MCS in *Escherichia coli*

The fact that shuttle plasmid pLK1-MCS constructed in Experimental Example 1 is stably replicated in *Escherichia coli* was confirmed by transforming the shuttle plasmid pLK1-MCS with *Escherichia coli* TOP10 containing pAN1 plasmid to obtain methylated pLK1-MCS in Experimental Example <2-1>.

<Experimental Example 3> Expression of foreign genes using shuttle plasmids

<3-1> Construction of pLK1-IPA2 plasmid

According to Korean Patent Publication No. 10-2011-0032375, it is possible for the secondary alcohol dehydrogenase of *Clostridium beijerinckii*, NRRL B593 to convert acetone into isopropanol. Accordingly, it was evaluated whether or not the novel shuttle plasmid obtained by recombining the secondary alcohol dehydrogenase gene (IPA-HydG) as a foreign gene to the novel shuttle plasmid of the present invention expresses the secondary alcohol dehydrogenase gene and thus converts acetone into alcohols. A region encoding secondary alcohol dehydrogenase was obtained by PCR reaction using as a template pTHL1-Cm-IPA2 plasmid used in Korean Patent Publication No. 10-2011-0032375 and using as primer base sequences of SEQ ID NO: 11 and SEQ ID NO: 12 (Table 4). The obtained region encoding secondary alcohol dehydrogenase was cloned into the novel plasmid of the present invention, pLK1-MCS, to construct pLK1-IPA2 plasmid. The constructed pLK1-IPA2 plasmid was introduced into *Clostridium acetobutylicum* PJC4BK strain to obtain a transformed *Clostridium acetobutylicum* PJC4BK (pLK1-IPA) (Fig. 4).

TABLE 4

SEQ ID NO: 11	CACAGGCCTATGAAAGGTTTTGCAATGCTAGGTATTAAT
SEQ ID NO: 12	ATATCTAGATTATTATCACCTCTGCAACCACAGCCACC

In this Experimental Example, the plasmids used in the transformation are all methylated in *Escherichia coli* TOP10 strain transformed with pAN1 plasmid (having genes for methylating inner cytosine in case that GCNGC sequence is present) before electroporation so that the plasmids were not affected by the restriction system of *Clostridium acetobutylicum* strain.

<3-2> Identification of production of isopropanol using *Clostridium acetobutylicum* PJC4BK (pLK1-IPA2)

It was evaluated whether or not the recombined secondary alcohol dehydrogenase

was normally expressed to convert acetone into isopropanol in case the recombinant strain prepared in <3-1> is batch cultured.

First, the recombinant *Clostridium* PJC4BK (pLK1-IPA2) strain prepared in <3-1> of <Experimental Example 3> was streaked on solid CGM, followed by culturing anaerobically at 37°C overnight. A single colony was inoculated into a 50 ml disposable tube (Falcon, USA) containing 40 ml of CGM, and then cultured anaerobically until OD600 reached 1 at 37°C. The seed culture was inoculated into 400 ml of CGM containing 1% glucose, followed by standing, and then culturing anaerobically until OD600 reached 1 at 37°C. The 400 ml culture solution was then inoculated into a fermenter containing 1.6 L of liquid CGM containing 8% glucose. As a control group, *Clostridium acetobutylicum* PCJ4BK (pTHL-Cm-IPA2) and *Clostridium acetobutylicum* PCJ4BK were used.

pH was maintained at 5.0 during anaerobic culture using ammonium hydroxide (NH₄OH) and anaerobic conditions were maintained by injecting nitrogen at a speed of 20 ml/min. The concentration of the produced butanol and mixed solvent was analyzed every three hours after the initiation of glucose culture. The analysis of butanol and mixed solvent was performed using a gas chromatograph (Agilent, USA). The analysis conditions are as summarized in Table 5.

TABLE 5

Injector temperature	320°C
Detector temperature	320°C
Injector split ratio	20/1
Injection volume	0.1ul
Oven condition	80°C/15min
Air flow	300mL/min
H ₂ flow	30mL/min
Column	Supelco CarboWAX

As a result, it was confirmed that *Clostridium acetobutylicum* PJC4BK (pLK1-IPA2) had isopropanol producing capability comparable or higher to that of *acetobutylicum* PJC4BK (pTHL-Cm-IPA2) used as a control group (Table 6).

Consequently, it was confirmed that the shuttle plasmid pLK1-MCS had segregational stability and foreign gene expression capability comparable or higher to those of prior shuttle plasmids.

5 TABLE 6

Strain	Plasmid	Acetone (g/L)	IPA (g/L)	Ethanol (g/L)	Butanol (g/L)	Total (g/L)
<i>Clostridium acetobutylicum</i> PJC4BK	-	2.606		2.641	15.296	20.543
<i>Clostridium acetobutylicum</i> PJC4BK	pTHL-Cm- IPA2	0.294	4.397	3.792	15.972	24.455
<i>Clostridium acetobutylicum</i> PJC4BK	pLK1-IPA2	0.371	4.328	3.953	16.051	24.703

Although some embodiments have been described herein, it should be understood by those skilled in the art that these embodiments are given by way of illustration only, and that various modifications, variations, and alterations can be made without departing from the spirit and scope of the invention. Therefore, the scope of the invention should be limited only by the accompanying claims and equivalents thereof.

WHAT IS CLAIMED IS:

1. A method for producing a transformed *Clostridium acetobutylicum* comprising:
preparing a shuttle plasmid which is replicable in both *Clostridium acetobutylicum* and *Escherichia coli* and comprises:
a base sequence of a first replication origin replicable in *Escherichia coli* which comprises SEQ ID NO: 9; and
a base sequence of a second replication origin from a pUB110 plasmid which comprises SEQ ID NO: 3;
wherein the shuttle plasmid is replicable in both *Clostridium acetobutylicum* and *Escherichia coli* under an environment with an absence of antibiotics, and
wherein the shuttle plasmid is replicable in both *Clostridium acetobutylicum* and *Escherichia coli* under an environment with a presence of antibiotics, and
introducing the shuttle plasmid into *Clostridium acetobutylicum*.
2. The method according to claim 1,
wherein the shuttle plasmid further comprises a base sequence from a pUB110 plasmid encoding a replication protein which comprises SEQ ID NO: 4.
3. The method according to claim 1,
wherein the shuttle plasmid further comprises a first antibiotic resistance gene operable to be expressed in *Escherichia coli*.
4. The method according to claim 3,
wherein the shuttle plasmid further comprises a second antibiotic resistance gene operable to be expressed in *Clostridium acetobutylicum*.
5. The method according to claim 2, wherein the amino acid sequence of the replication protein is SEQ ID NO: 5.
6. The method according to claim 1, wherein, after preparing the shuttle plasmid, a foreign gene is cloned into the shuttle plasmid and the shuttle plasmid into which the foreign gene has been cloned is introduced into *Clostridium acetobutylicum*.

7. A transformed *Clostridium acetobutylicum* comprising a shuttle plasmid which is replicable in both *Clostridium acetobutylicum* and *Escherichia coli* and comprises:

a base sequence of a first replication origin replicable in *Escherichia coli* which comprises SEQ ID NO: 9; and

a base sequence of a second replication origin from a pUB110 plasmid which comprises SEQ ID NO: 3, and

wherein the shuttle plasmid is replicable in both *Clostridium acetobutylicum* and *Escherichia coli* under an environment with an absence of antibiotics, and

wherein the shuttle plasmid is replicable in both *Clostridium acetobutylicum* and *Escherichia coli* under an environment with a presence of antibiotics.

8. A method for producing a culture comprising:

culturing the transformed *Clostridium acetobutylicum* according to claim 7; and

harvesting a culture.

Fig.1

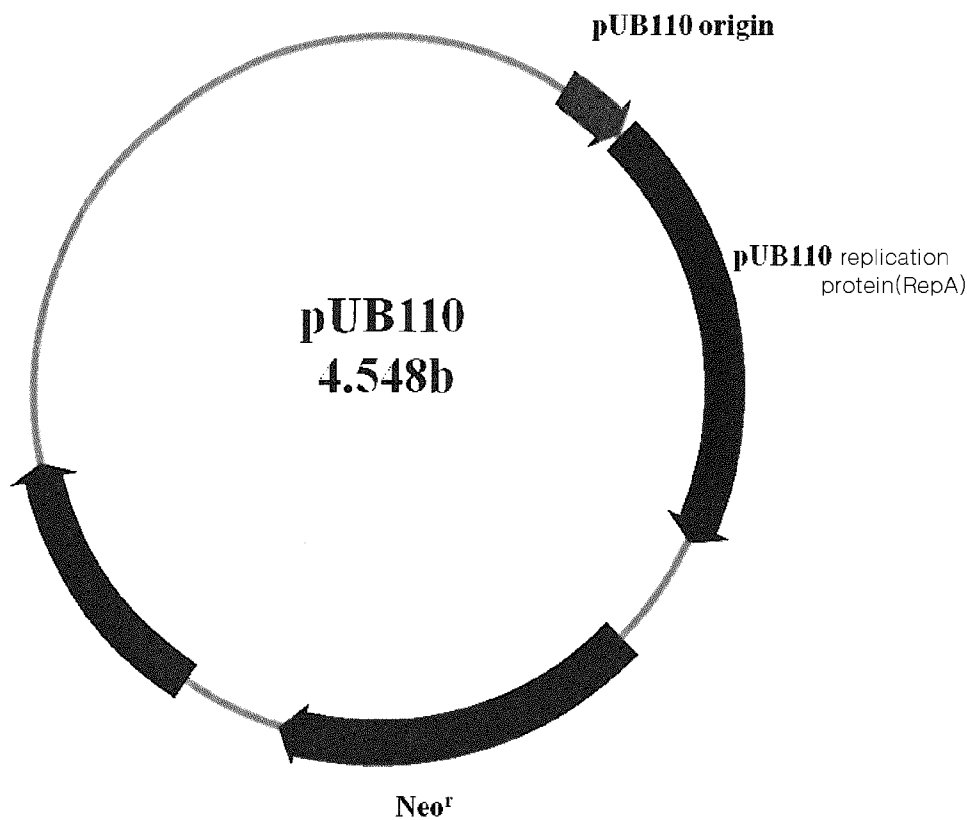


Fig.2

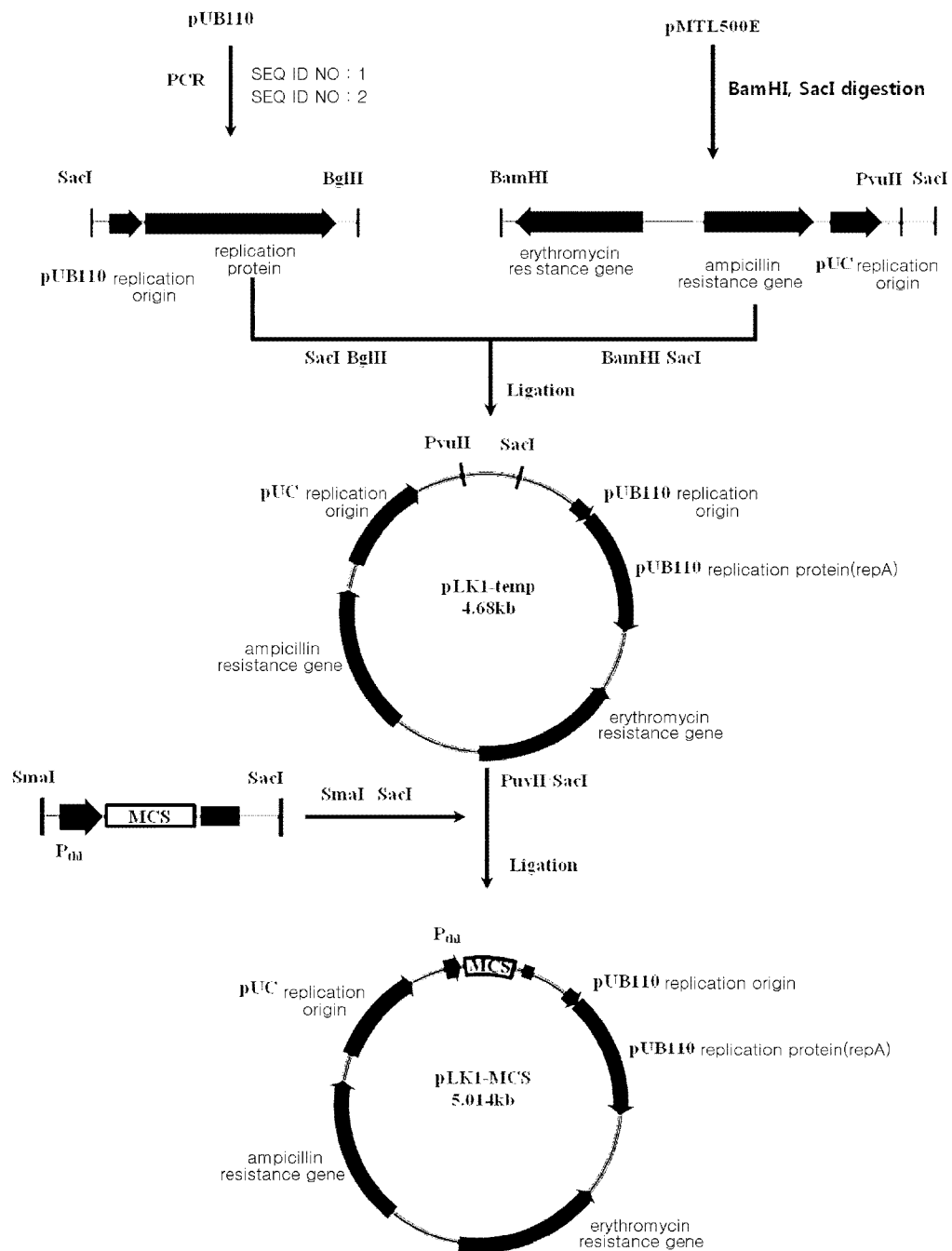


Fig.3

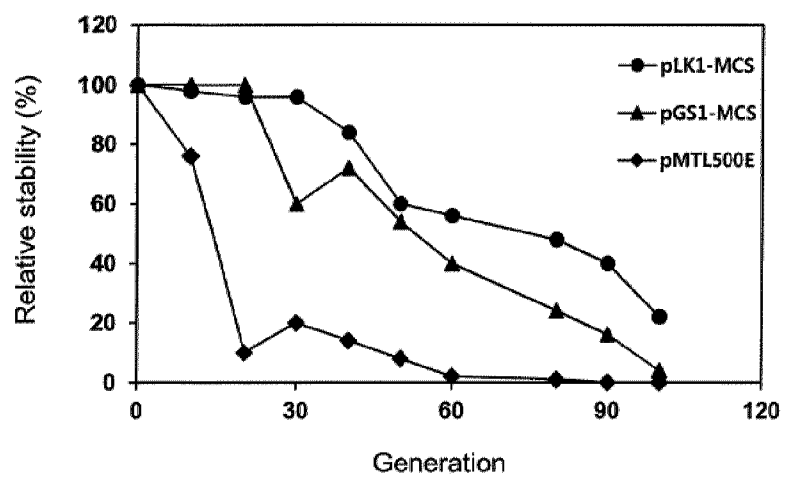


Fig.4

