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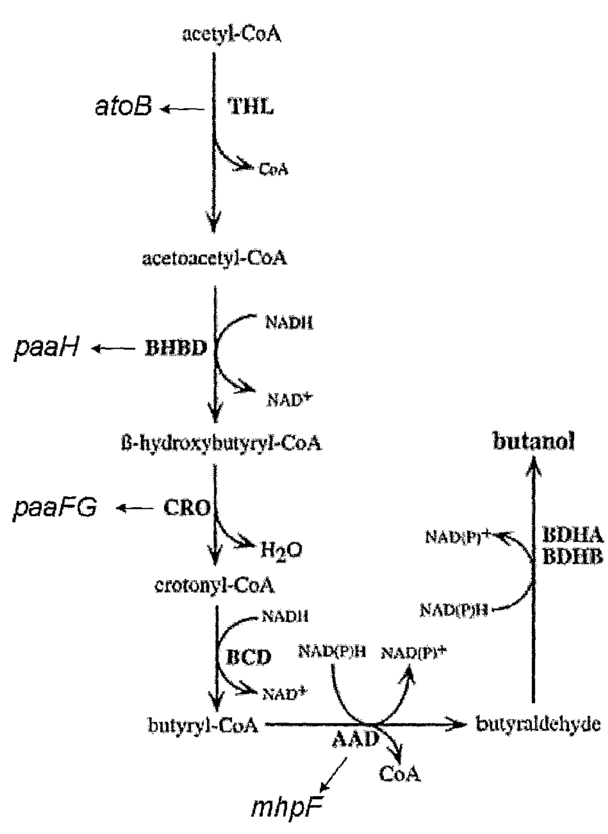

(57) Abstract: The present invention relates to a recombinant mutant microorganism having enhanced butanol producing capacity and a method for producing butanol using the same. In the microorganism, genes coding for enzymes responsible for the biosynthesis of lactate, ethanol and/or acetate are deleted or attenuated and genes coding for enzymes involved in butanol biosynthesis are introduced and amplified.

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ENHANCED BUTANOL PRODUCING MICROORGANISMS AND METHOD FOR PREPARING BUTANOL USING THE SAME

5

TECHNICAL FIELD

The present invention relates to recombinant mutant microorganisms having enhanced butanol-producing ability in which genes coding for enzymes
10 responsible for the biosynthesis of lactate, ethanol and/or acetate are deleted and genes coding for enzymes involved in butanol biosynthesis are introduced, and a method for producing butanol using the same.

BACKGROUND ART

15

With the great increase in oil prices and growing concern about global warming and greenhouse gases, biofuels have recently gained increasing attention with respect to the production thereof using microorganisms. Particularly, biobutanol has an advantage over bioethanol in that it is more highly miscible with fossil
20 fuels thanks to the low oxygen content thereof. Recently emerging as a substitute fuel for gasoline, biobutanol has rapidly increased in market size. The U.S. market for biobutanol amounts to 370 million gal per year, with a price of 3.75 $/gal. Butanol is superior to ethanol as a replacement for petroleum gasoline. With high energy density, low vapor pressure, a gasoline-like octane
25 rating and low impurity content, it can be blended into existing gasoline at much higher proportions than ethanol without compromising performance, mileage, or organic pollution standards. The mass production of butanol by microorganisms can confer economic and environmental advantages of decreasing the import of crude oil and greenhouse gas emissions.

30

Butanol can be produced through anaerobic ABE (acetone-butanol-ethanol) fermentation by Clostridial strains (Jones, D.T. and Woods, D.R., *Microbiol. Rev.*, 50:484, 1986; Rogers, P., *Adv. Appl. Microbiol.*, 31:1, 1986; Lesnik, E.A. *et al.*, *Necleic Acids Research*, 29: 3583, 2001). This biological method was the main
5 technology for the production of butanol and acetone for more than 40 years, until the 1950s. Clostridial strains are difficult to improve further because of complicated growth conditions thereof and the insufficient provision of molecular biology tools and omics technology therefor.

10 Thus, it is suggested that microorganisms such as *E. coli* that can grow rapidly under typical conditions and be manipulated using various omics technologies be developed as butanol-producing strains. Particularly, *E. coli* species, to which little metabolic engineering and omics technology have been applied for the development of butanol-producing strains, have vast potential for development
15 into butanol-producing strains.

Therefore, there is a need for the development of a microorganism having high butanol producing ability, especially a recombinant *E. coli*, by metabolic engineering such as metabolic network reconstitution by gene deletion, insertion
20 and amplification of desired genes, unlike the prior art wild type *Clostridium acetobutylicum*.

Recombinant bacteria capable of producing butanol, into which a butanol biosynthesis pathway is introduced, and butanol production using the same have
25 been disclosed (US 2007/0259410 A1; US 2007/0259411 A1), but the production efficiency is modest.

The present inventors have made extensive efforts to develop a microorganism having a high butanol productivity by metabolic engineering, and as a result,
30 constructed a recombinant microorganism by deleting or attenuating genes coding

for enzymes involved in the biosynthesis of lactate, ethanol and acetate and introducing or amplifying genes coding for enzymes responsible for butanol biosynthesis, and confirmed that the butanol production was remarkably increased by the recombinant mutant microorganism, thereby completing the present invention.

SUMMARY OF THE INVENTION

It is a main object of the present invention to provide a recombinant mutant microorganism capable of producing butanol at high efficiency and a preparation method thereof.

It is another object of the present invention to provide a method for producing butanol using the recombinant mutant microorganism.

In order to achieve the above objects, in one aspect, the present invention provides a method for preparing a recombinant mutant microorganism having high butanol productivity, the method comprises: deleting or attenuating at least one selected from the group consisting of genes coding for enzymes involved in lactate biosynthesis, genes coding for enzymes involved in acetate biosynthesis, and genes coding for enzymes involved in ethanol biosynthesis in a microorganism; and introducing or amplifying at least one gene coding for an enzyme involved in butanol biosynthesis into the microorganism.

In another aspect, the present invention provides a recombinant mutant microorganism having high butanol productivity, in which at least one selected from the group consisting of genes coding for enzymes involved in lactate biosynthesis, genes coding for enzymes involved in acetate biosynthesis, and genes coding for enzymes involved in ethanol biosynthesis is deleted or attenuated; and at least one gene coding for an enzyme involved in butanol

biosynthesis is introduced or amplified.

In an embodiment of this aspect, a *lacI* gene (coding for a lac operon repressor) is further deleted in the microorganism so as to enhance the expression of the gene coding for the enzyme involved in butanol biosynthesis.

In another embodiment, the gene coding for enzyme involved in the lactate biosynthesis may be *ldhA* (coding for lactate dehydrogenase), the gene coding for enzyme involved in the acetate biosynthesis may be *pta* (coding for phosphoacetyltransferase), and the gene coding for enzyme involved in the ethanol biosynthesis may be *adhE* (coding for alcohol dehydrogenase).

In the present invention, the enzyme involved in butanol biosynthesis is selected from the group consisting of thiolase (THL), 3-hydroxybutyryl-CoA dehydrogenase (BHBD), crotonase (CRO), butyryl-CoA dehydrogenase (BCD), butyraldehyde dehydrogenase (AAD), butanol dehydrogenase (BDH), and combinations thereof.

In the present invention, the THL may be encoded by a gene selected from the group consisting of *thl*, *thiL*, *phaA*, and *atoB*, and the BCD may be encoded by a *bcd* gene derived from *Pseudomonas* sp., a *bcd* gene derived from *Clostridium* sp., and a *ydbM* gene derived from *Bacillus* sp. When the gene coding for BCD is a *bcd* gene derived from *Clostridium* sp., a chaperone-encoding gene (*groESL*) and a BCD co-factor-encoding gene (*etfAB*) are further introduced into the microorganism. Also, the gene coding for the BHBD may be a *hbd* gene derived from *Clostridium* sp. or a *paaH* gene derived from *E. coli*. The gene coding for the CRO may be a *crt* gene derived from *Clostridium* sp. or a *paaFG* gene derived from *E. coli*. The gene coding for the AAD may be an *adhE* gene derived from *Clostridium* sp. or a *mhpF* gene derived from *E. coli*.

In the present invention, the gene coding for the enzyme involved in the butanol biosynthesis may be introduced into the host cell by an expression vector containing a strong promoter. This strong promoter may be selected from the group consisting of a trc promoter, a tac promoter, a T7 promoter, a lac promoter and a trp promoter. The expression vector containing the strong promoter may further contain a gene coding for an enzyme selected from the group consisting of 3-hydroxybutyryl-CoA dehydrogenase, thiolase, butyraldehyde dehydrogenase, crotonase, butanol dehydrogenase, butyryl-CoA dehydrogenase and combinations thereof.

In still another aspect, the present invention provides a recombinant mutant microorganism having high butanol productivity, in which genes coding for enzymes involved in lactate biosynthesis, genes coding for enzymes involved in acetate biosynthesis, and genes coding for enzymes involved in ethanol biosynthesis are deleted or attenuated; and genes coding for thiolase (THL), 3-hydroxybutyryl-CoA dehydrogenase (BHBD), crotonase (CRO), butyryl-CoA dehydrogenase (BCD), butyraldehyde dehydrogenase (AAD), butanol dehydrogenase (BDH), a chaperone protein (*groESL*), and BCD co-factors (*etfAB*) are introduced or amplified.

In further still another aspect, the present invention provides a method for producing butanol, the method comprises: culturing the recombinant mutant microorganism to produce butanol; and recovering the butanol from the culture broth.

Other features, advantages, and embodiments of the present invention will be obvious from the following detailed description and the accompanying claims.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 is a diagram showing a butanol biosynthesis pathway in *Clostridium acetobutylicum*;

5 FIG. 2 shows a construction process and a genetic map of pKKhbdadhEthiL (pKKHAT) vector.

FIG. 3 shows a construction process and a genetic map of pKKhbdadhEatoB (pKKHAA) vector.

FIG. 4 shows a construction process and a genetic map of pKKhbdadhEphaA 10 (pKKHAP) vector.

FIG. 5 shows a construction process and a genetic map of pKKhbdydbMadhEphaA (pKKHYAP) vector.

FIG. 6 shows a construction process and a genetic map of pKKhbdbcdPA01adhEphaA (pKKHPAP) vector.

15 FIG. 7 shows a construction process and a genetic map of pKKhbdbcdKT2440adhEphaA (pKKHKAP) vector.

FIG. 8 shows a construction process and a genetic map of pKKhbdgroESLadhEphaA (pKKHGAP) vector.

FIG. 9 shows a construction process and a genetic map of 20 pTrc184bcdbdhABcrt (pTrc184BBC) vector.

FIG. 10 shows a butanol biosynthesis pathway in the case where a part of genes derived from *C. acetobutylicum* involved in a butanol biosynthesis pathway, was substituted by genes derived from *E. coli*.

FIG. 11 shows a construction process and a genetic map of 25 pKKmhpFpaaFGHatoB (pKKMPA) vector.

FIG. 12 shows a construction process and a genetic map of pTrc184bcdetfABbdhABgroESL (pTrc184BEBG) vector.

DETAILED DESCRIPTION OF THE INVENTION, AND PREFERRED EMBODIMENTS

The term "deletion", as used herein in relation to a gene, means that the gene
5 cannot be expressed or, if it is expressed, cannot lead to enzyme activity, due to the mutation, substitution, deletion or insertion of any number of nucleotides from a single base to an entire piece of the gene, resulting in the blockage of the biosynthesis pathway in which an enzyme encoded by gene is involved.

10 By the term "attenuation", as used herein in relation to a gene, it is meant that the activity of the enzyme expressed by the gene is decreased by the mutation, substitution, deletion, or insertion of any number of nucleotides, ranging from a single base to entire pieces of the gene, resulting in the blockage of a part or a critical part of the biosynthesis pathway in which an enzyme encoded by gene is
15 involved.

The term "amplification", as used herein in relation to a gene, is intended to refer to an increase in the activity of the enzyme corresponding to the gene due to the mutation, substitution, deletion or insertion of any number of nucleotides from a
20 single base to partial pieces of the gene, or by the introduction of an exogenous gene coding for the same enzyme.

The present invention employs the butanol biosynthesis pathway of *Clostridium acetobutylicum* as a model for producing butanol in the recombinant
25 microorganism (FIG. 1). When account is taken of both the pathway of FIG. 1 and the pathway of *E. coli*, enzymes including thiolase (THL), 3-hydroxybutyryl-CoA dehydrogenase (BHBD), crotonase (CRO), butyryl-CoA dehydrogenase (BCD), butyraldehyde dehydrogenase (AAD) and butanol dehydrogenase (BDH) are believed to be involved in the biosynthesis of butanol.

30

The gene *thl* derived from *Clostridium* sp. has already been identified to effectively express THL in *E. coli* (Bermejo, L.L. *et al.*, *Appl. Environ. Microbiol.*, 64:1079, 1998). In addition to *thl*, the gene *thiL* is known to encode THL in *Clostridium* sp. (Nolling, J. *et al., J. Bacteriol.,* 183:4823, 2001). THL
5 functions to convert acetyl-CoA into acetoacetyl-CoA. In an example of the present invention, *phaA* derived from *Ralstonia* sp. or *atoB* derived from *E. coli* was identified to perform the same function as *thl* or *thiL*. Accordingly, as long as it is expressed to show THL activity in the host cells, any gene coding for THL, even if exogenous, can be used without limitations.

10

Also, Bennett *et al.* reported that among enzymes necessary for the production of butyryl-CoA from acetoacetyl-CoA, BHBD and CRO except for BCD are expressed in *E. coli* (Boynton, Z.L. *et al.*, *J. Bacteriol.*, 178:3015, 1996). Accordingly, *hbd* and *crt*, both of which are derived from *Clostridium* sp., are
15 introduced as genes encoding BHBD and CRO, respectively, in the recombinant microorganism according to the present invention. Both the genes, although exogenous, can be used without limitations, as long as they are expressed and show the same activity in the host cells. In an example of the present invention, butanol was also produced even when *hbd* and *crt* derived from *Clostridium* sp.
20 were substituted respectively with *paaH* (gene coding for 3-hydroxy-acyl-CoA dehydrogenase) and *paaFG* (a gene coding for enoyl-CoA hydratase) derived from *E. coli*.

According to the article, however, it is reported that *E. coli* has no BCD function
25 because of the poor expression of BCD or its cofactors (electron transfer flavoproteins putatively coded by the *Clostridium acetobutylicum* genes (*etfB* and *etfA*)) therein, or no *in vitro* activity is observed because of the poor stability of BCD or its cofactors.

30 In accordance with the present invention, low-level expression of butyryl-CoA

dehydrogenase can be overcome by the introduction of *bcd* derived from *Pseudomonas aeruginosa* or *Pseudomonas putida*, or *ydbM* derived from *Bacillus subtilis*. As long as it is expressed to show BCD activity in the host cells, a BCD gene, even though exogenous, can be used without limitations.

5

In an alternative embodiment, *bcd* derived from *Clostridium acetobutylicum* may be introduced together with a chaperone-encoding gene (*groESL*), so as to solve the problem of low-level expression of butyryl-CoA dehydrogenase. When the *bcd* of *Clostridium acetobutylicum* and the chaperone-encoding gene (*groESL*)
10 was introduced into *E. coli* host cells, butanol productivity thereof is increased as demonstrated in the example of the present invention. In addition to the *bcd* derived from *Clostridium acetobutylicum* and the chaperone-encoding gene (*groESL*), the introduction of a gene coding for BCD cofactors was found to significantly increase butanol production capacity, as demonstrated in an example
15 of the present invention.

Previously, the present inventors reported that although a gene coding for BDH is not introduced, a host cell (e.g., *E. coli*) which harbors a gene encoding an enzyme (AdhE, which converts butyryl-CoA to butanol), can produce butanol
20 using butyryl-CoA serving as an intermediate.

In the present invention, the *bdhAB* derived from *Clostridium* sp. is introduced as a BDH-encoding gene in order to improve the yield of conversion from butyryl-CoA to butanol. BDH encoding genes derived from microorganisms other than
25 *bdhAB* derived from *Clostridium* sp. may be used without limitations as long as they are expressed to show the same BDH activity.

Improvement in conversion from butyryl-CoA to butanol can be brought about by introducing the AAD-encoding gene, *adhE*, derived from *Clostridium* sp., in
30 accordance with the present invention. ADD-encoding genes derived from

microorganisms other than *adhE* derived from *Clostridium* sp. can be used without limitations as long as they are expressed to show the same AAD activity. It is well known that *mhpF* derived from *E. coli* encodes acetaldehyde dehydrogenase (Ferrandez, A. *et al.*, *J. Bacteriol.*, 179:2573, 1997). When *adhE*
5 derived from *Clostridium* sp. is substituted with *mhpF* (coding for acetaldehyde dehydrogenase or butyraldehyde) derived from *E. coli*, butanol can be produced, as demonstrated in an example of the present invention.

In consideration of the pathway of FIG. 1 and the pathway of *E. coli*, it is
10 understood that butanol production can be improved by shutting the biosynthesis pathways for acetate, ethanol and lactate, which compete with the butanol biosynthesis pathway. These competing pathways are shut down in the host cells of interest before introducing genes involved in the butanol biosynthesis pathway in accordance with the present invention.

15

In practice, genes coding for enzymes responsible for the biosynthesis of lactate, acetate and/or ethanol in *E. coli* wild-type W3110 are attenuated or deleted so as to construct a mutant *E. coli* which has enhanced butanol productivity in accordance with the present invention.

20

In detail, *ldhA* (coding for lactate dehydrogenase which is involved in the biosynthesis of lactate), *pta* (coding for phosphoacetyltransferase which is involved in the biosynthesis of acetate) and *adh* (coding for alcohol dehydrogenase which is involved in the biosynthesis of ethanol) are deleted. It
25 should be understood that as long as the competing biosynthesis pathways can be shot down, the deletion of genes other than these genes is within the scope of the present invention.

Afterwards, *lacI* (coding for lac operon repressor) was additionally deleted, so as
30 to increase the expression level of the genes encoding the enzymes responsible

for butanol biosynthesis.

In greater detail, *E. coli* WLLPA, which lacks the three genes (*lahA*, *pta* and *adh*) plus *lacI*, and *E. coli* WLL, which lacks *ldhA* and *lacI*, were constructed, followed by introducing genes encoding the enzymes responsible for butanol biosynthesis, including THL, BHBD, CRO, BCD, cofactors of BCD, and BHD, thereinto, thus constructing recombinant mutant microorganisms having excellent butanol productivity.

The THL-encoding gene, the BHBD-encoding gene, the CRO-encoding gene, the BCD-encoding gene, the BCD cofactor-encoding gene, the AAD-encoding gene and the BDH-encoding gene may be introduced into a host cell by an expression vector containing a strong promoter. Examples of the strong promoter useful in the present invention include, but are not limited to, a trc promoter, a tac promoter, a T7 promoter, a lac promoter and a trp promoter.

Finally, the genes encoding thiolase (THL), 3-hydroxybutyryl-CoA dehydrogenase (BHBD), crotonase (CRO), butyryl-CoA dehydrogenase (BCD), butyraldehyde dehydrogenase (AAD), butanol dehydrogenase (BDH), a chaperone protein (*groESL*) and BCD cofactors (*etfAB*) are introduced into *E. coli* strains, in which genes encoding the enzymes involved in the biosynthesis of lactate, acetate, and/or ethanol are attenuated or deleted, to prepare recombinant mutant *E. coli*, thus confirming that butanol productivity is remarkably improved in the recombinant mutant *E. coli*.

<u>Examples</u>

A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as the limit of the present invention.

Although, in the following examples, *E. coli* W3110 was used as a host microorganism, it will be obvious to those skilled in the art that other *E. coli* strains, bacteria, yeasts and fungi can also be used as host cells by deleting target gene to be deleted and introducing genes involved in butanol biosynthesis, in order to produce butanol.

Further, although genes derived from a specific strain are exemplified as target genes to be introduced in the following examples, it is obvious to those skilled in the art that as long as they are expressed to show the same activity in the host cells, any genes may be employed without limitations.

Also, it should be noted that although only specific culture media and methods are exemplified in the following example, saccharified liquid, such as whey, CSL (corn steep liquor), etc, and the other media, and various culture methods, such as fed-batch culture, continuous culture, etc. (Lee *et al., Bioprocess Biosyst. Eng.*, 26:63, 2003; Lee *et al., Appl. Microbiol. Biotechnol.*, 58:663, 2002; Lee *et al., Biotechnol. Lett.*, 25:111, 2003; Lee *et al., Appl. Microbiol. Biotechnol.*, 54:23, 2000; Lee *et al., Biotechnol. Bioeng.*, 72:41, 2001) also fall within the scope of the present invention.

Example 1: Preparation of recombinant mutant microorganism having high butanol productivity

1-1: Deletion of *lacI* gene

In *E. coli* W3110 (ATTC 39936), the *lacI* gene coding for the lac operon repressor, which functions to inhibit the transcription of an lac operon required for the metabolism of lactose, was deleted through one-step inactivation (Warner *et al., PNAS*, 6:97(12):6640, 2000) using primers of SEQ ID NOS: 1 and 2, thus

removing antibiotic resistance from the bacteria.

[SEQ ID NO: 1] lacI_1stup: 5'-gtgaaaccagtaacgttatacgatgtcgcagagtatgccgg tgtctcttagattgcagcattacacgtcttg-3'

[SEQ ID NO: 2] lacI_1stdo: 5'-tcactgcccgctttccagtcgggaaacctgtcgtgccagctg
5 cattaatgcacttaacggctgacatggg-3'

1-2: Deletion of *ldhA*, *pta* and *adhE* genes

In the *lacI* -knockout *E. coli* W3110 of Example 1-1, *ldhA* (coding for lactate
10 dehydrogenase), *pta* (coding for phosphotransacetylase) and *adhE* (coding for alcohol dehydrogenase) were further deleted by one-step inactivation using primers of SEQ ID NOS: 3 to 8.

That is, the three genes were deleted from *E. coli* W3110 competent cells lacking
15 *lacI*, prepared in Example 1-1, thus constructed a novel mutant WLLPA strain.

Additionally, *ldhA* (coding for lactate dehydrogenase) was deleted in the *lacI*-knockout *E. coli* W3110 competent cells of Example 1-1 through the one step inactivation with the aid of primers of SEQ ID NOS: 3 and 4, thus constructed a
20 novel mutant WLL strain.

[SEQ ID NO: 3] ldhA1stup: 5'-atgaaactcgccgtttatagcacaaaacagtacgacaagaag tacctgcagattgcagcattacacgtcttg-3'

[SEQ ID NO: 4] ldhA1stdo: 5'-ttaaaccagttcgttcgggcaggtttcgcctttttccagattgct taagtcacttaacggctgacatggga-3'

25 [SEQ ID NO: 5] pta1stup: 5'-gtgtcccgtattattatgctgatccctaccggaaccagcgtcggtc tgacgattgcagcattacacgtcttg-3'

[SEQ ID NO: 6] pta1stdo: 5'-ttactgctgctgtgcagactgaatcgcagtcagcgcgatggtgta gacgaacttaacggctgacatggg-3'

[SEQ ID NO: 7] adhE1stup: 5'-cgtgaatatgccagtttcactcaagagcaagtagacaaaatctt
30 ccgcgcgattgcagcattacacgtcttg-3'

[SEQ ID NO: 8] adhE1stdo: 5'-taatcacgaccgtagtaggtatccagcagaatctgtttcagctc ggagatcacttaacggctgacatggg-3'

1-3: Construction of pKKhbdadhEthiL (pKKHAT) vector

5

Genes necessary for the butanol biosynthesis pathway, including *hbd* (coding for 3-hydroxybutyryl-CoA dehydrogenase), *adhE* (coding for butyraldehyde dehydrogenase: the same spell, but different in function from the *adhE* (coding for alcohol dehydrogenase) of 1-2) and *thiL* (coding for thiolase) was amplified 10 using primers of SEQ ID NOS: 9 to 14 with the chromosomal DNA of *Clostridium acetobutylicum* (KCTC 1724) serving as a template, and they were sequentially cloned into a pKK223-3 expression vector (Pharmacia Biotech), thus constructed a recombinant expression vector, named pKKhbdadhEthiL (pKKHAT) (FIG. 2).

15 [SEQ ID NO: 9] hbdf: 5'-acgcgaattcatgaaaaaggtatgtgttat-3'

[SEQ ID NO: 10] hbdr: 5'-gcgtctgcaggagctcctgtctctagaatttgataatggggattctt-3'

[SEQ ID NO: 11] adhEf: 5'-acgctctagatataaggcatcaaagtgtgt-3'

[SEQ ID NO: 12] adhEr: 5'-gcgtgagctccatgaagctaatataatgaa-3'

[SEQ ID NO: 13] thiLf: 5'-acgcgagctctatagaattggtaaggatat-3'

20 [SEQ ID NO: 14] thiLr: 5'-gcgtgagctcattgaacctccttaataact-3'

1-4: Construction of pKKhbdadhEatoB (pKKHAA) vector

To clone the *atoB* (coding for acetyl-CoA acetyltransferase) of *Escherichia coli* 25 W3110 into the pKKhbdadhE vector (FIG. 2), PCR was performed on the chromosomal DNA of *Escherichia coli* W3110 using primers of SEQ ID NOS: 15 and 16, with 24 cycles of denaturing at 95°C for 20 sec, annealing at 55°C for 30 sec and extending at 72°C for 90 sec. The PCR product (*atoB*) obtained was digested with *Sac*I and inserted into the pKKhbdadhE vector digested with the 30 same restriction enzyme (*Sac*I), thus constructed a novel recombinant vector,

named pKKhbdadhEatoB (pKKHAA) (FIG. 3).

[SEQ ID NO: 15] atof: 5'-atacgagctctacggcgagcaatggatgaa-3'

[SEQ ID NO: 16] ator: 5'-gtacgagctcgattaattcaaccgttcaat-3'

5 1-5: Construction of pKKhbdadhEphaA (pKKHAP) vector

To clone the *phaA* (coding for thiolase) of *Ralstonia eutropha* (KCTC 1006) into the pKKhbdadhE vector, PCR was performed using primers of SEQ ID NOS: 17 and 18, with the chromosomal DNA of *Ralstonia eutropha* serving as a template.
10 The PCR product (*phaA*) obtained was cleaved with *Sac*I and inserted into the pKKhbdadhE vector digested with the same restriction enzyme (*Sac*I), thus constructed a novel recombinant vector, named pKKhbdadhEphaA (pKKHAP) (FIG. 4).

[SEQ ID NO: 17] phaAf: 5'-agtcgagctcaggaaacagatgactgacgttgtcatcgt-3'

15 [SEQ ID NO: 18] phaAr: 5'-atgcgagctcttatttgcgctcgactgcca-3'

1-6: Construction of pKKhbdydbMadhEphaA (pKKHYAP) vector

To clone the *ydbM* (coding for hypothetical protein) of *Bacillus subtilis* (KCTC
20 1022) into the pKKhbdadhE vector, PCR was performed using primers of SEQ ID NOS: 19 and 20 with the chromosomal DNA of *Bacillus subtilis* serving as a template. The PCR product (*ydbM*) obtained was cleaved with *Xba*I and inserted into the pKKhbdadhEphaA vector digested with the same restriction enzyme (*Xba*I), thus constructed a novel recombinant vector, named
25 pKKhbdydbMadhEphaA (pKKHYAP) (FIG. 5).

[SEQ ID NO: 19] ydbMf: 5'-agcttctagagatgggttacctgacatata-3'

[SEQ ID NO: 20] ydbMr: 5'-agtctctagattatgactcaaacgcttcag-3'

1-7: Construction of pKKhbdbcdPA01adhEphaA (pKKHPAP) vector

30

To clone the *bcd* (coding for butyryl-CoA dehydrogenase) of *Pseudomonas aeruginosa* PA01 (KCTC 1637) into the pKKhbdadhEphaA vector, PCR was performed using primers of SEQ ID NOS: 21 and 22 with the chromosomal DNA of *Pseudomonas aeruginosa* PA01 serving as a template. The PCR product
5 (*bcd*) obtained was cleaved with *Xba*I and inserted into the pKKhbdadhEphaA (pKKHAP) vector digested with the same restriction enzyme (*Xba*I), thus constructed a novel recombinant vector, named pKKhbdbcdPA01adhEphaA (pKKHPAP) (FIG. 6).

[SEQ ID NO: 21] bcdPA01f: 5'-agcttctagaactgctccttggacagcgcc-3'
10 [SEQ ID NO: 22] bcdPA01r: 5'-agtctctagaggcaggcaggatcagaacca-3'

1-8: Construction of pKKhbdbcdKT2440adhEphaA (pKKHKAP) vector

To clone the *bcd* (coding for butyryl-CoA dehydrogenase) of *Pseudomonas*
15 *putida* KT2440 (KCTC 1134) into the pKKhbdadhEphaA vector, PCR was performed using primers of SEQ ID NOS: 23 and 24 with the chromosomal DNA of *Pseudomonas putida* KT2440 serving as a template. The PCR product (*bcd*) obtained was cleaved with *Xba*I and inserted into the pKKhbdadhEphaA vector digested with the same restriction enzyme (*Xba*I), thus constructed a novel
20 recombinant vector, named pKKhbdbcdKT2440adhEphaA (pKKHKAP) (FIG. 7).

[SEQ ID NO: 23] bcdKT2440f: 5'-agcttctagaactgttccttggacagcgcc-3'
[SEQ ID NO: 24] bcdKT2440r: 5'-agtctctagaggcaggcaggatcagaacca-3'

1-9: Construction of pKKhbdgroESLadhEphaA (pKKHGAP) vector

25

PCR was performed using primers of SEQ ID NOS: 25 and 26 with the chromosomal DNA of *Clostridium acetobutylicum* serving as a template. The PCR product (*groESL*) obtained was cleaved with *Xba*I and inserted into the pKKhbdadhEphaA vector digested with the same restriction enzyme (*Xba*I), thus
30 constructed a novel recombinant vector, named pKKhbdgroESLadhEphaA

(pKKHGAP) (FIG. 8).

[SEQ ID NO: 25] groESLf: 5'-agcttctagactcaagattaacgagtgcta-3'

[SEQ ID NO: 26] groESLr: 5'-tagctctagattagtacattccgcccattc-3'

5 1-10: Construction of pTrc184bcdbdhABcrt vector

PCR was performed using primers of SEQ ID NOS: 27 and 28, with the chromosomal DNA of *Clostridium acetobutylicum* serving as a template. The PCR product (*bcd*) obtained was digested with *Nco*I and *Kpn*I and cloned into a 10 pTrc99A expression vector (Amersham Pharmacia Biotech), thus constructed a recombinant vector named pTrc99Abcd. After the pTrc99Abcd vector was digested with *Bsp*HI and *Eco*RV, the DNA fragment thus excised was inserted into pACYC184 (New England Biolabs) which was previously treated with the same restriction enzymes (*Bsp*HI and *Eco*RV), thus constructed a recombinant 15 expression vector for expressing the *bcd* gene, named pTrc184bcd (FIG. 9).

[SEQ ID NO: 27] bcdf: 5'-agcgccatggattttaatttaacaag-3'

[SEQ ID NO: 28] bcdr: 5'-agtcggtacccctccttaaattatctaaaa-3'

PCR was performed using primers of SEQ ID NOS: 29 and 30, with the 20 chromosomal DNA of *Clostridium acetobutylicum* serving as a template. The PCR product (*bdhAB*) obtained was digested with *Bam*HI and *Pst*I and inserted into the pTrc184bcd expression vector digested with the same restriction enzymes (*Bam*HI and *Pst*I), thus constructed a recombinant vector, named pTrc184bcdbdhAB (pTrc184BB), which contain both *bcd* and *bdhAB*.

25 [SEQ ID NO: 29] bdhABf: 5'-acgcggatccgtagtttgcatgaaatttcg-3'

[SEQ ID NO: 30] bdhABr: 5'-agtcctgcagctatcgagctctataatggctacgcccaaac-3'

PCR was performed using primers of SEQ ID NOS: 31 and 32, with the chromosomal DNA of *Clostridium acetobutylicum* serving as a template. The 30 PCR product (*crt*) obtained was digested with *Sac*I and *Pst*I and inserted into the

pTrc184bcdbdhAB vector digested with the same restriction enzymes (*Sac*I and *Pst*I), thus constructed a recombinant vector, named pTrc184bcdbdhABcrt (pTrc184BBC), which contain all of the *bcd* gene, the *bdhAB* gene and the *crt* gene (FIG. 9).

5 [SEQ ID NO: 31] crtf: 5'-actcgagctcaaaagccgagattagtacgg-3'

[SEQ ID NO: 32] crtr: 5'-gcgtctgcagcctatctatttttgaagcct-3'

1-11: Preparation of butanol-producing microorganisms

10 *E. coli* W3110 (WLLPA) lacking *lacI*, *ldhA*, *pta* and *adhE* and *E. coli* W3110 (WLL) lacking *lacI* and *ldhA*, respectively prepared in Examples 1-1 and 1-2, were transformed with the pTrc184bcdbdhABcrt (pTrc184BBC) vector of Example 1-10 and the vector selected from the group consisting of pKKhbdadhEthiL (pKKHAT), pKKhbdadhEatoB (pKKHAA), 15 pKKhbdydbMadhEphaA (pKKHYAP), pKKhbdadhEphaA (pKKHAP), pKKhbdbcdPA01adhEphaA (pKKHPAP), pKKhbdbcdKT2440adhEphaA (pKKHKAP) and pKKhbdgroESLadhEphaA (pKKHGAP) constructed in Examples 1-3 to 1-9, thus prepared recombinant mutant microorganisms (WLLPA+pKKHPAP+pTrc184BBC, WLL+pKKHAT+pTrc184BBC, 20 WLL+pKKHAA+pTrc184BBC, WLL+pKKHAP+pTrc184BBC, WLL+pKKHYAP+pTrc184BBC, WLL+pKKHPAP+pTrc184BBC, WLL+pKKHKAP+pTrc184BBC, and WLL+pKKHGAP+pTrc184BBC) capable of producing butanol.

25 1-12: Assay for butanol productivity

The butanol-producing microorganisms prepared in Example 1-11 were selected on LB plates containing 50 μg/ml ampicillin and 30 μg/ml chloramphenicol. For the selection of the WLLPA+pKKHPAP+pTrc184BBC strain, kanamycin was 30 added in an amount of 30 μg/ml to the LB plates. The recombinants were

precultured at 37°C for 12 hr in 10 ml of LB broth. After being autoclaved, 100 mL of LB broth maintained at 80°C or higher in a 250 mL flask was added with glucose (5g/L) and cooled to room temperature in an anaerobic chamber purged with nitrogen gas. 2 mL of the preculture was inoculated into the flask and
5 cultured at 37°C for 10 hr. Then, 2.0 liters of a medium containing 20 g of glucose, 2 g of KH_2PO_4, 15 g of $(NH_4)_2SO_4 \cdot 7H_2O$, 20 mg of $MnSO_4 \cdot 5H_2O$, 2 g of $MgSO_4 \cdot 7H_2O$, 3 g of yeast extract, and 5 ml of a trace metal solution (10g $FeSO_4 \cdot 7H_2O$, 1.35g $CaCl_2$, 2.25g $ZnSO_4 \cdot 7H_2O$, 0.5g $MnSO_4 \cdot 4H_2O$, 1g $CuSO_4 \cdot 5H_2O$, 0.106g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.23g $Na_2B_4O_7 \cdot 10H_2O$,
10 and 35% HCl 10 mℓ per liter of distilled water) per liter of distilled water in a 5 L fermenter (LiFlus GX, Biotron Inc., Korea) was autoclaved and cooled from 80°C or higher to room temperature with nitrogen supplied at a rate of 0.5 vvm for 10 hr. In the fermenter, the culture was carried out at 37℃, 200 rpm with shaking at 200 rpm. During the cultutivation, pH to be maintained at 6.8 by
15 automatic feeding with 25%(v/v) NH_4OH and nitrogen gas was supplied at a rate of 0.2 vvm (air volume/working volume/minute).

When the glucose of the medium was completely exhausted, as measured using a glucose analyzer (STAT, Yellow Springs Instrument, Yellow Springs, Ohio, USA),
20 the medium was analyzed for butanol concentration using gas chromatography (Agillent 6890N GC System, Agilent Technologies Inc., CA, USA) equipped with a packed column (Supelco CarbopackTM B AW/6.6% PEG 20M, 2 m × 2 mm ID, Bellefonte, PA, USA).

25 As a result, as shown in Table 1, wild-type *E. coli* W3110 did not produce butanol, whereas it was produced from the recombinant mutant microorganisms according to the present invention. In addition, all of the genes encoding thiolase (*thiL, phaA, atoB*) were observed to show activities. Particularly, the butyryl-CoA dehydrogenase of *Pseudomonas aeruginosa* or *Pseudomonas putida* is superior to
30 that of *Clostridium acetobutylicum* in terms of activity, as demonstrated by

butanol productivity.

Table 1

Strains	Containing genes	Butanol (mg/L)
W3110	-	ND[1]
WLL+pKKHAT+ pTrc184BBC	*hbd, adhE, thiL, bcd, bdhAB, crt*	1.2
WLL+pKKHAA+pTrc184BBC	*hbd, adhE, atoB, bcd, bdhAB, crt*	1.3
WLL+pKKHAP+pTrc184BBC	*hbd, adhE, phaA, bcd, bdhAB, crt*	1.4
WLL+pKKHYAP+pTrc184BBC	*hbd, adhE, ydbM, phaA, bcd, bdhAB, crt*	1.7
WLL+pKKHPAP+pTrc184BBC	*hbd, adhE, bcdPA01, phaA, bcd, bdhAB, crt*	3.1
WLLPA+pKKHPAP+pTrc184BBC		4.5
WLL+pKKHKAP+pTrc184BBC	*Hbd, adhE, bcdKT2440, phaA, bcd, bdhAB, crt*	9.1
WLL+pKKHGAP+pTrc184BBC	*hbd, adhE, groESL, phaA, bcd, bdhAB, crt*	13.5

[1] Not detected.

5

Also, the butanol productivity was greatly increased by the co-introduction of the chaperone-encoding gene (*groESL*) and the *bcd* derived from *Clostridium acetobutylicum* (WLL+pKKHGAP+pTrc184BBC). Accordingly, the chaperone protein is found to greatly promote the activity of butyryl-CoA dehydrogenase, as
10 demonstrated from the fact that when *groESL* was introduced, together with the *bcd* derived from *Clostridium acetobutylicum*, the butanol productivity increased more that 10-fold.

Previously, the present inventors reported that when the recombinant *E. coli* into
15 which genes responsible for butanol biosynthesis were introduced, the *E. coli* strain in which only *lacI* was deleted could produce butanol. As is apparent from the data of Table 1, butanol production is further increased when *ldhA* in addition to *lacI* is deleted. Moreover, the additional deletion of *pta* and *adhE* was shown to further improve the butanol productivity. Taken together, the data
20 obtained above demonstrate that the blockage of the lactate biosynthesis pathway, the acetate biosynthesis pathway and/or the ethanol biosynthesis pathway, all of

which compete with the butanol biosynthesis pathway, makes a contribution to butanol production.

Example 2: Production of butanol from recombinant microorganisms introduced with genes derived from *E. coli* and *C. acetobutylicum*

In this example, when the genes derived from *C. acetobutylicum*, responsible for the butanol biosynthesis pathway, were partially substituted with genes derived from *E. coli*, butanol productivity was measured (FIG. 10). In detail, when *adhE*, *crt, hbd* and *thiL* derived from *Clostridium* sp. were substituted with genes derived from *E. coli*, respectively, the resulting recombinant microorganisms were measured for butanol productivity.

2-1: Construction of pKKmhpFpaaFGHatoB vector

PCR was performed using primers of SEQ ID NOS: 33 to 38, with the chromosomal DNA of *E. coli* W3110 serving as a template, to amplify genes essential for the butanol biosynthesis pathway, including *mhpF* (coding for acetaldehyde dehydrogenase), *paaFG* (coding for enoyl-CoA hydratase), *paaH* (coding for 3-hydroxy-acyl-CoA dehydrogenase) and *atoB* (coding for acetyl-CoA acetyltransferase). These genes were sequentially cloned into a pKK223-3 expresison vector (Pharmacia Biotech), thus constructed a novel recombinant expression vector, named pKKmhpFpaaFGHatoB (pKKMPA) (FIG. 11).

[SEQ ID NO: 33] mhpFf: 5'-atgcgaattcatgagtaagcgtaaagtcgc-3'

[SEQ ID NO: 34] mhpFr: 5'-tatcctgcaggagctctctagagctagcttaccgttcatgccgcttct-3'

[SEQ ID NO: 35] paaFGHf: 5'-atacgctagcatgaactggccgcaggttat-3'

[SEQ ID NO: 36] paaFGHr: 5'-tatcgagctcgccaggccttatgactcata-3'

[SEQ ID NO: 37] atoBf: 5'-atacgagctctgcatcactgccctgctctt-3'

[SEQ ID NO: 38] atoBr: 5'-tgtcgagctccgctatcgggtgtttttatt-3'

2-2: Construction of pTrc184bcdetfABbdhABgroESL vector

PCR was performed using primers of SEQ ID NOS: 39 and 40, with the chromosomal DNA of *Clostridium acetobutylicum* serving as a template. The PCR product (*etfAB*) obtained was digested with *Kpn*I and *Bam*HI, followed by the insertion of the truncated PCR product into the pTrc184bcdbdhAB vextor digested with the same restriction enzymes (*Kpn*I and *Bam*HI), thus constructed a novel recombinant expression vector, named pTrc184bcdetfABbdhAB (pTrc184BEB), which contain all of the *bcd* gene, the *bdhAB* gene and the *etfAB* gene.

PCR was performed using primers of SEQ ID NOS: 41 and 42, with the chromosomal DNA of *Clostridium acetobutylicum* serving as a template. The PCR product obtained was digested with *Sac*I and *Pst*I, followed by the insertion of the truncated PCR product into the pTrc184bcdetfABbdhAB vector digested with the same restriction enzymes (*Sac*I and *Pst*I), thus constructed a novel recombinant expression vector, named pTrc184bcdetfABbdhABgroESL (pTrc184BEBG), which contain all of the *bcd* gene, the *bdhAB* gene, the *etfAB* gene and the *groESL* gene (FIG. 12).

[SEQ ID NO: 39] etfABf: 5'-atacggtaccaaatgtagcaatggatgtaa-3'
[SEQ ID NO: 40] etfABr: 5'-gtacggatcccttaattattagcagcttta-3'
[SEQ ID NO: 41] groESL1: 5'-atgcgagctcaaaaagcgagaaaaaccata-3'
[SEQ ID NO: 42] groESL2: 5'-gtacctgcagattagtacattccgcccatt-3'

2-3: Preparation of butanol-producing microorganism

E. coli W3110 (WLLPA), lacking *lacI*, *ldhA*, *pta* and *adhE,* and *E. coli* W3110 (WLL) lacking *lacI* and *ldhA*, respectively prepared in Examples 1-1 and 1-2, were transformed with the pKKMPA vector of Example 3-1 and the pTrc184bcdbdhAB (pTrc184BB) vector of Exmaple 1-10 or the pKKBEBG

vector of Example 3-2, thus prepared recombinant mutant microorganisms capable of producing butanol (WLL+pKKMPA+pTrc184BB, WLLPA+pKKMPA+pTrc184BB, WLL+pKKMPA+pTrc184BEBG, and WLLPA+pKKMPA+pTrc184BEBG).

5

2-4 Assay for butanol productivity

The butanol-producing microorganisms prepared in Example 2-3 were cultured in the same manner as in Example 1-13 and measured for butanol productivity

10 under the same conditions.

The results are summarized in Table 2, below. Compared to when only the butanol biosynthesis pathway of *C. acetobutylicum* was used, as shown in Table 2, butanol productivity was improved when *E. coli*-derived genes predicted to code

15 the corresponding enzymes (*adhE→mhpF, crt→paaFG, hbd→paaH, thiL→atoB*) and the *bcd* and *bdhAB* genes derived from *C. acetobutylicum* were used in combination. That is, four (butyraldehyde dehydrogenase, crotonase, BHBD and THL) of the enzymes from *Clostridium acetobutylicum* essential for butanol production in *E. coli* can be substituted with enzymes encoded by *mhpF*, *paaFG*,

20 *paaH* and *atoB* genes derived from *E. coli*, and these enzymes from *E. coli* were found to have higher activity than the corresponding enzymes from *C. acetobutylicum,* as demonstrated by the enhanced butanol production.

As demonstrated by the conspicuous increase in butanol productivity, the BCD

25 enzyme, known to have poor activity in *E. coli*, was found to recover its activity with the expression of the co-factor encoding gene (*etfAB*) and the chaperone encoding gene (*groESL*).

Table 2

Strains	**Containing genes**	**Butanol (mg/L)**

WLL+pKKMPA+pTrc184BB	*mhpF*, *paaFGH*, *atoB*, *bcd*, *bdhAB*	18.4
WLLPA+pKKMPA+pTrc184BB		32.4
WLL+pKKMPA+pTrc184BEBG	*mhpF*, *paaFGH*, *atoB*, *bcd*, *bdhAB*, *etfAB*, *groESL*	365.5
WLLPA+pKKMPA+pTrc184BEBG		627.8

INDUSTRIAL APPLICABILITY

As described in detail above, based on metabolic network reconstruction by gene deletion, metabolic engineering by amplification of desired genes and a method for increasing butyryl-CoA dehydrogenase activity, the present invention provides recombinant mutant microorganisms which have remarkably improved butanol productivity. Having advantages over *Clostridium acetobutylicum* in that they can be cultured easily and be further modified by manipulation of the metabolic pathways thereof, the recombinant mutant *E. coli* in accordance with the present invention is useful as a microorganism producing butanol for use in various industrial applications.

Although the present invention has been described in detail with reference to the specific features, it will be apparent to those skilled in the art that this description is only for a preferred embodiment and does not limit the scope of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

THE CLAIMS

What is Claimed is:

5 1. A method for preparing a recombinant mutant microorganism having high butanol productivity, the method comprises:

deleting or attenuating at least one selected from the group consisting of genes coding for enzymes involved in lactate biosynthesis, genes coding for enzymes involved in acetate biosynthesis, and genes coding for enzymes
10 involved in ethanol biosynthesis in a microorganism; and

introducing or amplifying at least one gene coding for an enzyme involved in butanol biosynthesis into said microorganism.

2. The method for preparing a recombinant mutant microorganism having high
15 butanol productivity according to claim 1, in which a *lacI* gene (coding for a lac operon repressor) is further deleted in the microorganism so as to enhance the expression of the gene coding for the enzyme involved in butanol biosynthesis.

3. The method for preparing a recombinant mutant microorganism having high
20 butanol productivity according to claim 1, wherein said microorganism is selected from the group consisting of a bacterium, a yeast, a fungus.

4. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claim 3, wherein said bacterium is *E. coli*.

25

5. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claim 1, wherein the gene coding for the enzyme involved in the lactate biosynthesis is *ldhA* (coding for lactate dehydrogenase).

30

6. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claim 1, wherein the gene coding for the enzyme involved in the acetate biosynthesis is *pta* (coding for phosphoacetyltransferase).

5

7. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claim 1, wherein the gene coding for the enzyme involved in the ethanol biosynthesis is *adhE* (coding for alcohol dehydrogenase).

10

8. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claims 1 or 2, wherein the enzyme involved in butanol biosynthesis is at least one selected from the group consisting of thiolase (THL), 3-hydroxybutyryl-CoA dehydrogenase (BHBD), crotonase (CRO),
15 butyryl-CoA dehydrogenase (BCD), butyraldehyde dehydrogenase (AAD), butanol dehydrogenase (BDH), and combinations thereof.

9. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claim 8, wherein the THL is encoded by a gene
20 selected from the group consisting of *thl*, *thiL*, *phaA*, and *atoB*.

10. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claim 8, wherein the BCD is encoded by a *bcd* gene derived from *Pseudomonas* sp. or a *ydbM* gene derived from *Bacillus* sp.

25

11. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claim 8, wherein the BCD is encoded by a *bcd* gene derived from *Clostridium* sp., and a chaperone-encoding gene is further introduced into the microorganism.

30

12. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claim 11, in which a BCD co-factor-encoding gene (*etfAB*) is further introduced into the microorganism.

5 13. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claim 11, wherein said chaperone-encoding gene is *groESL*.

14. The method for preparing a recombinant mutant microorganism having high
10 butanol productivity according to claim 8, wherein the gene coding for the BHBD is a *hbd* gene derived from *Clostridium* sp. or a *paaH* gene derived from *E. coli*.

15. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claim 8, wherein the gene coding for the CRO
15 is a *crt* gene derived from *Clostridium* sp. or a *paaFG* gene derived from *E. coli*.

16. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claim 8, wherein the gene coding for the AAD is an *adhE* gene derived from *Clostridium* sp. or a *mhpF* gene derived from *E.*
20 *coli*.

17. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claims 1 or 2, wherein the gene coding for the enzyme involved in the butanol biosynthesis is introduced into the microorganism
25 by an expression vector containing a strong promoter.

18. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claim 17, wherein the strong promoter is selected from the group consisting of a trc promoter, a tac promoter, a T7
30 promoter, a lac promoter and a trp promoter.

19. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claim 18, wherein the expression vector containing the strong promoter further contains a gene coding for an enzyme selected from the group consisting of 3-hydroxybutyryl-CoA dehydrogenase, thiolase, butyraldehyde dehydrogenase, crotonase, butanol dehydrogenase, butyryl-CoA dehydrogenase and combinations thereof.

20. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claim 19, wherein the expression vector further contains a chaperone-encoding gene and/or a BCD co-factor-encoding gene.

21. The method for preparing a recombinant mutant microorganism having high butanol productivity according to claim 19, wherein the expression vector is any one selected from the group consisting of pKKHAT, pKKHAA, pKKHYAP, pKKHAP, pKKHPAP, pKKHKAP, and pKKMPA; and any one selected from the group consisting of pTrc184BBC and pTrc184BEBG.

22. A recombinant mutant microorganism having high butanol productivity, in which at least one selected from the group consisting of genes coding for enzymes involved in lactate biosynthesis, genes coding for enzymes involved in acetate biosynthesis, and genes coding for enzymes involved in ethanol biosynthesis is deleted or attenuated; and at least one gene coding for an enzyme involved in butanol biosynthesis is introduced or amplified.

23. The recombinant mutant microorganism having high butanol productivity according to claim 22, in which a *lacI* gene (coding for a lac operon repressor) is further deleted in the microorganism so as to enhance the expression of the gene coding for the enzyme involved in butanol biosynthesis.

24. The recombinant mutant microorganism having high butanol productivity according to claim 22, wherein said microorganism is selected from the group consisting of a bacterium, a yeast, and a fungus.

5 25. The recombinant mutant microorganism having high butanol productivity according to claim 24, wherein said bacterium is *E. coli*.

26. The recombinant mutant microorganism having high butanol productivity according to claim 22, wherein the gene coding for the enzyme involved in the
10 lactate biosynthesis is *ldhA* (coding for lactate dehydrogenase).

27. The recombinant mutant microorganism having high butanol productivity according to claim 22, wherein the gene coding for the enzyme involved in the acetate biosynthesis is *pta* (coding for phosphoacetyltransferase).

15

28. The recombinant mutant microorganism having high butanol productivity according to claim 22, wherein the gene coding for the enzyme involved in the ethanol biosynthesis is *adhE* (coding for alcohol dehydrogenase).

20 29. The recombinant mutant microorganism having high butanol productivity according to claim 22, wherein the enzyme involved in butanol biosynthesis is at least one selected from the group consisting of thiolase (THL), 3-hydroxybutyryl-CoA dehydrogenase (BHBD), crotonase (CRO), butyryl-CoA dehydrogenase (BCD), butyraldehyde dehydrogenase (AAD), butanol dehydrogenase (BDH),
25 and combinations thereof.

30. The recombinant mutant microorganism having high butanol productivity according to claim 29, wherein the THL is encoded by a gene selected from the group consisting of *thl*, *thiL*, *phaA*, and *atoB*.

30

31. The recombinant mutant microorganism having high butanol productivity according to claim 29, wherein the BCD is encoded by a *bcd* gene derived from *Pseudomonas* sp. or a *ydbM* gene derived from *Bacillus* sp.

32. The recombinant mutant microorganism having high butanol productivity according to claim 29, wherein the BCD is encoded by a *bcd* gene derived from *Clostridium* sp., and a chaperone-encoding gene is further introduced into the microorganism.

33. The recombinant mutant microorganism having high butanol productivity according to claim 32, in which a BCD co-factor-encoding gene (*etfAB*) is further introduced into the microorganism.

34. The recombinant mutant microorganism having high butanol productivity according to claim 32, wherein said chaperone-encoding gene is *groESL*.

35. The recombinant mutant microorganism having high butanol productivity according to claim 29, wherein the gene coding for the BHBD is a *hbd* gene derived from *Clostridium* sp. or a *paaH* gene derived from *E. coli*.

36. The recombinant mutant microorganism having high butanol productivity according to claim 29, wherein the gene coding for the CRO is a *crt* gene derived from *Clostridium* sp. or a *paaFG* gene derived from *E. coli*.

37. The recombinant mutant microorganism having high butanol productivity according to claim 29, wherein the gene coding for the AAD is an *adhE* gene derived from *Clostridium* sp. or a *mhpF* gene derived from *E. coli*.

38. The recombinant mutant microorganism having high butanol productivity according to claim 22, wherein the gene coding for the enzyme involved in the

butanol biosynthesis is introduced into the microorganism by an expression vector containing a strong promoter.

39. The recombinant mutant microorganism having high butanol productivity according to claim 38, wherein the strong promoter is selected from the group consisting of a trc promoter, a tac promoter, a T7 promoter, a lac promoter and a trp promoter.

40. The recombinant mutant microorganism having high butanol productivity according to claim 39, wherein the expression vector containing the strong promoter further contains a gene coding for an enzyme selected from the group consisting of 3-hydroxybutyryl-CoA dehydrogenase, thiolase, butyraldehyde dehydrogenase, crotonase, butanol dehydrogenase, butyryl-CoA dehydrogenase and combinations thereof.

41. The recombinant mutant microorganism having high butanol productivity according to claim 40, wherein the expression vector further contains a chaperone-encoding gene and/or a BCD co-factor-encoding gene.

42. The recombinant mutant microorganism having high butanol productivity according to claim 40, wherein the expression vector is of any one selected from the group consisting of pKKHAT, pKKHAA, pKKHYAP, pKKHAP, pKKHPAP, pKKHKAP, and pKKMPA; and any one selected from the group consisting of pTrc184BBC and pTrc184BEBG.

43. A recombinant mutant microorganism having high butanol productivity, in which genes coding for enzymes involved in lactate biosynthesis, genes coding for enzymes involved in acetate biosynthesis, and genes coding for enzymes involved in ethanol biosynthesis are deleted or attenuated; and genes coding for thiolase (THL), 3-hydroxybutyryl-CoA dehydrogenase (BHBD), crotonase

(CRO), butyryl-CoA dehydrogenase (BCD), butyraldehyde dehydrogenase (AAD), butanol dehydrogenase (BDH), a chaperone protein (*groESL*), and BCD co-factors (*etfAB*) are introduced or amplified.

5 44. A method for producing butanol, the method comprises culturing the recombinant mutant microorganism of claims 22, 29 or 32 to produce butanol; and recovering the butanol from the culture broth.

45. A method for producing butanol, the method comprises culturing the 10 recombinant mutant microorganism of claim 43 to produce butanol; and recovering the butanol from the culture broth.

DRAWINGS

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FIG. 1

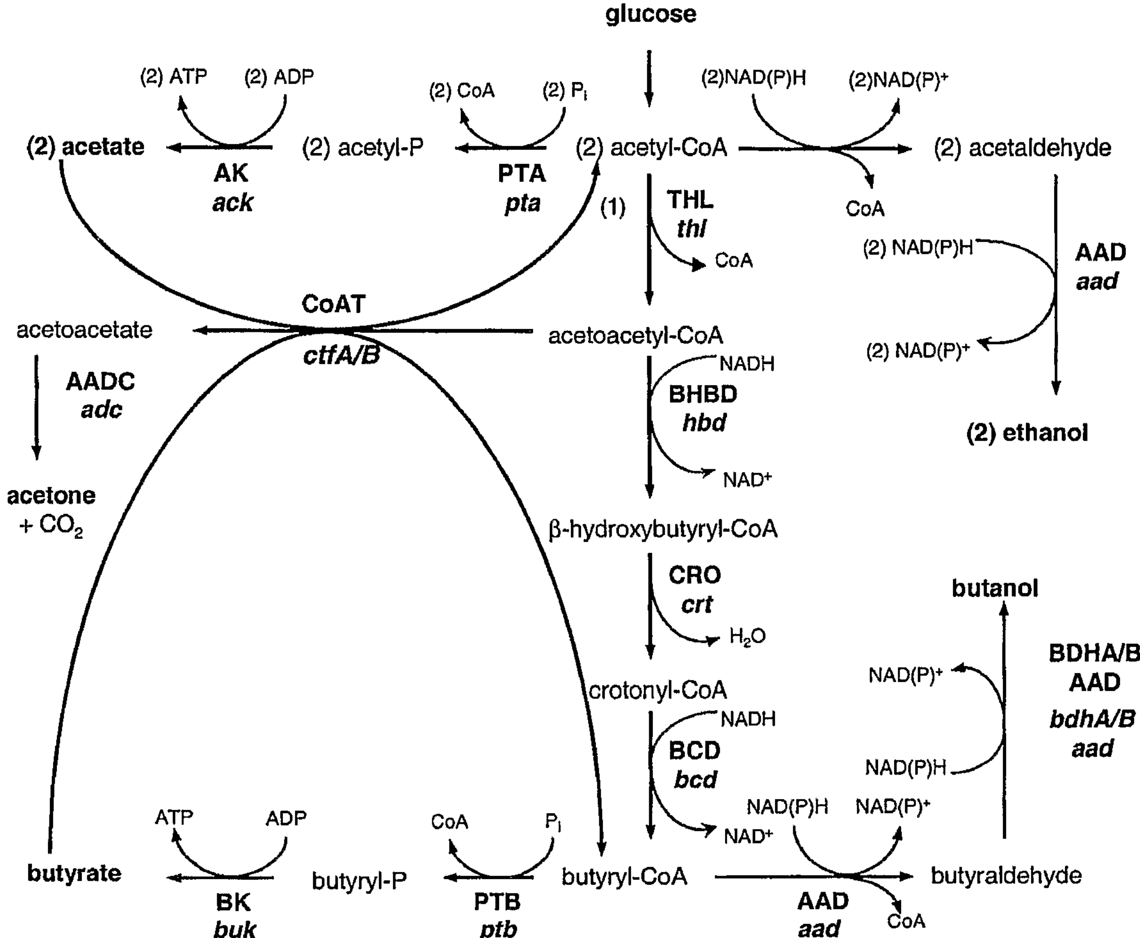


FIG. 2

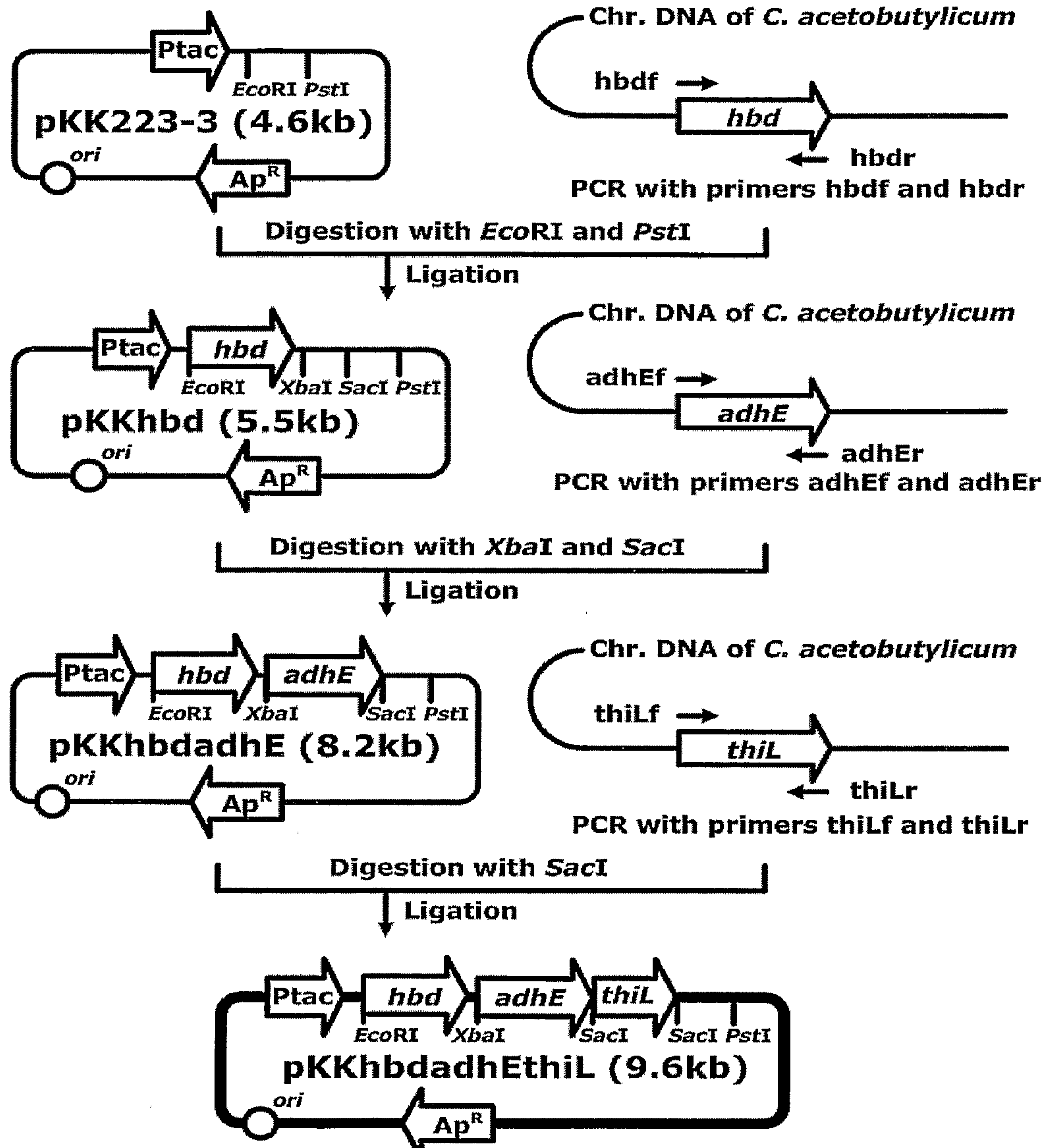


FIG. 3

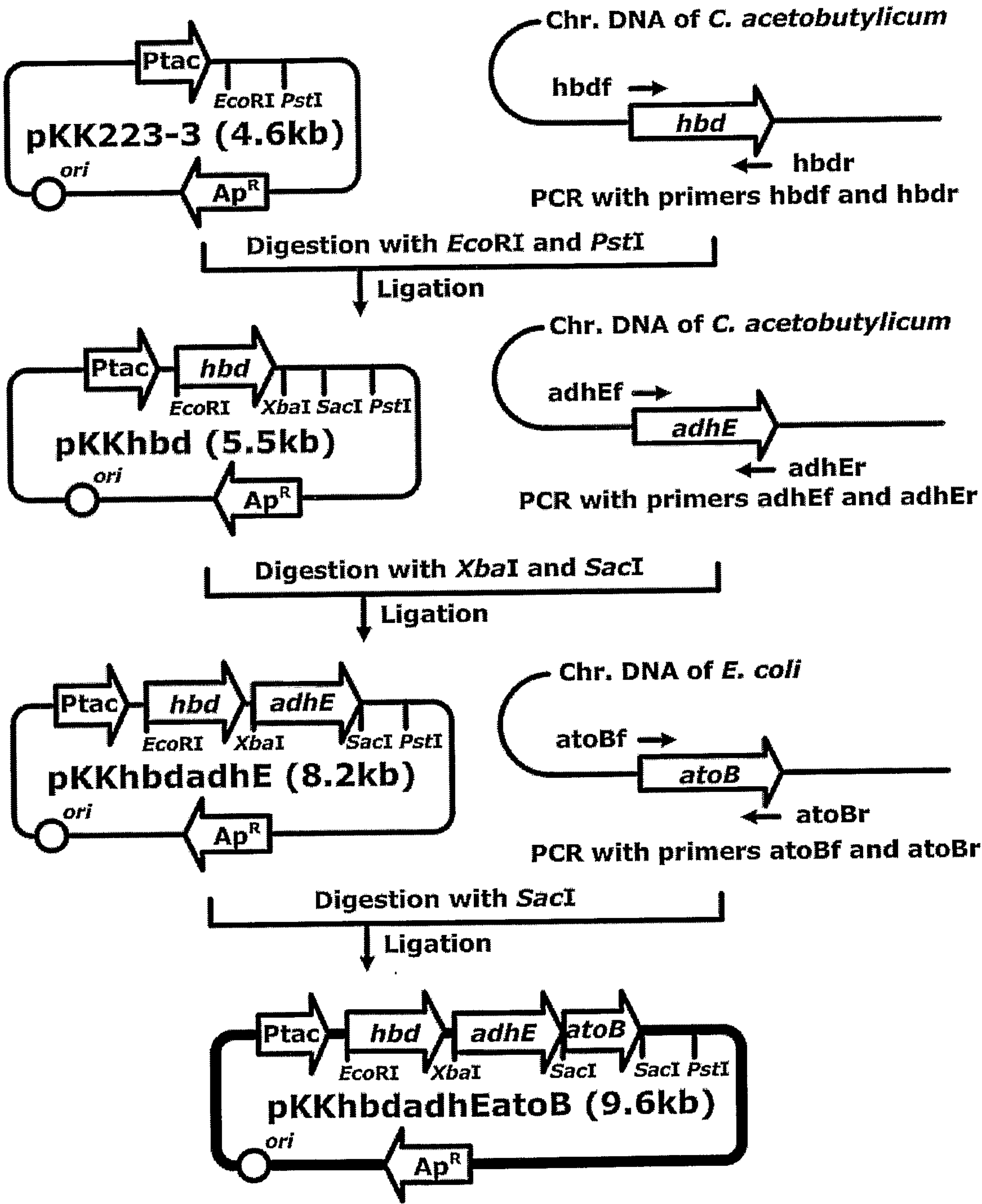


FIG. 4

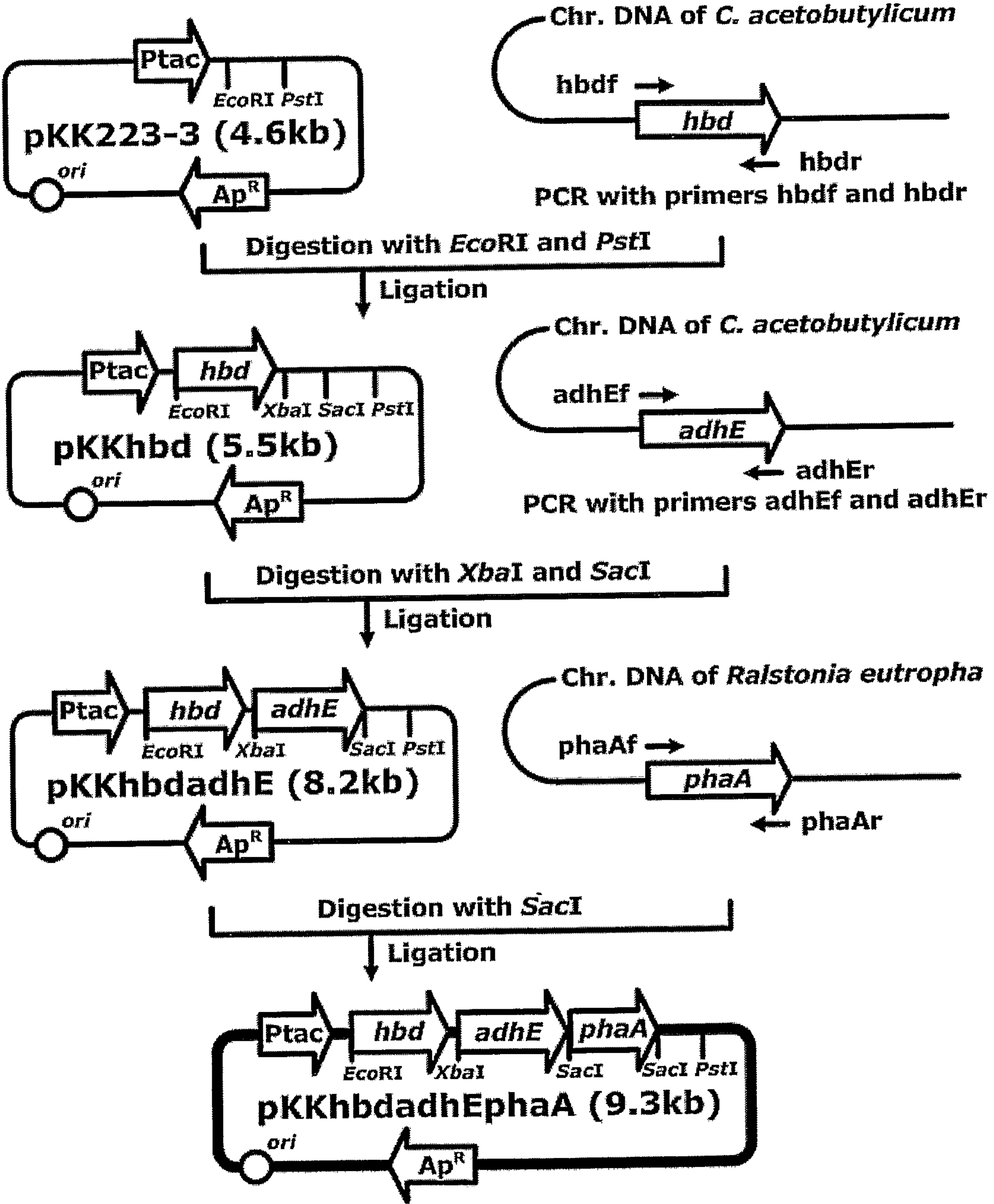


FIG. 5

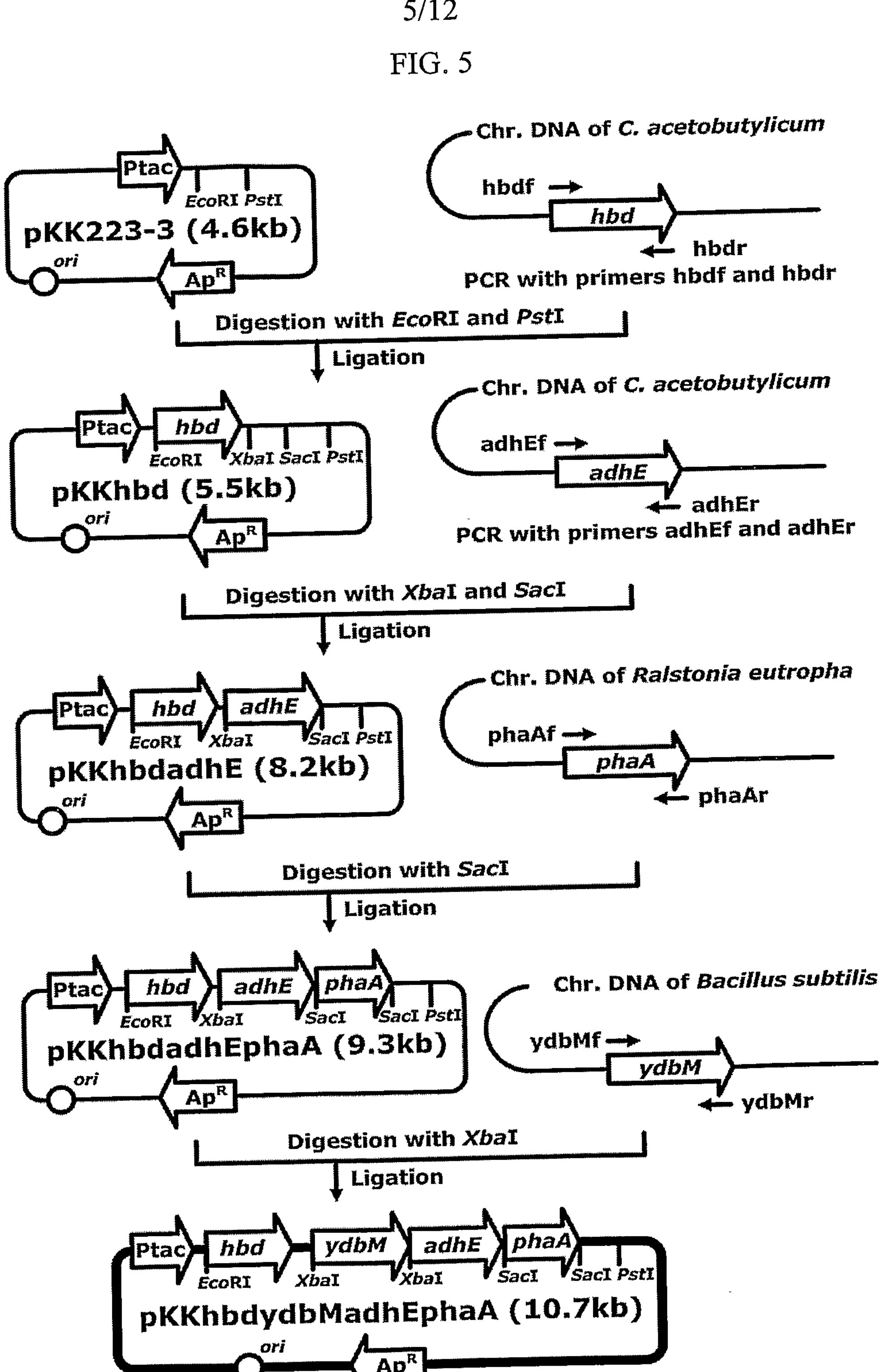


FIG. 6

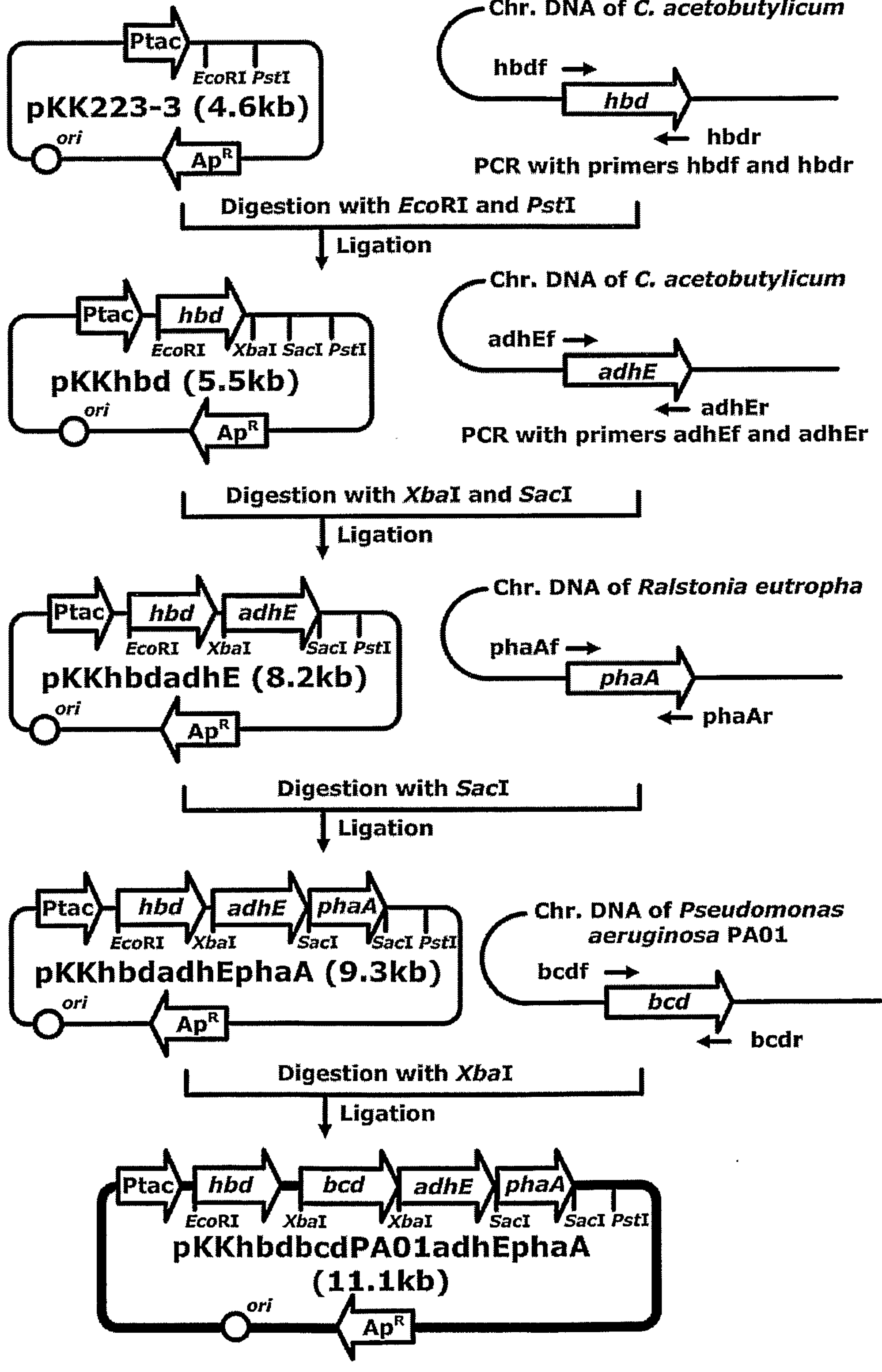


FIG. 7

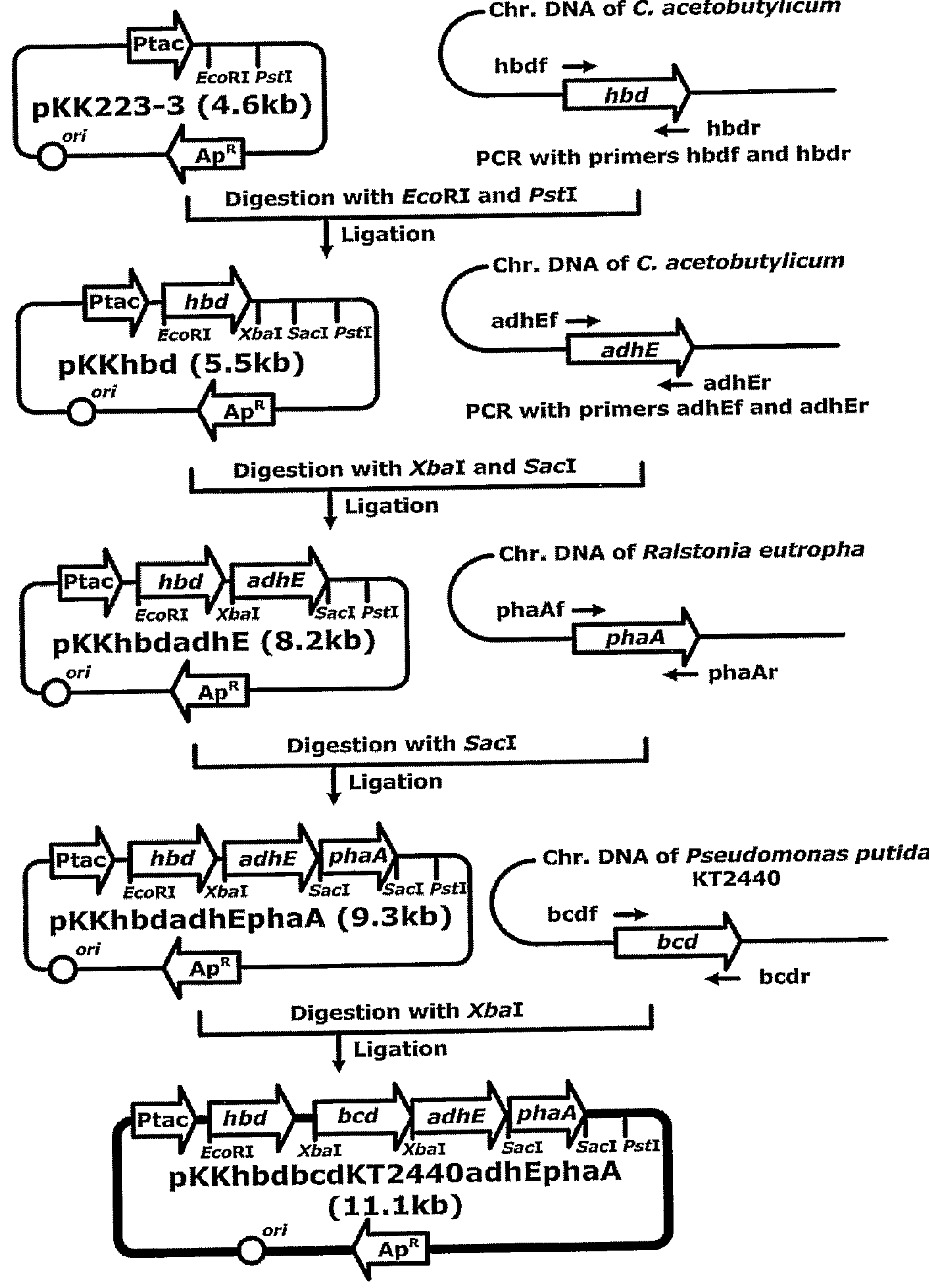


FIG. 8

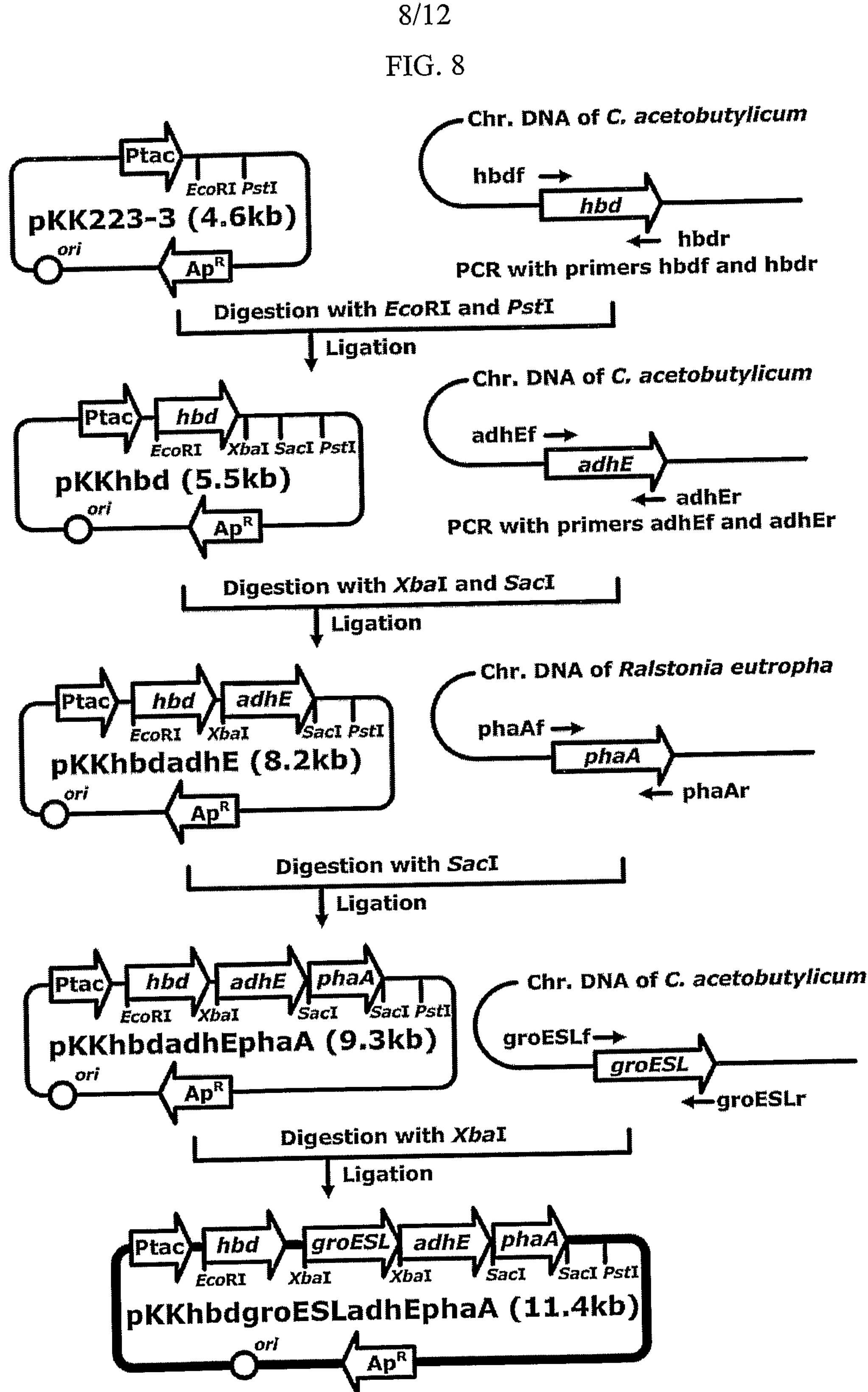


FIG. 9

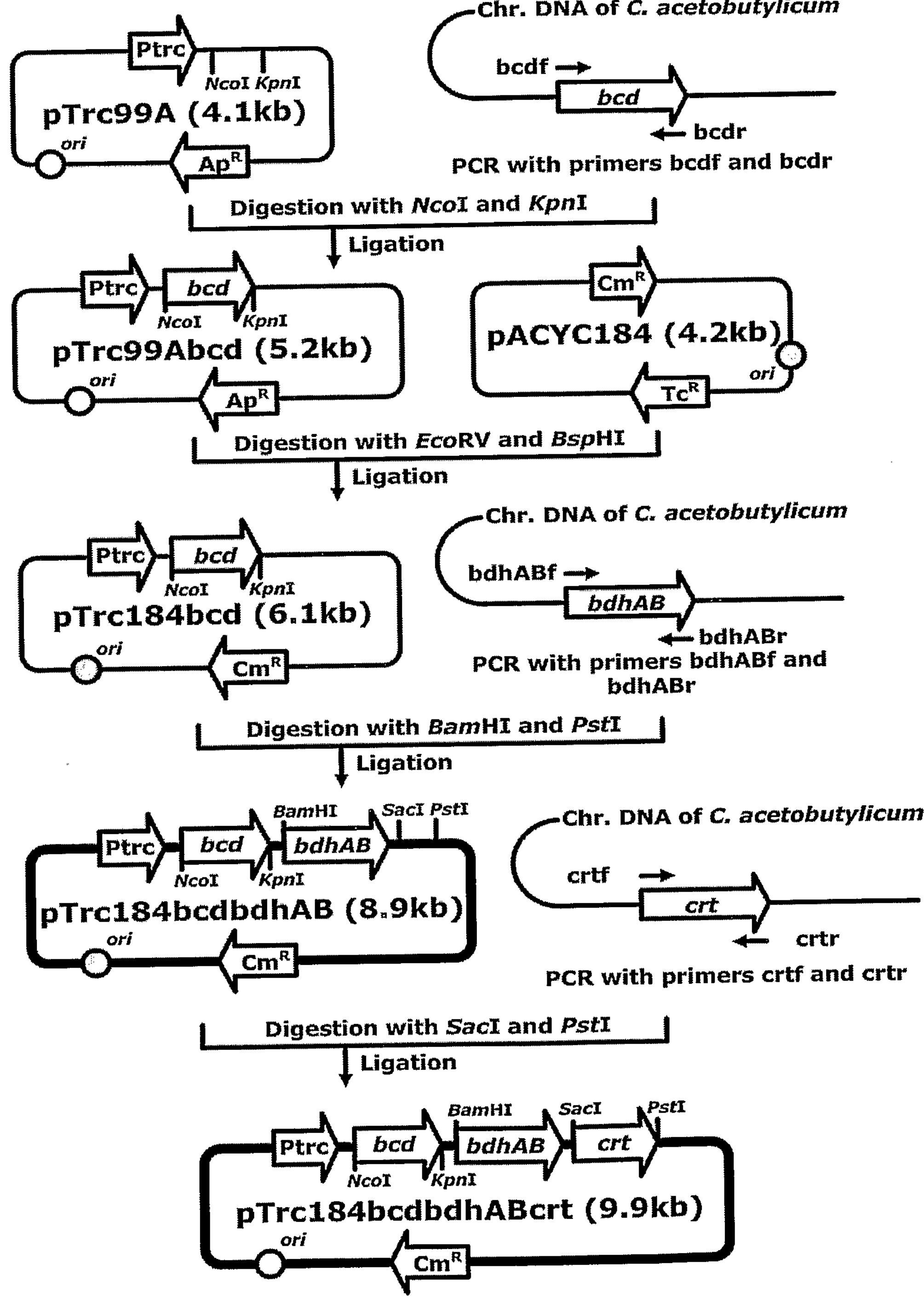
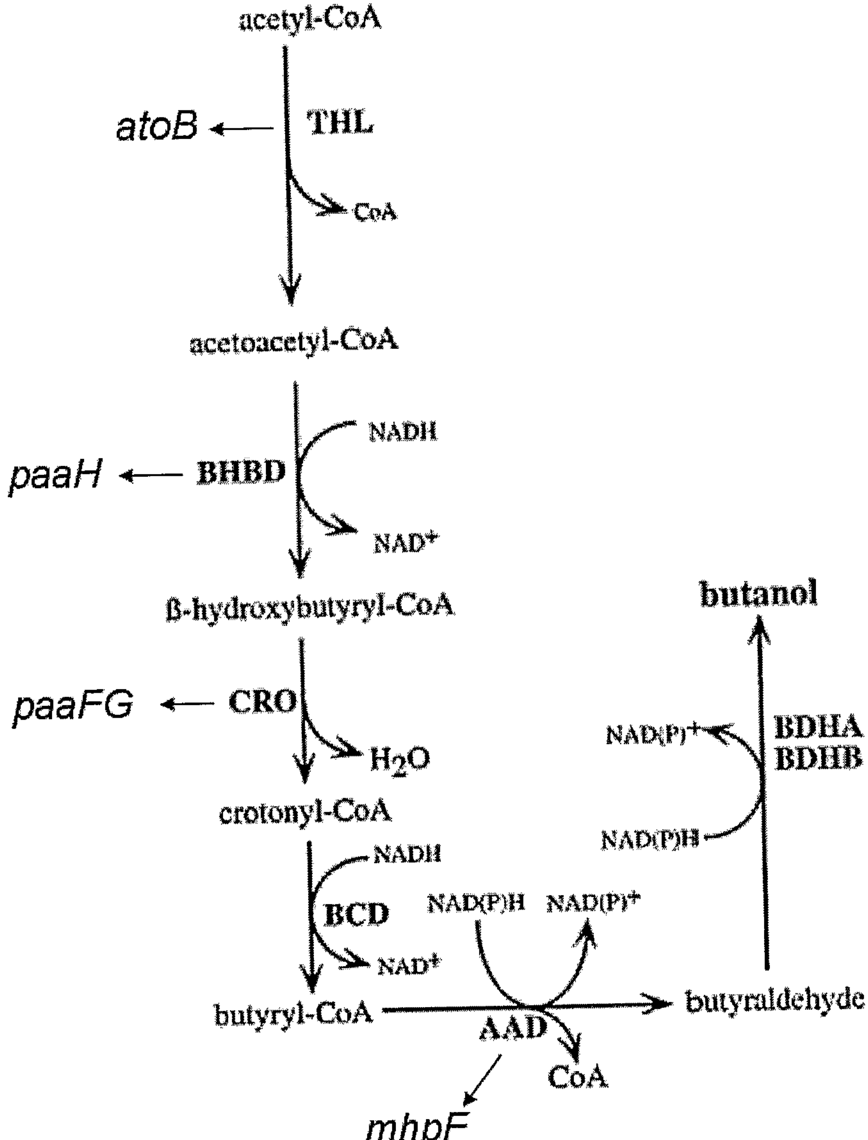


FIG. 10



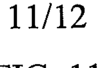


FIG. 11

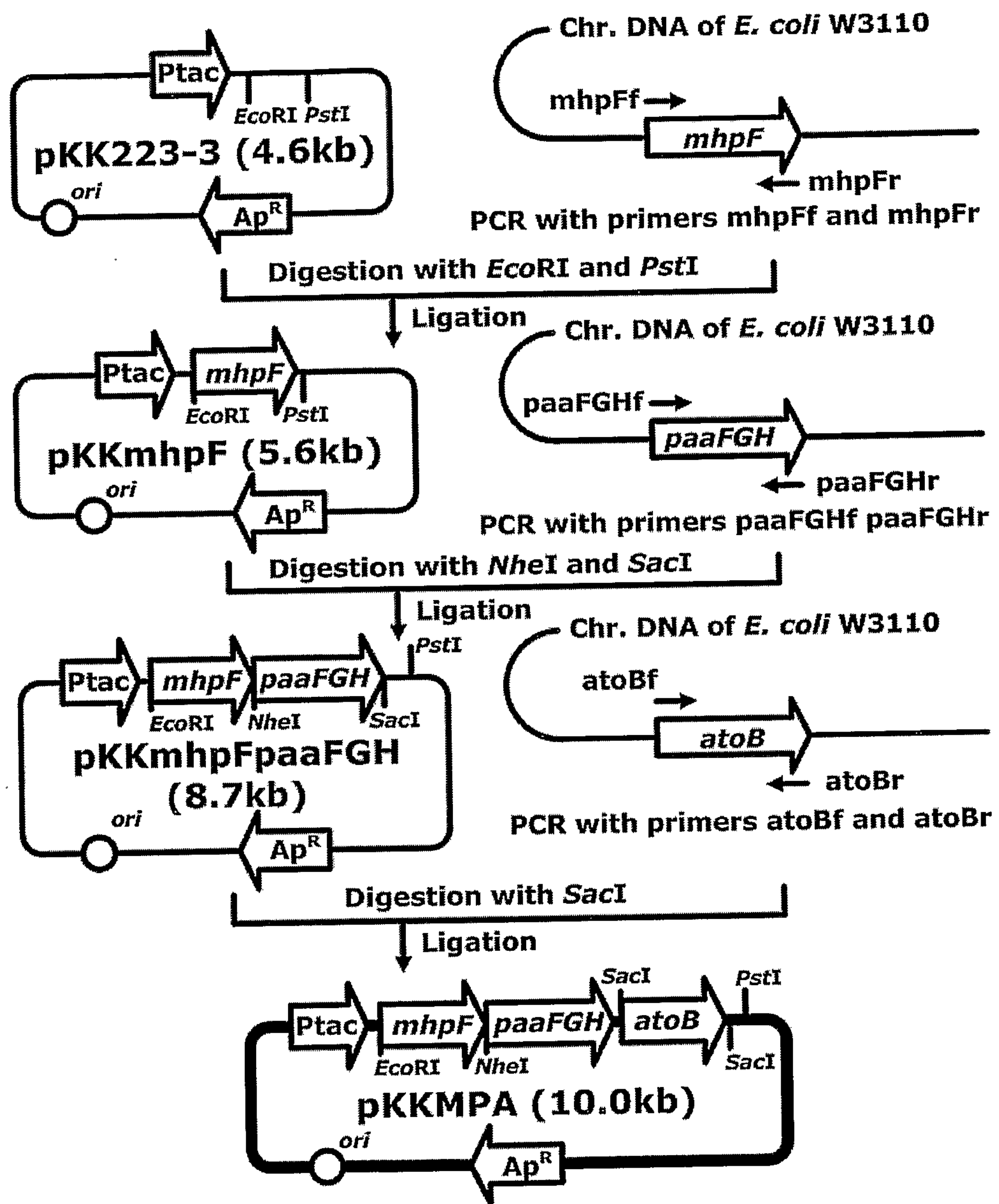
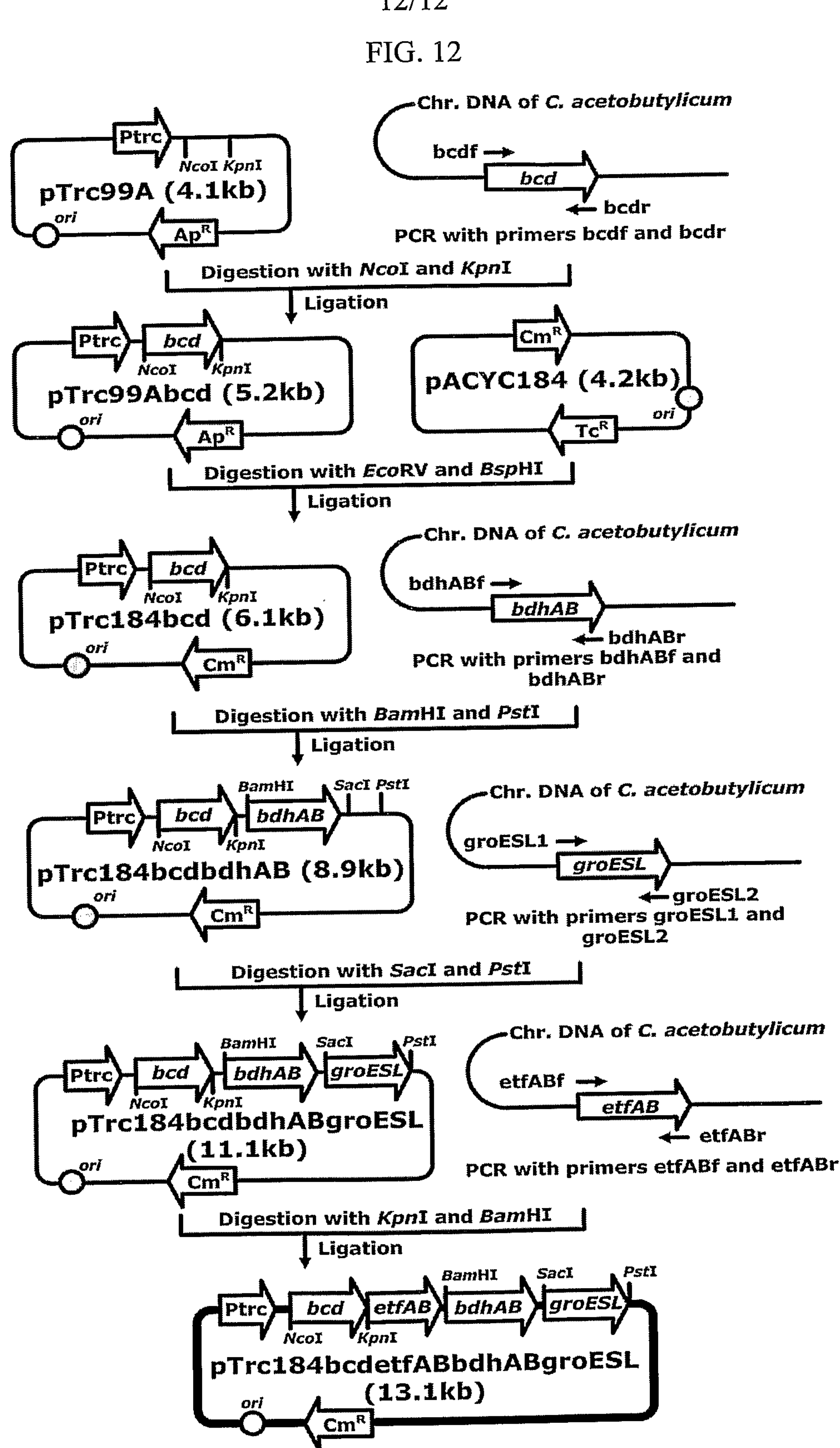


FIG. 12



INTERNATIONAL SEARCH REPORT

International application No. **PCT/KR2007/006525**

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/00(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 C12N 15/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Utility models and applications for Utility Models since 1975
Japanese Utility models and applications for Utility Models since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKIPASS(KIPO internal), PubMed, DELPHION, "butanol, product*, recombinant, and similar terms"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LISA FONTAINE et al. ' Molecular characterization and transcriptional analysis of adhE2, the gene encoding the NADH-dependent Aldehyde/Alcohol dehydrogenase responsible for butanol production in alcohologenic cultures of Clostridium acetobutylicum ATCC 824.' Journal of Bacteriology. 2002. vol.184(3), pp.821-830. See the whole document.	1-45
A	CHRISTOPHER A. TOMAS et al. Overexpression of groESL in Clostridium acetobutylicum results in increased solvent production and tolerance, prolonged metabolism, changes in the cell's transcriptional program.' Appl. Environ. Microbiol. 2003, vol.69(8), pp.4951-4965. See the whole document.	1-45
P,X	SHOTA ATSUMI et al. 'Metabolic engineering of Escherichia coli for 1-butanol production.' Metabolic Engineering. 2007. Sep. 13, vol. 10(1), pp.24-32. See pp.2, 4, 5.	1-45
T	MASAYUKI INUI et al. ' Expression of Clostridium acetobutylicum butanol synthetic genes in Escherichia coli. ' Applied Genetics and Molecular Biotechnology. 2008, vol.77, pp.1305-1316. See the whole document.	1-45

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 MARCH 2008 (14.03.2008)	**14 MARCH 2008 (14.03.2008)**

Name and mailing address of the ISA/KR

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Facsimile No. 82-42-472-7140

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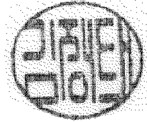
KIM Jung Tae

Telephone No. 82-42-481-5594



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2007/006525

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

   a. type of material
      - [x] a sequence listing
      - [ ] table(s) related to the sequence listing

   b. format of material
      - [ ] on paper
      - [x] in electronic form

   c. time of filing/furnishing
      - [x] contained in the international application as filed
      - [ ] filed together with the international application in electronic form
      - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: