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(73) Patenthaver: **CJ Cheiljedang Corporation, CJ Cheiljedang Center , 330 Dongho-ro , Jung-gu, Seoul 100-400, Sydkorea**

(72) Opfinder: **LEE, Kwang Ho, 809, Twin Vill, 28, Hwagok-ro 68-gil, Gangseo-gu, Seoul 157-840, Sydkorea**
PARK, Hye Min, 425, Hagye-ro, Jinyeong-eup, Gimhae-si, Gyeongsangnam-do 621-805, Sydkorea
LEE, Hyo Hyoung, 118-302 World Apt., 836, Geomdan-ro, Seo-gu, Incheon 404-775, Sydkorea
LEE, Seok Myung, 205-2202 Ssangyong Apt., 193, Dongnam-ro, Songpa-gu, Seoul 138-162, Sydkorea
HWANG, Young Bin, c/o CJ Cheiljedang Corporation, CJ Cheiljedang Center, 330, Dongho-ro, Jung-gu, Seoul, Sydkorea

(74) Fuldmægtig i Danmark: **Novagraaf Brevets, Bâtiment O2, 2 rue Sarah Bernhardt CS90017, F-92665 Asnières-sur-Seine cedex, Frankrig**

(54) Benævnelse: **MIKROORGANISMER FRA ESCHERICHIA COLI MED FORBEDRET PRODUKTION AF L-TRYPTOFAN OG FREMGANGSMÅDE TIL FREMSTILLING AF L-TRYPTOFAN DERMED**

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DESCRIPTION

Technical Field

[0001] The present invention relates to a microorganism of the genus *Escherichia* having enhanced L-tryptophan productivity, and a method of producing L-tryptophan using the same.

Background Art

[0002] L-tryptophan, an essential amino acid, has been widely used as a feed additive, a raw material for medical drugs such as infusion solutions, and a material for healthfoods, and has been produced by chemical synthesis, enzymatic reaction, fermentation, etc.

[0003] Recently, production of L-tryptophan is mainly carried out by microbial fermentation. In the initial stage of industrialization, analogue resistant strains obtained by chemical mutation have been mainly used. However, as gene recombination technologies rapidly developed in the 1990s and regulatory mechanisms were understood at the molecular level, the recombinant *E. coli* and *Corynebacterium* strains obtained by genetic engineering techniques have been mainly used.

[0004] The production of tryptophan by microorganisms starts with DAHP(3-deoxy-D-arobino-heptulosonate-7-phosphate) produced by the polymerization of PEP (PhospoEnolPyruvate) that is an intermediate of glycolysis, with E4P (erythrose-4-phosphate) that is an intermediate of the pentose phosphate pathway. Then, tryptophan is biosynthesized from chorismate through the common aromatic biosynthetic pathway. Specifically, tryptophan is synthesized by anthranilate synthase (EC 4.1.3.27) encoded by *trpE* gene, anthranilate synthase (EC 4.1.3.27) and anthranilate PRPP transferase (EC 2.4.1.28) encoded by *trpD* gene, indole-3-glycerol phosphate synthase (EC 4.1.1.48) and phosphoribosylanthranilate isomerase (EC 5.3.1.24) encoded by *trpC* gene, and tryptophan synthase (EC 4.2.1.20) encoded by *trpB* gene and *trpA* gene. The gene cluster *trpEDCBA* that mediates the above reaction is placed in the chromosome and have an operon structure containing a single regulatory region.

[0005] A tryptophan operon is actively transcribed so as to produce a sufficient amount of tryptophan required by the cell. However, if tryptophan level in the cell is high, a repressor binds to tryptophan and then the tryptophan operon is inactivated by the binding of the repressor to operon regulatory region, thereby the transcription is inhibited.

[0006] In addition, operons for biosynthesis of amino acids such as threonine, phenylalanine, leucine, tryptophan and histidine have another regulatory mechanism known as an attenuation(J Bacteriol. (1991) 173, 2328-2340) . As is known in the art with respect to the attenuation, under conditions deficient in amino acids, the structure of the mRNA corresponding specific sequence region between the promoter and the first gene of the operon on the chromosome, changes to a structure advantageous for the translation process to promote the expression of biosynthetic genes, but under conditions rich in the amino acids, the short transcribed mRNA forms a three-dimensional structure, named "hairpin structure, to inhibit the translation process (J Biol Chem., (1988) 263:609-612).

[0007] In the initial stage of the development of L-tryptophan-producing strains, it was a major object to increase the efficiency of production through the enhancement of enzyme activity either by releasing the feedback inhibition of tryptophan biosynthesis pathway enzymes caused by the final product, tryptophan or by increasing the copy number of the tryptophan operon genes on the chromosome or in the form of vector in order to enhance the expression of tryptophan biosynthetic enzymes (Appl. Environ. Microbiol., (1982) 43:289-297; Appl. Microbiol. Biotechnol., (1993) 40:301-305; Trends Biotechnol., (1996) 14:250-256).

[0008] Methods for imparting the ability to produce L-tryptophan to microorganisms include a method of imparting resistance to tryptophan analogues or anthranilate as the intermediate product by chemical mutation, or a method of modifying microorganisms by genetic engineering. Examples of the chemical mutation method include those described in Korean Patent Registration No. 1987-0001813, Korean Patent Registration No. 0949312 and the like, and examples of the modification method based on genetic engineering include various approaches which use a strain obtained by enhancing the transketolase-encoding tktA gene or the galactose permease-encoding *galP* gene in the aromatic amino acid biosynthesis pathway to increase the supply of E4P (erythrose4-phosphate) or PEP (phosphoenolpyruvate) and reducing the feedback inhibition of DAHP (3-deoxy-D-arabino-heptulosonate-7-phosphate) in order to enhance the aromatic biosynthetic pathway (Trends Biotechnol.,(1996)14:250-256, Microbial Cell Factories (2009) 8:19), or a strain obtained by additionally introducing tryptophan operon genes into the vector or chromosome (Appl. Environ. Microbiol.,(1982) 43:289-297, Appl. Microbiol. Biotechnol., (1993) 40:301-305).

[0009] However, even though the tryptophan operon was introduced with releasing the feedback inhibition of the biosynthetic enzymes, those approaches did not reached to an increase in the production yield of tryptophan, due to the regulatory mechanisms such as the inhibition or attenuation of the operon genes at transcription level.

[0010] US 4,371,614 discloses a bacterium which comprises a host of the genus Escherichia deficient in the enzyme tryptophanase carrying a plasmid with genetic information to control L-tryptophan production is useful for the fermentative production of L-tryptophan in high yields.

[0011] US 2009/239269 discloses a bacterium which belongs to the Enterobacteriaceae family and has an ability to produce an L-amino acid such as L-lysine, L-threonine and L-tryptophan and is modified to enhance glutamic acid decarboxylase activity. The bacterium is cultured in a medium to produce and accumulate L-amino acids in the medium or cells of the bacterium and the L-amino acid is then collected from the medium or the cells.

[0012] WO 2005/049808 discloses the production of L-threonine and L-isoleucine by culturing a bacterium which belongs to the genus Escherichia.

[0013] EP 0165614 discloses a hybrid plasmid comprising a tryptophan promoter and beta-galactosidase gene, e.g., pTREZI, that when contained in a host microorganism such as E. coli can produce beta-galactosidase in large amounts.

[0014] WO 84/00380 discloses an expression vector suitable for the production of fusion proteins. The vector includes a nucleotide sequence comprising in phase from the 5' end; a trp promoter-operator, a trpE ribosome binding site, a structural gene comprising a trpE gene, or a 5' portion thereof and a gene coding for a heterologous polypeptide wherein the nucleotide sequence does not include a trp attenuator. The structural gene may further include a 3' portion of a trpE gene downstream of the gene coding for the heterologous polypeptide.

Disclosure

Technical Problem

[0015] The present inventors have developed a method of releasing the inhibition or attenuation of tryptophan operon genes at transcription level in an L-tryptophan-producing strain, and a method capable of enhancing tryptophan biosynthetic enzymes using the same. In addition, in order to solve the problem in that the production yield of tryptophan-producing strains does not increase because of anthranilate accumulation as the tryptophan operon is enhanced, the present inventors have constructed a tryptophan-producing strain which has increased production yield and low level of anthranilate accumulation by expressing the gene cluster other than the gene encoding anthranilate synthase (TrpE) among the tryptophan operon genes as a form which is a desensitized regulatory mechanism such as the feedback inhibition or inhibition mechanism.

[0016] It is an object of the present invention to provide a microorganism of the genus *Escherichia* having enhanced L-tryptophan productivity by modifying so as to desensitize the inhibition or attenuation of the tryptophan operon and reduce the accumulation of anthranilate.

[0017] Another object of the present invention is to provide a method of producing L-tryptophan using the microorganism of the genus *Escherichia.*

Technical Solution

[0018] In order to accomplish the above objects, an embodiment of the present invention provides a recombinant microorganism of the genus *Escherichia* having an enhanced L-tryptophan productivity, wherein the recombinant microorganism has a modification to an expression regulatory region of the tryptophan operon, wherein the modification is a partial or full deletion of a leader peptide having a nucleotide sequence represented by SEQ ID NO: 2 in an expression regulatory region having a nucleotide sequence represented by SEQ ID NO: 1 of an endogenous tryptophan operon, wherein the recombinant microorganism is also modified to have increased chromosomal or intracellular copy number of the tryptophan operon genes trpD, trpC, trpB, and trpA, but not trpE, thereby enhancing the activity of the tryptophan biosynthetic gene cluster except for the anthranilate synthase that is encoded by the trpE gene as compared to the corresponding wild-type *Escherichia* microorganism.

[0019] Another embodiment of the present invention also provides a method for producing L-tryptophan, comprising culturing the above-described recombinant microorganism of the genus *Escherichia* under conditions suitable for the production of L-tryptophan.

Advantageous Effects

[0020] The recombinant microorganism produced according to the present invention eliminates the excessive accumulation of anthranilate therein and can be advantageously used to produce L-tryptophan in high yield.

Description of Drawings

[0021] FIG. 1 shows a schematic view representing the tryptophan operon genes , a regulatory region for the genes in the *E. coli* chromosome, and the deletion form of the gene described in the present invention.

1. A) Tryptophan operon genes, and a regulatory region thereof in the *E. coli* chromosome(Ptrp);

2. B) A Ptrp form;
3. C) A form that the *trpL* gene encoding leader peptide is deleted (DtrpL); and
4. D) A form that the *trpL* gene encoding the leader peptide and the attenuator are deleted (Dtrp_att).

[0022] FIG. 2 shows a pCL-GFP vector used to measure the intensity of an expression regulatory region of the tryptophan operon.

[0023] FIG. 3 shows the vector pINT17E-Patt-trpDCBA for introducing the tryptophan biosynthetic genes *trpDCBA* into the chromosome to increase the copy number of the genes.

Mode for Invention

[0024] Hereinafter, the present invention will be described in detail.

[0025] An embodiment of the present invention provides a recombinant microorganism of the genus *Escherichia* having enhanced L-tryptophan productivity, wherein the recombinant microorganism has a modification to an expression regulatory region of the tryptophan operon, wherein the modification is a partial or full deletion of a leader peptide having a nucleotide sequence represented by SEQ ID NO: 2 in an expression regulatory region having a nucleotide sequence represented by SEQ ID NO: 1 of an endogenous tryptophan operon, wherein the recombinant microorganism is also modified to have increased chromosomal or intracellular copy number of the tryptophan operon genes trpD, trpC, trpB, and trpA, but not trpE, thereby enhancing the activity of the tryptophan biosynthetic gene cluster except for the anthranilate synthase that is encoded by the trpE gene as compared to the corresponding wild-type *Escherichia* microorganism.

[0026] In a further embodiment the recombinant microorganism described herein has an additional modification to the expression regulatory region of the tryptophan operon, wherein the modification is a partial or full deletion of an endogenous attenuator having a nucleotide sequence represented by SEQ ID NO: 3 in the expression regulatory region having a nucleotide sequence represented by SEQ ID NO: 1.

[0027] In certain embodiments of the recombinant microorganism described herein the trpD gene encodes a polypeptide comprising the amino acid sequence represented by SEQ ID NO: 37, the trpC gene encodes a polypeptide comprising the amino acid sequence represented by SEQ ID NO: 38, the trpB gene encodes a polypeptide comprising the amino acid sequence represented by SEQ ID NO: 39, and the trpA gene encodes a polypeptide comprising the amino acid sequence represented by SEQ ID NO: 40.

[0028] As used herein, the term "tryptophan operon" or "Trp operon" means the entire operon including all the *trpEDCBA* genes. The tryptophan operon has a nucleotide sequence represented by SEQ ID NO: 9.

[0029] As used herein, the term "tryptophan biosynthetic gene cluster" means a gene cluster consisting of a combination of two or more of *trpD, trpC, trpB* and *trpA* that are tryptophan operon genes. Specifically, the tryptophan biosynthetic gene cluster may be a *trpDCBA* gene cluster having a nucleotide sequence represented by SEQ ID NO: 10. Herein, the *trpD* gene encodes a protein having an amino acid sequence represented by SEQ ID NO: 37; the *trpC* gene encodes a protein having an amino acid sequence represented by SEQ ID NO: 38; the *trpB* gene encodes a protein having an amino acid sequence represented by SEQ ID NO: 39; and the *trpA* gene encodes a protein having an amino acid sequence represented by SEQ ID NO: 40.

[0030] Enhancing the activity of the tryptophan biosynthetic gene cluster except for anthranilate synthase that is encoded by the *trpE* gene of the tryptophan operon is performed to solve the problem which the production yield of tryptophan does not increase due to accumulation of anthranilate as the tryptophan operon is enhanced.

[0031] An L-tryptophan-producing microorganisms may be any prokaryotic or eukaryotic microorganism as long as they have a L-tryptophan productivity. Examples of this microorganism may include microorganisms belonging to the genus *Escherichia,* the genus *Erwinia,* the genus *Serratia,* the genus *Providencia,* the genus *Corynebacterium* and the genus *Brevibacterium.* The microorganism of use in the invention is a microorganism belonging to the genus *Escherichia,* and more specifically *E. coli.* Most specifically, the *E. coli* strain of the present invention may be a strain which maintains 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (aroG) which is released for feedback inhibition, or which has enhanced activities of aromatic biosynthesis pathway enzymes such as 3-dehydroquinate synthetase(AroB), shikimate dehydrogenase (AroE), shikimate kinase (AroL), 5-enolpyruvylshikimate-3-phosphate synthase (AroA) or chorismate synthase (AroC), or enhanced activity of phosphoglycerate dehydrogenase (SerA) or transketolase (TktA) to enhance the supply of the intermediates, serine and PRPP, of the tryptophan biosynthesis pathway. Furthermore, the *E. coli* strain of the present invention may be a strain having inactivated prephenate dehydratase and chorismate mutase (PheA) in the aromatic biosynthesis pathway or inactivated prephenate dehydrogenase, chorismate mutase (tyrA), tryptophanase (tnaA) and tryptophan transporter (tnaB, mtr).

[0032] More specifically, the recombinant microorganism of the present invention is recombinant *E. coli* strain CA04-2004 (accession number: KCCM11246P).

[0033] As used herein, the term "expression regulatory region" of the endogenous tryptophan operon means a region including a promoter, a leader peptide and an endogenous attenuator. In accordance with the invention, the expression regulatory region has a nucleotide sequence represented by SEQ ID NO: 1.

[0034] As used herein, the term "leader peptide" means a low-molecular-weight peptide that is encoded by the upstream leader sequence of the start codon of the gene. In accordance with the invention, the leader peptide has a nucleotide sequence represented by SEQ ID NO: 2, and a polypeptide that is expressed by the leader peptide may be an amino acid sequence represented by SEQ ID NO: 4. This leader peptide functions to form the hairpin structure when the concentration of tryptophan is high, thereby promoting the structure-formation of the endogenous attenuator to terminate the transcription of tryptophan biosynthetic genes.

[0035] As used herein, the term "delete" means removing part or all of either a nucleotide sequence from the start codon to the stop codon of the target gene, or the nucleotide sequence of a regulatory region thereof, from the chromosome.

[0036] As used herein, the term "endogenous attenuator" means a region having a nucleotide sequence represented by SEQ ID NO: 3, which excludes the promoter and the leader peptide in the expression regulatory region that causes the attenuation mechanism.

[0037] Methods for enhancing the expression of genes include: 1) a method of increasing the chromosomal or intracellular copy number of the genes; or 2) a method of replacing the chromosomal promoter of the genes with a strong exogenous promoter or modifying the chromosomal promoter to a strong promoter.

[0038] Examples of the method of increasing the copy number include a method of introducing the gene into a vector to enhance the expression of the gene. Examples of a vector that may be used in the present invention include plasmid vectors such as pBR, pUC, pBluescriptII, pGEM, pTZ, pCL and pET-type plasmids. Vectors that may be used in the present invention are not particularly limited to, and any known expression vectors may be used. Specifically, pACYC177, pACYC184, pCL, pECCG117, pUC19, pBR322 or pMW118 vectors may be used. Most preferably, pACYC177, pCL and pCC1BAC vectors may be used.

[0039] Meanwhile, examples of strong exogenous promoters include, but are not limited to, known promoters such as trc, lac and tac promoters. In addition, modifying the chromosomal promoter to a strong promoter can be performed by deleting part or all of the leader peptide and/or further deleting part or all of the endogenous attenuator as described above, but is not limited thereto.

[0040] In a specific embodiment of the present invention, a recombinant L-tryptophan-producing microorganism of the genus *Escherichia* was produced by deleting part or all of the leader peptide having a nucleotide sequence represented by SEQ ID NO: 2 in the expression regulatory region having a nucleotide sequence represented by SEQ ID NO: 1 on the endogenous tryptophan operon, and deleting part or all of the endogenous attenuator having a nucleotide sequence represented by SEQ ID NO: 3 so as to increase the ability to produce L-tryptophan. In addition, the recombinant L-tryptophan-producing microorganism was produced by further enhancing the activities of the proteins having an amino acid sequence represented by SEQ ID NOS: 37, 38, 39 and 40, which are encoded by the tryptophan biosynthetic gene cluster trpDCBA excluding the trpE gene encoding anthranilate synthase by increasing the chromosomal or intracellular copy number of the tryptophan operon genes trpD, trpC, trpB, and trpA, but not trpE. The produced recombinant microorganism was deposited as the accession number KCCM11246P.

[0041] Another embodiment of the present invention also provides a method for producing L-tryptophan, comprising culturing the recombinant L-tryptophan-producing microorganism of the genus *Escherichia* having enhanced L-tryptophan productivity described herein.

[0042] In a specific aspect, the present invention provides a method comprising culturing a recombinant L-tryptophan-producing microorganism of the genus *Escherichia* having enhanced L-tryptophan productivity, which has been modified to delete part or all of the leader peptide having a nucleotide sequence represented by SEQ ID NO: 2 in the expression regulatory region having a nucleotide sequence represented by SEQ ID NO: 1 on an endogenous tryptophan operon and to delete part or all of the endogenous attenuator having a nucleotide sequence represented by SEQ ID NO: 3 so as to increase the ability to produce L-tryptophan, and further to enhance the activities of the proteins which is encoded by the tryptophan operon by enhancing the expression of tryptophan biosynthetic gene cluster trpDCBA excluding the trpE gene encoding anthranilate synthase by increasing the chromosomal or intracellular copy number of the tryptophan operon genes trpD, trpC, trpB, and trpA, but not trpE.

[0043] The media and culture conditions that are used in culture of the microorganism of the present invention may be any of those that are used in culture of microorganisms belonging to the genus Escherichia, but these should suitably satisfy the requirements of the microorganism of the present invention. Specifically, the microorganism of the present invention may be cultured in a conventional medium containing suitable carbon sources, nitrogen sources, amino acids, vitamins and the like under aerobic conditions while adjusting temperature, pH and the like.

[0044] Carbon sources that may be used in the present invention include carbohydrates such as glucose, fructose, sucrose, maltose, mannitol, sorbitol; alcohols such as sugar alcohol, glycerol, pyruvic acid, lactic acid and citric acid; and amino acids such as organic acid, glutamic acid, methionine and lysine. In addition, natural organic nutrient sources such as starch hydrolysates, molasses, blackstrap molasses, rice bran, cassava, bagasse and corn steep liquor may be used. Specifically, carbohydrates such as glucose and sterile pretreated molasses (i.e., molasses converted to reduced sugars) may be used. In addition, suitable amounts of other carbon sources may be used without limitation.

[0045] Nitrogen sources that may be used in the present invention include inorganic nitrogen sources such as ammonia, ammonium sulfate, ammonium chloride, ammonium acetate, ammonium carbonate, and ammonium nitrate; amino acids such as glutamic acid, methionine and glutamine; and organic nitrogen sources such as peptone, NZ-amine, meat extract, yeast extract, malt extract, corn steep liquor, casein hydrolysate, fish meal or its digested product, defatted soybean cake or its digested product, etc. These nitrogen sources may be used alone or in combination.

[0046] The medium may contain, as phosphorus sources, potassium phosphate monobasic, potassium phosphate dibasic and corresponding sodium-containing salts. Inorganic compounds that may be used in the present invention include sodium chloride, calcium chloride, iron chloride, magnesium sulfate, iron sulfate, manganese sulfate and calcium carbonate. In addition, the medium may contain amino acids, vitamins and suitable precursors. These sources or precursors may be added to the medium in a batch or continuous manner.

[0047] Compounds such as ammonium hydroxide, potassium hydroxide, ammonia, phosphoric acid and sulfuric acid may be added to the medium in a suitable manner during culture to adjust the pH of the culture medium. In addition, during the culture, a antifoaming agent such as fatty acid polyglycol ester may be used to suppress the formation of bubbles. Further, in order to maintain the culture medium in an aerobic state, oxygen or oxygen-containing gas may be injected into the culture medium. In addition, in order to maintain the culture medium in an anaerobic or non-aerobic state, no gas is injected, or nitrogen, hydrogen or carbon dioxide gas may be injected into the culture medium.

[0048] The culture medium is typically maintained at a temperature ranging from 27 °C to 37 °C, and more specifically from 30 °C to 35 °C. Culture of the microorganism may be continued until the desired level of the useful substance will be obtained. Specifically, the culture period may be 10-100 hours.

[0049] The method of the present invention may further comprise purifying or recovering the L-amino acid produced in the culture step. The purification or recovery process may be performed by purifying or recovering the desired L-amino acid from the culture medium using a suitable method, for example, a batch, continuous or fed-batch culture method.

[0050] Hereinafter, the present invention will be described in further detail with reference to examples. It is to be understood, however, that these examples are for illustrative purposes and are not intended to limit the scope of the present invention as defined in the claims.

Examples

Example 1: Construction of a fusion vector comprising GFP and an expression regulatory region from which a leader peptide was removed, in order to release an expression regulation for tryptophan biosynthesis

[0051] As shown in FIG. 1, in order to amplify an expression regulatory region comprising a deletion of the *trpL* gene encoding a leader peptide (L) (the expression regulatory region is hereinafter referred to as "DtrpL", corresponding to FIG. 1C) in an expression regulatory region of the tryptophan operon which is composed of a promoter (P), the leader peptide (L) and an attenuator (A), polymerase chain reaction (hereinafter referred to as "PCR") was performed using the chromosomal DNA of an *E. coli* W3110 strain (purchased from the American Type Culture Collection (ATCC); GenBank accession number AC000091) as a template.

[0052] Specifically, a 155bp fragment having a KpnI restriction enzyme site in the 5' region was amplified by PCR with Pfu polymerase using primers 1 and 2 under the following conditions: 30 cycles, each consisting of denaturation at 94 °C for 1 min, annealing at 58 °C for 30 sec, and extension at 72 °C for 30 sec. Meanwhile, a 105bp fragment having an *EcoRV* restriction enzyme site in the 3' region was amplified by PCR using primers 3 and 4 under the above-described conditions. The obtained DNA fragments were recovered using GeneAll^{R} Expin™ GEL SV kit (Seoul, Korea), and then used as a template for crossover PCR.

[0053] In order to make the DtrpL, crossover PCR was performed using the two DNA fragments as a template and primers 1 and 4. Specifically, a 245bp fragment (SEQ ID NO: 5) was amplified by PCR under the above-described conditions. The amplified fragment was treated with the restriction enzymes *Kpn*I and *EcoR*V, and then ligated with pCL1920GFP (SEQ ID NO: 8) which was treated with the same restriction enzymes, thereby constructing pCL-DtrpL GFP.

[0054] In order to amplify an expression regulatory region comprising a deletion of the genes encoding the leader peptide (L) and the attenuator (A) (the expression regulatory region is hereinafter referred to as "Dtrp_att") in the expression regulatory region of the tryptophan operon, a 148bp fragment (SEQ ID NO: 6) having a *Kpn*I restriction enzyme site in the 5' region and an *EcoRV* restriction enzyme site in the 3' region was amplified by PCR using the chromosomal DNA of the *E. coli* W3110 strain as a template and primers 1 and 5. The amplified fragment was treated with the restriction enzymes *Kpn*I and *EcoRV,* and then ligated with pCL1920GFP which was treated with the same restriction enzymes, thereby constructing pCL-Dtrp_att-GFP.

[0055] In addition, in order to make a vector having a wild-type expression regulatory region for use as a control in subsequent experiments, a 290bp fragment having a *Kpn*I restriction enzyme site in the 5' region and an *EcoRV* restriction enzyme site in the 3' region was amplified by PCR using the chromosomal DNA of the *E. coli* W3110 strain as a template and primers 1 and 4. The amplified fragment was treated with the restriction enzymes *Kpn*I and *EcoRV,* and then ligated with pCL1920GFP which was treated with the same restriction enzymes, thereby constructing pCL-Ptrp-GFP.

Primer 1:
5' TTAGGTACCGGCGCACTCCCGTTCTGGATA 3' (SEQ ID NO: 11);

Primer 2:
5' ACTGCCCGTTGTCGATACCCTTTTTACGT 3' (SEQ ID NO: 12);

Primer 3:
5' TCGACAACGGGCAGTGTATTCACCATG 3' (SEQ ID NO: 13);

Primer 4:
5' AATGATATCTGTTATTCTCTAATTTTGTT 3' (SEQ ID NO: 14);

Primer 5:
5' AATGATATCACCCTTTTTACGTGAACTTG 3' (SEQ ID NO: 15).

Example 2: Measurement of expression level of GFP

[0056] Each of the pCL-DtrpL_GFP, pCL-Dtrp_att-GFP and pCL-Ptrp_GFP vectors prepared in Example 1 was transformed into wild-type *E. coli* W3110 and the tryptophan-producing strain *E. coli* KCCM10812P, and then the intensities of GFP in the strains were measured.

[0057] The parent strain *E. coli* KCCM10812P (Korean Patent Registration No. 10-0792095) used in this Example is a strain derived from an *E. coli variant* having L-phenylalanine productivity (KFCC 10066, Korean Patent Publication No. 1985-0001232). Specifically, KCCM10812P is a recombinant *E. coli* strain having L-tryptophan productivity, wherein the strain has been modified to recover tryptophan auxotrophy, to inactivate the *pheA, trpR, mtr* and *tnaAB* genes, and to mutate the *aroG* and *trpE* genes .

[0058] Specifically, each of the strains was inoculated into 25 ml of M9 medium (containing 0.5% glucose + 2 g/L yeast extract and further containing 0.1g/L tyrosine and 0.1g/L phenylalanine in the case of KCCM10812) in a 250 ml flask at a volume ratio of 1/100 (v/v) and cultured at 37 °C until a predetermined OD was reached. The cultured strains were recovered by centrifugation and washed once with 1xTE, and GFP therein was measured using Synergy HT Multi-Mode Microplate Reader (Biotek, USA).

[0059] The results of the measurement are shown in Table 1 below. OD1 and OD3 in Table 1 indicate the OD values measured at 600 nm using UV mini-1240 spectrophotometer (Shimadzu) after diluting each of the culture products to a suitable concentration.

[0060] As shown in FIG. 1, in the case of the wild-type W3110 strain, with respect to that of Ptrp as 1, the relative intensity of Dtrp_att (comprising a deletion of the leader peptide and the attenuator) was about 7 fold at an OD value of 1 (OD1) and 10 fold at an OD value of 3 (OD3), and the relative intensity of DtrpL comprising a deletion of only the leader peptide was about 1.5-2 fold higher than that of the wild-type regulatory region (Ptrp). In comparison with this, in the case of the L-tryptophan-producing strain KCCM 10812P, with respect to that of Ptrp taken as 1, the relative intensity of Dtrp_att (comprising a deletion of the leader peptide and the attenuator) was about 19 fold at an OD value of 1 (OD1) and 27 fold at an OD value of 3 (OD3), and the relative intensity of DtrpL comprising a deletion of only the leader peptide was about 4 fold higher than that of the wild-type regulatory region (Ptrp). Such results indicate that the deletion of the leader peptide or the attenuator leads to an increase in expression, even though this increase in expression in the wild-type strain is weaker than in the L-tryptophan producing strain.

Table 1

Strain	Promoter	GFP measurement (fold)	
		OD1	OD3
W3110	Dtrp_att	6.5±0.7	9.6±1.1
	DtrpL	1.5±0.2	2.4±0.3
	Ptrp	1	1
KCCM10812P	Dtrp_att	18.9±1.3	27±2.0
	DtrpL	3.8±0.5	3.9±0.8
	Ptrp	1	1

<u>Example 3: Construction of vectors having tryptophan operon (trpEDCBA) whose expression regulatory region was replaced</u>

[0061] Based on the results of Example 2, in order to construct an *E. coli* strain whose tryptophan operon genes were enhanced using a vector, a 6564bp fragment (SEQ ID NO: 9) was amplified using the chromosomal DNA of the parent strain *E. coli* KCCM10812P as a template and primers 6 and 7 under the above-described PCR conditions.

[0062] The amplified DNA fragment was recovered using GeneAll^R Expin™ GEL SV kit (Seoul, Korea), and then treated with the restriction enzymes EcoRV and *Hind*III. For cloning with the prepared DNA fragment, each of the pCL-Dtrp_att-GFP, pCL-DtrpL_GFP and pCL-Ptrp_GFP vector was treated with EcoRV and *Hind*III to remove the GFP region, thereby obtaining 4291bp fragments. Each of the prepared vectors was ligated with the insert, and then introduced into *E. coli* DH5a by transformation, thereby constructing pCL-Dtrp att-trpEDCBA, pCL-DtrpL_trpEDCBA and pCL-Ptrp_trpEDCBA vectors.

Primer 6:
5' CCCGATATCATGCAAACACAAAAACCGAC 3' (SEQ ID NO: 16);

Primer 7:
5' GGGAAGCTTAAAGGATCCGTGGGATTAACTGCGCGTCGCCGCTTT 3' (SEQ ID NO: 17) .

Example 4: Construction of vectors whose expression regulatory region was replaced and which had tryptophan biosynthetic gene cluster (trpDCBA) excluding trpE

[0063] In order to construct vectors by replacing the GFP region of the pCL-Dtrp_att-GFP, pCL-DtrpL_GFP and pCL-Ptrp_GFP vectors prepared in Example 1 with trpDCBA, each of the pCL Dtrp_att-GFP, pCL-DtrpL_GFP and pCL-Ptrp GFP vectors was treated with EcoRV and *Hind*III to remove the GFP region, thereby obtaining 4291bp fragments.

[0064] Then, in order to construct *E. coli* strains whose the trpDCBA genes of the tryptophan operon were enhanced using a vector, a 5002-bp fragment (SEQ ID NO: 10) was amplified by PCR using the chromosomal DNA of the parent strain *E. coli* KCCM10812P as a template and primers 7 and 8.

[0065] The amplified DNA fragment was recovered using GeneAll^R Expin™ GEL SV kit (Seoul, Korea), and then treated with the restriction enzymes EcoRV and *HindIII*. The prepared vector and insert were ligated with each other, and then introduced into *E. coli* DH5a by transformation, thereby constructing pCL-Dtrp att-trpDCBA, pCL-DtrpL_trpDCBA and pCL-Ptrp_trpDCBA vectors.
Primer 8:
5' AAAGATATCATGGCTGACATTCTGCTGCT 3' (SEQ ID NO: 18).

Example 5: Construction of vectors having low copy number of tryptophan operon genes having various expression regulatory regions

[0066] A typical vector that is expressed with low copy number in *E. coli* is pCC1BAC (Epicentre, USA). In order to express the tryptophan operon genes with low copy number using this vector, the pCL-Dtrp_att-trpEDCBA, pCL-DtrpL_trpEDCBA, pCL-Ptrp_trpEDCBA, pCL-Dtrp_att-trpDCBA, pCL-DtrpL_trpDCBA and pCL-Ptrp_trpDCBA prepared in Examples 3 and 4 were digested with the restriction enzyme *HindIII*.

[0067] The resulting DNA fragments were electrophoresed on agarose, and then cut according to their size and recovered using GeneAll^R Expin™ GEL SV kit (Seoul, Korea) . Next, each of the fragments was ligated with the pCC1BAC vector (digested at the *Hind*III site), and then introduced into *E. coli* DH5a by transformation.

[0068] Each of the transformed strains was smeared on LB Cm solid medium (LB + chloramphenicol agar plate), and strains having Cm resistance were selected, thereby constructing pBAC-Dtrp_att-trpEDCBA, pBAC-DtrpL_trpEDCBA, pBAC-Ptrp_trpEDCBA, pBAC-Dtrp_att-trpDCBA, pBAC-DtrpL_trpDCBA and pBAC-Ptrp_trpDCBA vectors.

Example 6: Construction of *E. coli* strain in which pheA gene was inactivated

[0069] In order to construct a strain close to a tryptophan-producing strain from the wild type *E. coli* W3110 strain, the *pheA* gene (NCBI gene ID: 12934467) encoding chorismate mutase/prephenate dehydratase (CM-PDT) was inactivated by deletion through homologous recombination. CM-PDT is an enzyme in the first step of producing phenylalanine from chorismate, and deletion of the *pheA* gene was used to inhibit the phenylalanine biosynthesis pathway. For this deletion, the one-step inactivation method (developed by Datsenko KA et al.), mutagenesis technique using lambda red recombinase, was used (One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products, Datsenko KA, Wanner BL., Proc Natl Acad Sci U S A. 2000 Jun 6;97(12):6640-5). As a marker for confirming insertion into the genes, the chloramphenicol-resistant gene of pUCprmfmloxC was used (Korean Patent Laid-Open Publication No. 2009-0075549).

[0070] An about 1200bp gene fragment was amplified by PCR using the vector pUCprmfmloxP as a template and primers 9 and 10, which have a portion of the *pheA* gene and a portion of the nucleotide sequence of the chloramphenicol-resistant gene of the pUCprmfmloxP vector.

Primer 9:
5'-GGCCTCCCAAATCGGGGGGCCTTTTTTATTGATAACAAAAAGGCAACACTAGGTGACACTATAGAACGCG - 3' (SEQ ID NO: 19);

Primer 10:
5'-AACAGCCCAATACCTTCATTGAACGGGTGATTTCCCCTAACTCTTTCAATTAGTGGATCTGATGGGTACC -3' (SEQ ID NO: 20).

[0071] The DNA fragment obtained by PCR amplification was electrophoresed on 0.8% agarose gel, and then eluted and used as a template in secondary PCR. Secondary PCR was performed so that the 5' and 3' regions of the primary DNA fragment had 20 pairs of complementary nucleotide bases. In addition, an about 1300bp gene fragment was amplified by PCR using the eluted primary PCR product as a template and primers 11 and 12, which include the 5' and 3' regions of the *pheA* gene. The resulting DNA fragment was electrophoresed on 0.8% agarose gel, and then eluted and used in recombination.

Primer 11:
5'-GAATGGGAGGCGTTTCGTCGTGTGAAACAGAATGCGAAGACGAACAATAAGGCCTCCCAAATCGGGGGGC -3' (SEQ ID NO: 21);

Primer 12:
5-GGCACCTTTTCATCAGGTTGGATCAACAGGCACTACGTTCTCACTTGGGTAACAGCCCAATACCTTCATT -3' (SEQ ID NO: 22).

[0072] According to the method developed by Datsenko KA et al., the W3110 *E. coli* strain transformed with the pKD46 vector was made as competent status, and then transformed with the 1300bp gene fragment obtained by PCR. The strain having resistance to chloramphenicol was selected on LB medium. PCR was performed using primers 13 and 14, and the PCR amplification product had a size of about 2500 bp, indicating that the *pheA* gene was deleted in the strain.

Primer 13:
5'- TTGAGTGTATCGCCAACGCG -3' (SEQ ID NO: 23);

Primer 14:
5'- AAAGCCGCGTGTTATTGCGT -3' (SEQ ID NO: 24).

[0073] The pKD46 vector was removed from the primary recombinant strain having chloramphenicol resistance, and then a pJW168 vector was introduced into the strain, and the chloramphenicol marker gene was removed from the strain (Gene, (2000) 247,255-264). The resulting strain was an about 500bp amplification product obtained by PCR using primers 13 and 14, indicating that the target gene deletion was achieved. The constructed strain was named "*E. coli* W3110 trpΔ1".

Example 7: Construction of *E. coli* strain from which tnaAB gene was inactivated

[0074] From the *E. coli* W3110 trpΔ1 strain constructed in Example 6, the *tnaAB* operon(NCBI gene ID: 12933600, 12933602) consisting of the *tnaA* gene encoding tryptophanase and the *tnaB* gene encoding tryptophan importer was deleted by homologous recombination. Due to this deletion, the degradation pathway of tryptophan after its production can be blocked, and the influx of tryptophan which is secreted to the medium into the cells can be prevented, thereby imparting the properties of tryptophan-producing strains. For this deletion, an about 1200bp gene fragment was amplified by PCR in the same manner as described in Example 6 using the vector pUCprmfmloxP as a template together with primers 15 and 16, which have a portion of the tnaAB gene and a portion of the nucleotide sequence of the chloramphenicol-resistant gene of the pUCprmfmloxP vector. In addition, the DNA fragment obtained by PCR amplification was further amplified by PCR in the same manner as described in Example 6 using primers 17 and 18, thereby obtaining a 1300bp gene fragment.

Primer 15:
5'-TTAGCCAAATTTAGGTAACACGTTAAAGACGTTGCCGAACCAGCACAAAAAGGTGACACTATAGAACGCG - 3' (SEQ ID NO: 25);

Primer 16:
5'-ATGAAGGATTATGTAATGGAAAACTTTAAACATCTCCCTGAACCGTTCCGTAGTGGATCTGATGGGTACC -3' (SEQ ID NO: 26);

Primer 17:
5'-
TGATTTCCTGAGAGGCAAGAAGCCAGCGAATGGCTGGCTTCTTGAAGGATTTAGCCAAATTTAGGTAACA -3' (SEQ ID NO: 27);

Primer 18:
5'-AATCGGTATAGCAGATGTAATATTCACAGGGATCACTGTAATTAAAATAAATGAAGGATTATGTAATGGA -3' (SEQ ID NO: 28).

[0075] In order to delete the *tnaAB* genes, the *E. coli* strain W3110 trpΔ1 that the vector pKD46 was introduced was made competent was constructed in the same manner described in Example 6, and then the 1300bp gene fragment obtained by PCR was transformed into the *E. coli* strain. The strain having resistance to chloramphenicol was selected on LB medium. PCR was performed using primers 19 and 20, and the PCR amplification product had a size of about 5400 bp, indicating that the *tnaAB* genes were deleted in the strain.

Primer 19:
5'- CGGGATAAAGTAAAACCAGG -3' (SEQ ID NO: 29);

Primer 20:
5'- CGGCGAAGGTAAGTTGATGA -3' (SEQ ID NO: 30).

[0076] The pKD46 vector was removed from the primary recombinant strain having chloramphenicol resistance in the same manner described in Exampl 6, and then the chloramphenicol marker gene was removed from the strain. The resulting strain was an about 550bp amplification product obtained by PCR using primers 19 and 20, indicating that the desired gene deletion was achieved. The constructed strain was named "*E. coli* W3110 trpΔ2".

Example 8: Identification of L-tryptophan productivity of strains having tryptophan operon having various expression patterns

[0077] The *E. coli* strains transformed with the vectors prepared according to the methods described in Examples 3, 4 and 5. The effects of the *E. Coli* variant were evaluated using W3110 trpΔ2 prepared in Examples 6 and 7 as a parent strain, and its carbon source was glucose.

[0078] In order to evaluate the titer, each strain was inoculated by a platinum loop and cultured overnight on LB solid medium. Then, one platinum loop of each strain was inoculated into 25 mL of glucosecontaining medium, the composition of the medium is shown in Table 2 below. After inoculation, each strain was incubated at 37 °C and 200 rpm for 48 hours. The results are shown in Table 3 below. All the results were recorded as the average of three flask results.

Table 2

Composition	Concentration (per liter)
Glucose	2 g
$KH2PO_4$	1 g
$(NH_4)_2SO_4$	12 g
NaCl	1 g
$Na_2HPO_4 \cdot H_2O$	5 g
$MgSO_4 \cdot H_2O$	1 g
$MnSO_4 \cdot H_2O$	15 mg
$CuSO_4 \cdot H_2O$	3 mg
$ZnSO_4 \cdot H_2O$	30 mg
Sodium citrate	1 g

Composition	Concentration (per liter)
Yeast extract	1 g
Phenylalanine	0.15 g
pH	6.8

Table 3

Parent strain	Vector		L-tryptopha n (g/L) **	Anthranilat e (mg/L)**
W3110 trp Δ2	pCL1920	pCC1BAC	0.1	13
	pCL-Ptrp_trpEDCBA	pBAC-Ptrp_trpEDCBA	0.4	56
		pBAC-DtrpL_trpEDCBA	0.4	53
		pBAC-Dtrp att-trpEDCBA	0.5	61
	pCL-DtrpL_trpEDCBA	pBAC-Ptrp_trpEDCBA	0.4	68
		pBAC-DtrpL_trpEDCBA	0.5	73
		pBAC-Dtrp att-trpEDCBA	0.4	74
	pCL-Dtrp_att-trpEDCBA	pBAC-Ptrp trpEDCBA	0.6	89
		pBAC-DtrpL_trpEDCBA	0.5	95
		pBAC-Dtrp att-trpEDCBA	0.7	98
	pCL-Ptrp-trpEDCBA	pBAC-Ptrp_trpEDCBA	0.5	34
		pBAC-DtrpL_trpEDCBA	0.6	35
		pBAC-Dtrp att-trpEDCBA	0.6	40
	pCL-DtrpL-trpEDCBA	pBAC-Ptrp_trpDCBA	0.5	45
		pBAC-DtrpL_trpDCBA	0.5	42
		pBAC-Dtrp att-trpDCBA	0.6	38
	pCL-Dtrp_att-trpEDCBA	pBAC-Ptrp_trpDCBA	0.7	36
		pBAC-DtrpL_trpDCBA	0.8	28
		pBAC-Dtrp att-trpDCBA	1.0	29

[0079] As can be seen from the results in Table 3 above, in the case in which the parent strain *E. coli* W3110 trpΔ2 was transformed with a combination of various vectors, if only the tryptophan operon was continuously enhanced, no positive effect on the production yield of tryptophan appeared while anthranilate accumulated. On the contrary, the strain modified to enhance the Trp operon and trpDCBA showed a positive effect on the production yield of tryptophan together with a decrease in the accumulation of anthranilate, compared to the strain in which only tryptophan operon was enhanced. Thus, it was confirmed that a decrease in the accumulation of anthranilate is an effective way to increase the production yield of L-tryptophan in tryptophan-producing strains.

Example 9: Identification of L-tryptophan productivity of strains having tryptophan operon having various expression patterns

[0080] The vectors constructed according to the methods described in Examples 3, 4 and 5 were introduced into the L-tryptophan-producing parent strain *E. coli* KCCM10812P according to the combination shown in Table 5 below.The titers of the strains were evaluated using glucose as a carbon source. As a result, it appeared that not only the enhancement of trpDCBA, but also the enhancement of the tryptophan operon, is important, similar to the results of Example 8. Thus, the effects on the tryptophan-producing strains were evaluated.

[0081] In order to evaluate the titer, each strain was inoculated by a platinum loop and cultured overnight on LB solid medium. Then, one platinum loop of each strain was inoculated into 25 mL of glucosecontaining medium, the composition of the medium is shown in Table 4 below. After inoculation, each strain was incubated at 37 °C and 200 rpm for 48 hours. The results are shown in Table 5 below. All the results were recorded as the average of three flask results.

Table 4

Composition	Concentration (per liter)
Glucose	60 g
K_2HPO_4	1 g
$(NH_4)_2SO_4$	10 g
NaCl	1 g
$MgSO_4 \cdot H_2O$	1 g
Sodium citrate	5 g
Yeast extract	2 g
Calcium carbonate	40 g
Sodium citrate	5 g
Phenylalanine	0.15 g
Tyrosine	0.1 g
pH	6.8

Table 5

Vector		OD	Glucose consumption (g/L)*	L-tryptophan (g/L) **	Anthranilate (mg/L) **
pCL	pBAC				
pCL1920	pCC1BAC	13.5	53.0	7.0	1005
pCL-Ptrp_trpEDCBA	pBAC-Ptrp_trpEDCBA	14.0	52.1	7.2	1053
	pBAC-DtrpL_trpEDCBA	14.2	51.0	7.5	1157
	pBAC-Dtrp_att-trpEDCBA	13.8	52.6	7.1	1263

Vector		OD	Glucose consumption (g/L)*	L-tryptophan (g/L) **	Anthranilate (mg/L) **
pCL	pBAC				
pCL-DtrpL_trpEDCBA	pBAC-Ptrp_trpEDCBA	13.9	50.0	7.5	1170
	pBAC-DtrpL_trpEDCBA	13.7	51.6	7.3	1290
	pBAC-Dtrp_att-trpEDCBA	13.6	49.8	7.8	1485
pCL-Ptrp att-trpEDCBA	pBAC-Ptrp_trpEDCBA	13.8	49.8	7.5	1358
	pBAC-DtrpL_trpEDCBA	13.1	47.6	7.6	1501
	pBAC-Dtrp_att-trpEDCBA	12.7	45.3	7.5	1853
pCL-	pBAC-	14.2	52.1	7.5	950
Ptrp_trpEDCBA	Ptrp_trpDCBA				
	pBAC-DtrpL_trpDCBA	14.6	51.3	7.2	813
	pBAC-Dtrp_att-trpDCBA	14.3	52.7	7.1	687
pCL-DtrpL_trpEDCBA	pBAC-Ptrp_trpDCBA	13.9	50.6	7.5	953
	pBAC-DtrpL_trpDCBA	13.7	51.7	7.6	852
	pBAC-Dtrp_att-trpDCBA	13.6	51.3	7.7	715
pCL-Dtrp att-trpEDCBA	pBAC-Ptrp_trpDCBA	13.2	51.6	8.0	1085
	pBAC-DtrpL_trpDCBA	13.9	50.9	8.6	867
	pBAC-Dtrp_att-trpDCBA	13.5	51.2	9.5	783

* measured at 33 hours
** measured at 48 hours

[0082] As can be seen from the results in Table 5 above, in the case in which the parent strain *E.coli* KCCM10812P was transformed with a combination of various vectors, if only the tryptophan operon was continuously enhanced, no positive effect on the production yield of tryptophan appeared while anthranilate accumulated. On the contrary, it appears that the strain modified by enhancing the operon using the pCL vector and enhancing trpDCBA using the pBAC vector showed a positive effect on the production yield of tryptophan together with a decrease in the accumulation of anthranilate, compared to the strain in which only tryptophan operon was enhanced. Thus, it was confirmed that a decrease in the accumulation of anthranilate is an effective way to increase the production yield of L-tryptophan in tryptophan-producing strains.

Example 10: Construction of strain wherein the copy number of the tryptophan biosynthetic gene cluster trpDCBA in the chromosome was increased and the accumulation of anthranilate decreased

[0083] Based on the results of Example 9, in order to increase the copy number of the tryptophan biosynthetic gene cluster trpDCBA in the chromosome, a vector was constructed.

[0084] Specifically, pCL-Dtrp att-trpDCBA described in Example 5 was cleaved with the restriction enzymes *EcoRI* and *BamH*I to obtain Dtrp_att-trpDCBA, and then ligated with pINT17E treated with the same restriction enzymes, thereby obtaining pINT17E-Patt-trpDCBA. In order to introduce pINT17E-Patt-trpDCBA into the tryptophan-producing parent strain *E. coli* KCCM10812P to increase the copy number of the tryptophan biosynthetic gene cluster trpDCBA, pKD46 that is used in the one-step inactivation method (developed by Datsenko KA et al.), a mutagenesis technique using lambda red recombinase, according to Example 6. As a marker for confirming insertion into the genes, the chloramphenicol-resistant gene of pUCprmfmloxC was used. Specifically, the parent strain, inwhich pKD46 was introduced, transformed with pINT17E-Patt-trpDCBA, and then cultured at 37 °C for 1-2 days to obtain colonies. To confirm whether pINT17E-Patt-trpDCBA was correctly inserted into the chromosome of the obtained colonies, about 2000-bp fragment was amplified by PCR using primers 21 and 22.

Primer 21:
5' TATTTGCTGTCACGAGCAGG 3' (SEQ ID NO: 31);

Primer 22:
5' AGTTCCGGCATACAACCGGCTT 3' (SEQ ID NO: 32).

[0085] pKD46 was removed from the primary recombinant strain having chloramphenicol resistance, and then pJW168 plasmid was introduced to remove the chloramphenicol marker gene from the strain (Gene, (2000) 247, 255-264). An about 5000-bp amplification product obtained by PCR using primers 23 and 24, and an about 6500-bp amplification product obtained by PCR using primers 25 and 26, it demonstrated that trpDCBA is continuously place following the tryptophan operon which endogenously place on the chromosome. This strain was named "KCCM10812P/ trpDCBA".

Primer 23:
5' TAATACGACTCACTATAGGG 3' (SEQ ID NO: 33);

Primer 24:
5' CTGTTGGGCGGAAAAATGAC 3' (SEQ ID NO: 34);

Primer 25:
5' TGATCGCCAGGGTGCCGACG 3' (SEQ ID NO: 35);

Primer 26:
5' CCCTATAGTGAGTCGTATTA 3' (SEQ ID NO: 36).

[0086] In order to additionally insert one copy into the above-prepared strain in which the copy number of trpDCBA was increased, pKD46 was introduced into the above-prepared KCCM10812P/trpDCBA strain. Then the pINT17E-Patt-trpDCBA vector was introduced into KCCM10812P/trpDCBA/pKD46, thereby constructing a strain having two copies of trpDCBA inserted into the chromosome. This constructed strain was named "KCCM10812P/2trpDCBA". This strain was deposited with the Korean Culture Center of Microorganisms (361-221, Hongje 1-dong, Seodaemun-gu, Seoul, Korea), an international depository authority, on December 29, 2011 under the accession number KCCM11246P.

Example 11: Examination of effect of L-tryptophan-producing strain having increased activities of proteins that are encoded by the tryptophan biosynthetic gene cluster trpDCBA

[0087] According to the method described in Example 10, the titer of KCCM10812P/trpDCBA was evaluated using glucose as a carbon source. The KCCM10812P/trpDCBA was obtained by further introducing trpDCBA into the tryptophan-producing strain *E. coli* KCCM10812P to enhance the activities of some enzymes of tryptophan biosynthesis pathway.

[0088] To evaluate the titer, the strain was inoculated by a platinum loop and cultured overnight on LB solid medium. Then, one platinum loop of the strain culture was inoculated into 25 ml of a flask titer medium, the composition of the medium is shown in Table 4 above. After inoculation, the strain was cultured at 37 °C and 200 rpm for 48 hours. The results are shown in Table 6 below. All the results were recorded as the average of three flask results.

Table 6

Strain	OD	Glucose consumption (g/L)*	L-tryptophan (g/L)**	Anthranilate (mg/L)
KCCM10812P	14.0	54.0	7.2	1020
KCCM10812P/ trpDCBA	14.5	54.5	7.9	630
KCCM10812P/2 trpDCBA	13.3	55.2	8.2	320

* measured at 33 hours
** measured at 48 hours

[0089] As can be seen in Table 6 above, when one copy of the tryptophan biosynthetic gene cluster trpDCBA was inserted into the chromosome, the concentration of anthranilate decreased by 39% compared to that in the parent strain. howver two copies were inserted into the chromosome, the concentration of anthranilate decreased by 69% compared to that in the parent strain.

[0090] In addition, the concentrations of L-tryptophan in the two strains increased by 10% and 13%, respectively. As shown in Table 6 above, when the copy number of trpDCBA was increased, the consumption rate of glucose slightly decreased in some cases, but the enhancement of the tryptophan biosynthetic gene cluster has positive effects on an increase in the concentration of L-tryptophan and a decrease in the concentration of anthranilate.

Accession Number

[0091]

Depository authority: Korean Culture Center of Microorganisms(international)

Accession Number: KCCM11246P

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<110> CJ Cheiljedang Corporation

<120> A MICROORGANISM OF ESCHERICHIA COLI GENUS HAVING ENHANCED L-TRYPTOPHAN PRODUCING ACTIVITY AND METHOD FOR PRODUCING L-TRYPTOPHAN USING THE SAME

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Leu Ala Asp Gln Leu Arg Ser Asn Gly His Asn Val Val Ile Tyr Arg
            20                  25                  30

Asn His Ile Pro Ala Gln Thr Leu Ile Glu Arg Leu Ala Thr Met Ser
        35                  40                  45

Asn Pro Val Leu Met Leu Ser Pro Gly Pro Gly Val Pro Ser Glu Ala
    50                  55                  60

Gly Cys Met Pro Glu Leu Leu Thr Arg Leu Arg Gly Lys Leu Pro Ile
65                  70                  75                  80

Ile Gly Ile Cys Leu Gly His Gln Ala Ile Val Glu Ala Tyr Gly Gly
                85                  90                  95

Tyr Val Gly Gln Ala Gly Glu Ile Leu His Gly Lys Ala Ser Ser Ile
            100                 105                 110

Glu His Asp Gly Gln Ala Met Phe Ala Gly Leu Thr Asn Pro Leu Pro
        115                 120                 125

Val Ala Arg Tyr His Ser Leu Val Gly Ser Asn Ile Pro Ala Gly Leu
    130                 135                 140

Thr Ile Asn Ala His Phe Asn Gly Met Val Met Ala Val Arg His Asp
145                 150                 155                 160

Ala Asp Arg Val Cys Gly Phe Gln Phe His Pro Glu Ser Ile Leu Thr
                165                 170                 175

Thr Gln Gly Ala Arg Leu Leu Glu Gln Thr Leu Ala Trp Ala Gln Gln
            180                 185                 190

Lys Leu Glu Pro Ala Asn Thr Leu Gln Pro Ile Leu Glu Lys Leu Tyr
        195                 200                 205

Gln Ala Gln Thr Leu Ser Gln Gln Glu Ser His Gln Leu Phe Ser Ala
    210                 215                 220

Val Val Arg Gly Glu Leu Lys Pro Glu Gln Leu Ala Ala Ala Leu Val
225                 230                 235                 240

Ser Met Lys Ile Arg Gly Glu His Pro Asn Glu Ile Ala Gly Ala Ala
                245                 250                 255

Thr Ala Leu Leu Glu Asn Ala Ala Pro Phe Pro Arg Pro Asp Tyr Leu
            260                 265                 270

Phe Ala Asp Ile Val Gly Thr Gly Gly Asp Gly Ser Asn Ser Ile Asn
        275                 280                 285

Ile Ser Thr Ala Ser Ala Phe Val Ala Ala Ala Cys Gly Leu Lys Val
    290                 295                 300

Ala Lys His Gly Asn Arg Ser Val Ser Ser Lys Ser Gly Ser Ser Asp
305                 310                 315                 320

Leu Leu Ala Ala Phe Gly Ile Asn Leu Asp Met Asn Ala Asp Lys Ser
                325                 330                 335
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Arg Gln Ala Leu Asp Glu Leu Gly Val Cys Phe Leu Phe Ala Pro Lys
            340                 345                 350

Tyr His Thr Gly Phe Arg His Ala Met Pro Val Arg Gln Gln Leu Lys
        355                 360                 365

Thr Arg Thr Leu Phe Asn Val Leu Gly Pro Leu Ile Asn Pro Ala His
    370                 375                 380

Pro Pro Leu Ala Leu Ile Gly Val Tyr Ser Pro Glu Leu Val Leu Pro
385                 390                 395                 400

Ile Ala Glu Thr Leu Arg Val Leu Gly Tyr Gln Arg Ala Ala Val Val
                405                 410                 415

His Ser Gly Gly Met Asp Glu Val Ser Leu His Ala Pro Thr Ile Val
            420                 425                 430

Ala Glu Leu His Asp Gly Glu Ile Lys Ser Tyr Gln Leu Thr Ala Glu
        435                 440                 445

Asp Phe Gly Leu Thr Pro Tyr His Gln Glu Gln Leu Ala Gly Gly Thr
    450                 455                 460

Pro Glu Glu Asn Arg Asp Ile Leu Thr Arg Leu Leu Gln Gly Lys Gly
465                 470                 475                 480

Asp Ala Ala His Glu Ala Ala Val Ala Ala Asn Val Ala Met Leu Met
                485                 490                 495

Arg Leu His Gly His Glu Asp Leu Gln Ala Asn Ala Gln Thr Val Leu
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Glu Val Leu Arg Ser Gly Ser Ala Tyr Asp Arg Val Thr Ala Leu Ala
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Ala Arg Gly
    530
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<211> 452

<212> PRT

<213> Escherichia coli

<400> 38

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

Gln Pro Ser Thr Arg His Phe Tyr Asp Ala Leu Gln Gly Ala Arg Thr
         35                  40                  45

Ala Phe Ile Leu Glu Cys Lys Lys Ala Ser Pro Ser Lys Gly Val Ile
     50                  55                  60

Arg Asp Asp Phe Asp Pro Ala Arg Ile Ala Ala Ile Tyr Lys His Tyr
 65                  70                  75                  80

Ala Ser Ala Ile Ser Val Leu Thr Asp Glu Lys Tyr Phe Gln Gly Ser
                 85                  90                  95

Phe Asn Phe Leu Pro Ile Val Ser Gln Ile Ala Pro Gln Pro Ile Leu
            100                 105                 110

Cys Lys Asp Phe Ile Ile Asp Pro Tyr Gln Ile Tyr Leu Ala Arg Tyr
        115                 120                 125

Tyr Gln Ala Asp Ala Cys Leu Leu Met Leu Ser Val Leu Asp Asp Asp
    130                 135                 140

Gln Tyr Arg Gln Leu Ala Ala Val Ala His Ser Leu Glu Met Gly Val
145                 150                 155                 160

Leu Thr Glu Val Ser Asn Glu Glu Glu Gln Glu Arg Ala Ile Ala Leu
                165                 170                 175

Gly Ala Lys Val Val Gly Ile Asn Asn Arg Asp Leu Arg Asp Leu Ser
            180                 185                 190
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Ile Asp Leu Asn Arg Thr Arg Glu Leu Ala Pro Lys Leu Gly His Asn
        195                 200                 205

Val Thr Val Ile Ser Glu Ser Gly Ile Asn Thr Tyr Ala Gln Val Arg
    210                 215                 220

Glu Leu Ser His Phe Ala Asn Gly Phe Leu Ile Gly Ser Ala Leu Met
225                 230                 235                 240

Ala His Asp Asp Leu His Ala Ala Val Arg Arg Val Leu Leu Gly Glu
                245                 250                 255

Asn Lys Val Cys Gly Leu Thr Arg Gly Gln Asp Ala Lys Ala Ala Tyr
            260                 265                 270

Asp Ala Gly Ala Ile Tyr Gly Gly Leu Ile Phe Val Ala Thr Ser Pro
        275                 280                 285

Arg Cys Val Asn Val Glu Gln Ala Gln Glu Val Met Ala Ala Ala Pro
    290                 295                 300

Leu Gln Tyr Val Gly Val Phe Arg Asn His Asp Ile Ala Asp Val Val


305                 310                 315                 320

Asp Lys Ala Lys Val Leu Ser Leu Ala Ala Val Gln Leu His Gly Asn
                325                 330                 335

Glu Glu Gln Leu Tyr Ile Asp Thr Leu Arg Glu Ala Leu Pro Ala His
            340                 345                 350

Val Ala Ile Trp Lys Ala Leu Ser Val Gly Glu Thr Leu Pro Ala Arg
        355                 360                 365

Glu Phe Gln His Val Asp Lys Tyr Val Leu Asp Asn Gly Gln Gly Gly
    370                 375                 380

Ser Gly Gln Arg Phe Asp Trp Ser Leu Leu Asn Gly Gln Ser Leu Gly
385                 390                 395                 400

Asn Val Leu Leu Ala Gly Gly Leu Gly Ala Asp Asn Cys Val Glu Ala
                405                 410                 415

Ala Gln Thr Gly Cys Ala Gly Leu Asp Phe Asn Ser Ala Val Glu Ser
            420                 425                 430

Gln Pro Gly Ile Lys Asp Ala Arg Leu Leu Ala Ser Val Phe Gln Thr
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Leu Arg Ala Tyr
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<211> 397

<212> PRT

<213> Escherichia coli

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

Val Ser Ala Gln Lys Asp Pro Glu Phe Gln Ala Gln Phe Asn Asp Leu
         35                  40                  45

Leu Lys Asn Tyr Ala Gly Arg Pro Thr Ala Leu Thr Lys Cys Gln Asn
     50                  55                  60

Ile Thr Ala Gly Thr Asn Thr Thr Leu Tyr Leu Lys Arg Glu Asp Leu
 65                  70                  75                  80

Leu His Gly Gly Ala His Lys Thr Asn Gln Val Leu Gly Gln Ala Leu
                 85                  90                  95

Leu Ala Lys Arg Met Gly Lys Thr Glu Ile Ile Ala Glu Thr Gly Ala
            100                 105                 110
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```
Gly Gln His Gly Val Ala Ser Ala Leu Ala Ser Ala Leu Leu Gly Leu
        115                 120                 125

Lys Cys Arg Ile Tyr Met Gly Ala Lys Asp Val Glu Arg Gln Ser Pro
    130                 135                 140

Asn Val Phe Arg Met Arg Leu Met Gly Ala Glu Val Ile Pro Val His
145                 150                 155                 160

Ser Gly Ser Ala Thr Leu Lys Asp Ala Cys Asn Glu Ala Leu Arg Asp
                165                 170                 175

Trp Ser Gly Ser Tyr Glu Thr Ala His Tyr Met Leu Gly Thr Ala Ala
            180                 185                 190

Gly Pro His Pro Tyr Pro Thr Ile Val Arg Glu Phe Gln Arg Met Ile
        195                 200                 205

Gly Glu Glu Thr Lys Ala Gln Ile Leu Glu Arg Glu Gly Arg Leu Pro
    210                 215                 220

Asp Ala Val Ile Ala Cys Val Gly Gly Gly Ser Asn Ala Ile Gly Met
225                 230                 235                 240

Phe Ala Asp Phe Ile Asn Glu Thr Asn Val Gly Leu Ile Gly Val Glu
                245                 250                 255

Pro Gly Gly His Gly Ile Glu Thr Gly Glu His Gly Ala Pro Leu Lys
            260                 265                 270

His Gly Arg Val Gly Ile Tyr Phe Gly Met Lys Ala Pro Met Met Gln
        275                 280                 285

Thr Glu Asp Gly Gln Ile Glu Glu Ser Tyr Ser Ile Ser Ala Gly Leu
    290                 295                 300

Asp Phe Pro Ser Val Gly Pro Gln His Ala Tyr Leu Asn Ser Thr Gly
305                 310                 315                 320

Arg Ala Asp Tyr Val Ser Ile Thr Asp Asp Glu Ala Leu Glu Ala Phe
                325                 330                 335

Lys Thr Leu Cys Leu His Glu Gly Ile Ile Pro Ala Leu Glu Ser Ser
            340                 345                 350

His Ala Leu Ala His Ala Leu Lys Met Met Arg Glu Asn Pro Asp Lys
        355                 360                 365

Glu Gln Leu Leu Val Val Asn Leu Ser Gly Arg Gly Asp Lys Asp Ile
    370                 375                 380

Phe Thr Val His Asp Ile Leu Lys Ala Arg Gly Glu Ile
385                 390                 395
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<210> 40
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<213> Escherichia coli

<400> 40

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Met Glu Arg Tyr Glu Ser Leu Phe Ala Gln Leu Lys Glu Arg Lys Glu
  1               5                  10                  15

Gly Ala Phe Val Pro Phe Val Thr Leu Gly Asp Pro Gly Ile Glu Gln
             20                  25                  30

Ser Leu Lys Ile Ile Asp Thr Leu Ile Glu Ala Gly Ala Asp Ala Leu
         35                  40                  45

Glu Leu Gly Ile Pro Phe Ser Asp Pro Leu Ala Asp Gly Pro Thr Ile
     50                  55                  60

Gln Asn Ala Thr Leu Arg Ala Phe Ala Ala Gly Val Thr Pro Ala Gln
 65                  70                  75                  80

Cys Phe Glu Met Leu Ala Leu Ile Arg Gln Lys His Pro Thr Ile Pro
                 85                  90                  95

Ile Gly Leu Leu Met Tyr Ala Asn Leu Val Phe Asn Lys Gly Ile Asp
            100                 105                 110
```

```
Glu Phe Tyr Ala Gln Cys Glu Lys Val Gly Val Asp Ser Val Leu Val
        115                 120                 125

Ala Asp Val Pro Val Glu Glu Ser Ala Pro Phe Arg Gln Ala Ala Leu
    130                 135                 140

Arg His Asn Val Ala Pro Ile Phe Ile Cys Pro Pro Asn Ala Asp Asp
145                 150                 155                 160

Asp Leu Leu Arg Gln Ile Ala Ser Tyr Gly Arg Gly Tyr Thr Tyr Leu
                165                 170                 175

Leu Ser Arg Ala Gly Val Thr Gly Ala Glu Asn Arg Ala Ala Leu Pro
            180                 185                 190

Leu Asn His Leu Val Ala Lys Leu Lys Glu Tyr Asn Ala Ala Pro Pro
        195                 200                 205

Leu Gln Gly Phe Gly Ile Ser Ala Pro Asp Gln Val Lys Ala Ala Ile
    210                 215                 220

Asp Ala Gly Ala Ala Gly Ala Ile Ser Gly Ser Ala Ile Val Lys Ile
225                 230                 235                 240

Ile Glu Gln His Ile Asn Glu Pro Glu Lys Met Leu Ala Ala Leu Lys
                245                 250                 255

Val Phe Val Gln Pro Met Lys Ala Ala Thr Arg Ser
            260                 265
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REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

1. Rekombinant mikroorganisme af slægten *Escherichia*, som har forbedret L-tryptofanproduktivitet, hvor den rekombinante mikroorganisme har en modifikation af et ekspressionsregulerende område af tryptofanoperonet, hvor modifikationen er en delvis eller fuld sletning af et lederpeptid, der har en nukleotidsekvens, der er repræsenteret af SEQ ID NO: 2 i et ekspressionsregulerende område, der har en nukleotidsekvens, der er repræsenteret af SEQ ID NO: 1 fra et endogent tryptofanoperon, hvor den rekombinante mikroorganisme også modificeres til at have øget kromosomalt eller intracellulært kopieringsantal for tryptofanoperongenerne trpD, trpC, trpB og trpA, men ikke trpE, hvilket derved forstærker aktiviteten af den biosyntetiske genklynge for tryptofan med undtagelse af den anthranilatsyntase, der kodes af trpE-genet, i forhold til den tilsvarende *Escherichia*-mikroorganisme af vild type.

2. Den rekombinante mikroorganisme af slægten Escherichia ifølge krav 1, hvor den rekombinante mikroorganisme har en yderligere modifikation af det ekspressionsregulerende område af tryptofanoperonet, hvor modifikationen er en delvis eller fuld sletning af en endogen attenuator, der har en nukleotidsekvens, der er repræsenteret af SEQ ID NO: 3 i det ekspressionsregulerende område, der har en nukleotidsekvens, der er repræsenteret af SEQ ID NO: 1.

3. Den rekombinante mikroorganisme ifølge krav 1, hvor trpD-genet koder et polypeptid, der omfatter den aminosyresekvens, der er repræsenteret af SEQ ID NO: 37, trpC-genet koder et polypeptid, der omfatter den aminosyresekvens, der er repræsenteret af SEQ ID NO: 38, trpB-genet koder et polypeptid, der omfatter den aminosyresekvens, der er repræsenteret af SEQ ID NO: 39, og trpA-genet koder et polypeptid, der omfatter den aminosyresekvens, der er repræsenteret af SEQ ID NO: 40.

4. Den rekombinante mikroorganisme ifølge krav 1, hvor den rekombinante mikroorganisme er en *E. coli*-stamme.

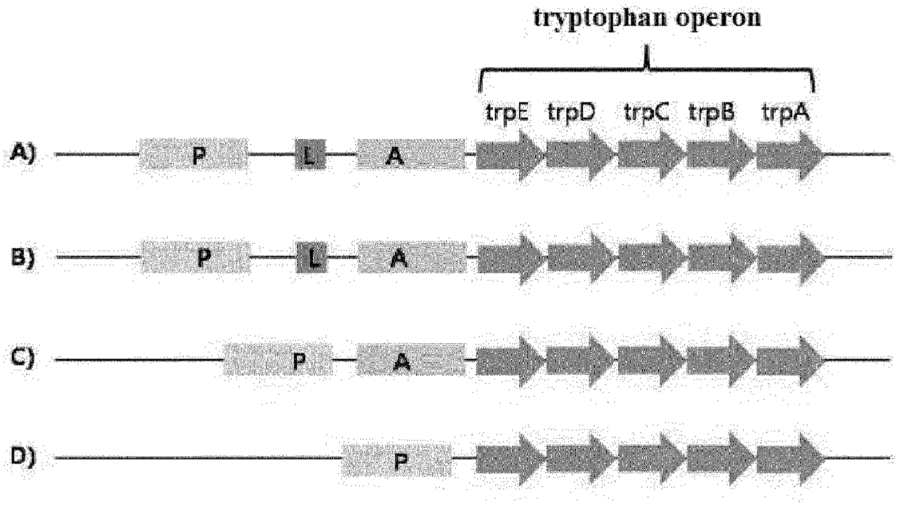
5. Fremgangsmåde til fremstilling af L-tryptofan, som omfatter dyrkning af den rekombinante mikroorganisme, som krævet i krav 1, under betingelser, der er egnede til fremstillingen af L-tryptofan.

6. Fremgangsmåden ifølge krav 5, hvor den rekombinante mikroorganisme har en yderligere modifikation af det ekspressionsregulerende område af tryptofanoperonet, hvor modifikationen er en delvis eller fuld sletning af en endogen attenuator, der har en nukleotidsekvens, der er repræsenteret af SEQ ID NO: 3 i det ekspressionsregulerende område, der har en nukleotidsekvens, der er repræsenteret af SEQ ID NO: 1.

7. Fremgangsmåden ifølge krav 5, hvor den rekombinante mikroorganisme er en *E. coli*-stamme.

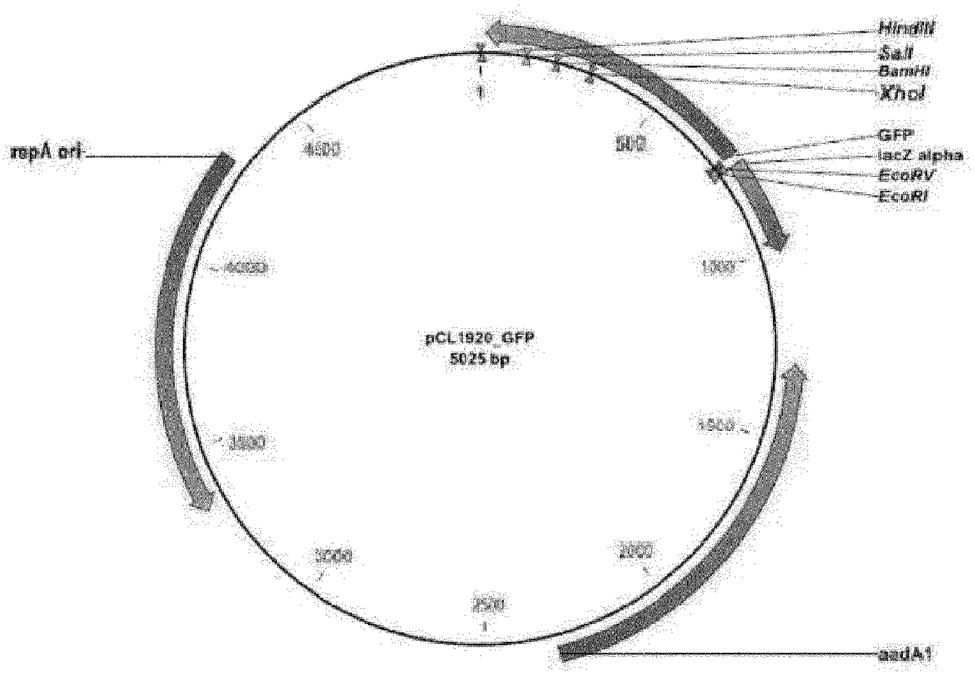
DRAWINGS

Fig. 1



P: promoter L: leader peptide A: attenuator

Fig. 2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XhoI
GFP
lacZ alpha
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EcoRI
repA ori
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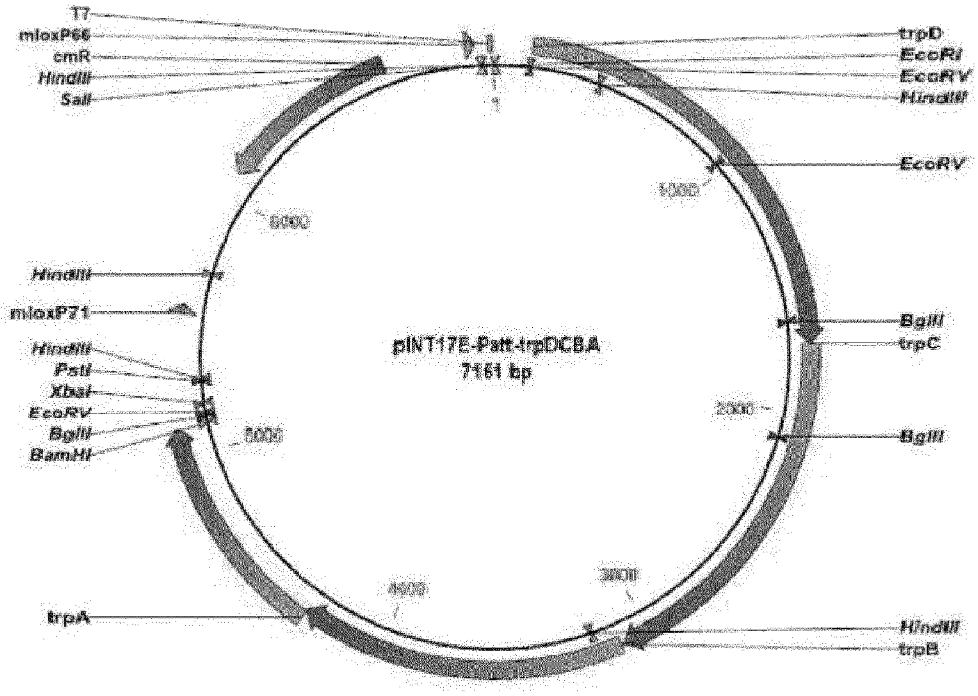
3500
4000
4500

Fig. 3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mloxP71
HindIII
PstI
XbaI
EcoRV
BglII
BamHI
5000
pINT17E-Patt-trpDCBA
7161 bp
BglII
trpC
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BglII
3000
4000
trpA
HindIII
trpB