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(54) Title: A METHOD FOR PRODUCING AN L-AMINO ACID USING A BACTERIUM OF THE ENTEROBACTERIACEAE FAMILY, HAVING ATTENUATED EXPRESSION OF GENE(S) ENCODING PEPTIDASE

(57) Abstract: The present invention provides a method for producing L-amino acids, such as L-arginine, L-citrulline, and L-lysine, using a bacterium of the *Enterobacteriaceae* family, particularly a bacterium belonging to the genus *Escherichia* or *Pantoea*, which has been modified to attenuate expression of one or more genes selected from the group consisting of *pepA*, *pepB*, and *pepD* genes.



DESCRIPTION

A METHOD FOR PRODUCING AN L-AMINO ACID USING A BACTERIUM OF THE *ENTEROBACTERIACEAE* FAMILY, HAVING ATTENUATED EXPRESSION OF GENE(S) ENCODING PEPTIDASE

Technical Field

The present invention relates to the microbiological industry, and specifically to a method for producing L-amino acids, such as L-arginine, L-citrulline, and L-lysine. The method uses a bacterium of the *Enterobacteriaceae* family which has been modified to attenuate expression of gene(s) encoding peptidase.

Background Art

Conventionally, L-amino acids are industrially produced by fermentation methods utilizing strains of microorganisms from natural sources, or mutants thereof. Typically, the microorganisms are modified to enhance production yields of L-amino acids.

Many techniques to enhance L-amino acid production yields have been reported, including by transforming microorganisms with recombinant DNA (see, for example, US patent No. 4,278,765). Other techniques for enhancing production yields include increasing the activities of enzymes involved in amino acid biosynthesis and/or desensitizing the target enzymes of the feedback inhibition caused by the produced L-amino acid (see, for example, WO 95/16042 or US patent Nos. 4,346,170; 5,661,012 and 6,040,160).

Other ways to enhance L-amino acid production yields is to attenuate expression of a gene or several genes which are involved in the degradation of the target L-amino acid, genes which divert the precursors of the target L-amino acid from the L-amino acid biosynthetic pathway, genes involved in the redistribution of the carbon, nitrogen, and phosphate fluxes, and genes coding for toxins, etc.

The *pepA* gene (synonym *carP*) from *Escherichia coli* encodes the multifunctional enzyme aminopeptidase A (PepA), which combines catalytic and regulatory properties. It has been shown that, apart from hydrolysis of N-terminal amino acid residues of proteins, aminopeptidase A is required for the site-specific DNA recombination and monomerization of plasmid multimers (Stirling et al. (1989) EMBO J., 8, 1623–1627; Charlier et al (1995) J. Mol. Biol., 250, 392–406). Another aminopeptidase A function is transcriptional regulation. PepA is a DNA binding protein and is involved in the transcriptional repression of the *carAB* operon, which

controls the synthesis of carbamoyl phosphate synthase (Roovers et al, (1988) J. Mol. Biol., 204, 857–865). Carbamoyl phosphate is an intermediate of L-arginine and pyrimidine biosynthetic pathways. Interaction between carbamoyl phosphate and L-ornithine results in L-citrulline formation, which is converted to L-arginine via L-argininosuccinate.

The *pepB* gene (synonyms *yhfI*, *b2523*, *ECK2520*) from *Escherichia coli* encodes aminopeptidase B (PepB), which is one of four cysteinylglycinases in *E. coli*. Aminopeptidase B cleaves Leu-Gly, Leu-Gly-Gly, Cys-Gly and Leu-Gly in vitro (Miller et al. (1994) Journal of Bacteriology, 176, 610-619, Hermsdorf et al. (1979) International Journal of Peptide and Protein Research, 13, 146-151, Suzuki et al (2001) Bioscience. Biotechnology. Biochemistry., 5,1549-1558.) In vivo, aminopeptidase B is, along with aminopeptidases A and N, and dipeptidase D, one of four cysteinylglycinases (Suzuki et al., (2001) Journal of Bacteriology 2001; 183(4) 1489-1490). Divalent cations, including some that are not effective stimulators of activity, stabilize aminopeptidase B against heat inactivation (Suzuki et al (2001) Bioscience. Biotechnology. Biochemistry., 5,1549-1558.).

The *pepD* gene (synonyms *pepH*, carnosinase) from *Escherichia coli* encodes peptidase D, which is a dipeptidase capable of breaking down a number of dipeptides with unblocked N termini, including cysteinylglycine (Schroeder et al., (1994) FEMS Microbiology Letters, 123, 153-159, Suzuki et al., (2001) Journal of Bacteriology, 183, 1489-1490). Peptidase D functions as a dimer of PepD monomers (Klein et al., (1986) Journal of General Microbiology, 132, 2337-2343). Transcription of *pepD* increases five fold during phosphate starvation (Henrich et al., (1992) Molecular General Genetics, 232, 117-125).

But currently, there have been no reports of attenuating expression of the gene(s) encoding peptidase for production of L-amino acids, such as L-arginine, L-citrulline, and L-lysine.

Disclosure of the Invention

Aspects of the present invention include enhancing the productivity of strains which are able to produce L-amino acids, such as L-arginine, L-citrulline, or L-lysine, and providing a method for producing such an L-amino acid using these strains.

The above aspects were achieved by finding that inactivation of gene encoding peptidase, *pepA*, *pepB*, and/or *pepD*, can enhance production of L-amino acid, such as L-arginine, and L-lysine. Furthermore, since L-citrulline is an intermediate in the L-arginine biosynthetic pathway, production of this L-amino acid can also be enhanced.

The present invention provides a bacterium of the *Enterobacteriaceae* family having an increased ability to produce an L-amino acid such as L-arginine and L-citrulline.

It is an aspect of the present invention to provide a bacterium of the *Enterobacteriaceae* family having the ability to produce L-amino acid, wherein said bacterium has been modified to attenuate expression of one or more genes selected from the group consisting of *pepA*, *pepB*, and *pepD* genes.

It is a further aspect of the present invention to provide the bacterium as described above, wherein expression of the gene(s) is/are attenuated by inactivation of the gene(s).

It is a further aspect of the present invention to provide the bacterium as described above, wherein the bacterium belongs to the genus *Escherichia*.

It is a further aspect of the present invention to provide the bacterium according to claim 3, wherein said bacterium is *Escherichia coli*.

It is a further aspect of the present invention to provide the bacterium as described above, wherein the bacterium belongs to the genus *Pantoea*.

It is a further aspect of the present invention to provide the bacterium as described above, wherein the L-amino acid is selected from the group consisting of L-arginine, L-citrulline, and L-lysine.

It is a further aspect of the present invention to provide the bacterium as described above, wherein the bacterium has been modified to attenuate expression of *pepA*, *pepB*, and *pepD* genes, and the L-amino acid is L-lysine.

It is a further aspect of the present invention to provide a method for producing an L-amino acid comprising:

cultivating the bacterium as described above in a medium, and
collecting the L-amino acid from the culture medium.

It is a further aspect of the present invention to provide a method as described above, wherein said L-amino acid is selected from the group consisting of L-arginine, L-citrulline, and L-lysine.

Brief Description of Drawings

Figure 1 shows the relative positions of pair of primers P1 and P2 on plasmid pMW118-attL-Cm-attR.

Figure 2 shows the construction of the chromosomal DNA fragment which includes the inactivated *pepA* gene.

Description of Embodiments

The present invention is described in detail below.

1. Bacterium

The bacterium in accordance with the presently disclosed subject matter is a bacterium of the *Enterobacteriaceae* family having ability to produce L-amino acid, such as L-arginine, L-citrulline, or L-lysine, wherein the bacterium has been modified to attenuate expression of one or more genes selected from the group consisting of *pepA*, *pepB*, and *pepD* gene encoding aminopeptidase.

The term “bacterium having ability to produce an L-amino acid” can mean a bacterium which is able to produce and secrete L-amino acid into a medium, when the bacterium is cultured in the medium.

The term “bacterium having ability to produce an L-amino acid” also can mean a bacterium which is able to produce and cause accumulation of L- amino acid such as L-arginine, L-citrulline, or L-lysine in a culture medium in an amount larger than a wild-type or parental strain of *E. coli*, such as *E. coli* K-12, and can mean that the microorganism is able to cause accumulation in a medium of an amount not less than 0.5 g/L, in another example not less than 1.0 g/L of L- amino acid. The term “L-amino acid” can include L-arginine, L-citrulline, and L-lysine.

The *Enterobacteriaceae* family includes bacteria belonging to the genera *Escherichia*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Pantoea*, *Photorhabdus*, *Providencia*, *Salmonella*, *Serratia*, *Shigella*, *Morganella*, *Yersinia*, etc.. Specifically, those classified as *Enterobacteriaceae* according to the taxonomy used in the NCBI (National Center for Biotechnology Information) database

(<http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=543>) can be used.

A bacterium belonging to the genus *Escherichia* or *Pantoea* are particular examples.

The phrase “a bacterium belonging to the genus *Escherichia*” can mean that the bacterium is classified in the genus *Escherichia* according to the classification known to a person skilled in the art of microbiology. An example of a bacterium belonging to the genus *Escherichia* is, but is not limited to, *Escherichia coli* (*E. coli*). The bacterium belonging to the genus *Escherichia* is not particularly limited, however for example, bacteria described by Neidhardt, F.C. et al. (*Escherichia coli* and *Salmonella typhimurium*, American Society for Microbiology, Washington D.C., 1208, Table 1) are encompassed.

The phrase “a bacterium belonging to the genus *Pantoea*” can mean that the bacterium is classified as the genus *Pantoea* according to the classification known to a

person skilled in the art of microbiology. Some species of *Enterobacter agglomerans* have been recently re-classified into *Pantoea agglomerans*, *Pantoea ananatis*, *Pantoea stewartii* or the like, based on nucleotide sequence analysis of 16S rRNA, etc..

The phrase “bacterium has been modified to attenuate expression of *pepA* gene encoding aminopeptidase A” can mean that the bacterium has been modified in such a way that the modified bacterium contains a reduced amount of the aminopeptidase A, or it can also mean that the bacterium is unable to synthesize the aminopeptidase A.

The phrase “bacterium has been modified to attenuate expression of *pepB* gene encoding aminopeptidase B” can mean that the bacterium has been modified in such a way that the modified bacterium contains a reduced amount of the aminopeptidase B, or it can also mean that the bacterium is unable to synthesize the aminopeptidase B.

The phrase “bacterium has been modified to attenuate expression of *pepD* gene encoding dipeptidase D” can mean that the bacterium has been modified in such a way that the modified bacterium contains a reduced amount of the dipeptidase D, or it can also mean that the bacterium is unable to synthesize the dipeptidase D.

The phrase “inactivation of a gene” can mean that the modified gene encodes a completely non-functional protein. It is also possible that the modified DNA region is unable to naturally express the gene due to the deletion of a part of the gene, the shifting of the reading frame of the gene, the introduction of missense/nonsense mutation(s), or the modification of an adjacent region of the gene, including sequences controlling gene expression, such as a promoter, enhancer, attenuator, ribosome-binding site, etc.

The presence or absence of a gene on the chromosome of a bacterium can be detected by well-known methods, including PCR, Southern blotting, and the like. In addition, the level of gene expression can be estimated by measuring the amount of mRNA transcribed from the gene using various well-known methods, including Northern blotting, quantitative RT-PCR, and the like. The amount of the protein encoded by the gene can be measured by well-known methods, including SDS-PAGE followed by an immunoblotting assay (Western blotting analysis), and the like.

The *pepA* gene (synonyms: *carP*, *b4260*, *ECK4253*) coding for aminopeptidase A from *Escherichia coli* has been elucidated (nucleotides complementary to nucleotides 4482463 to 4483974 in the sequence of GenBank Accession NC_000913.2). The *pepA* gene from *E. coli* is located on the chromosome between the *holC* and *lptF* genes. The nucleotide sequences of the *pepA* gene, and the amino acid sequence of PepA protein encoded by the *pepA* gene, are shown in SEQ ID NO: 1 and SEQ ID NO: 2, respectively.

The *pepB* gene (synonyms: *yfhI*, *b2523*, *ECK2520*) coding for aminopeptidase B from *Escherichia coli* has been elucidated (nucleotides complementary to nucleotides 2653097 to 2654380 in the sequence of GenBank Accession EG12310). The *pepB* gene from *E. coli* is located on the chromosome between the *iscX* and *sseB* genes. The nucleotide sequences of the *pepB* gene, and the amino acid sequence of PepB protein encoded by the *pepB* gene, are shown in SEQ ID NO: 7 and SEQ ID NO: 8, respectively.

The *pepD* gene (synonyms: *pepH*, *b0237*, *ECK0238*) coding for peptidase D from *Escherichia coli* has been elucidated (nucleotides complementary to nucleotides 254259 to 255716 in the sequence of GenBank Accession 10695). The *pepD* gene from *E. coli* is located on the chromosome between the *gpt* and *prfH* genes. The nucleotide sequences of the *pepD* gene, and the amino acid sequence of PepD protein encoded by the *pepD* gene, are shown in SEQ ID NO: 9 and SEQ ID NO: 10, respectively.

Since there may be some differences in DNA sequences between the genera or strains of the *Enterobacteriaceae* family, the gene to be inactivated on the chromosome is not limited to the gene shown in SEQ ID NO:1, 7, or 9, but may include genes homologous to SEQ ID No:1, 7, or 9, and which encode a variant protein. The phrase "variant protein" can mean a protein which has changes in the sequence, whether they are deletions, insertions, additions, or substitutions of amino acids, but still maintains the activity of the protein. The number of changes in the variant protein depends on the position in the three dimensional structure of the protein or the type of amino acid residues. It may be 1 to 30, in another example 1 to 15, and in another example 1 to 5. These changes in the variants can occur in regions of the protein which are not critical for the function of the protein. This is because some amino acids have high homology to one another so the three dimensional structure or activity is not affected by such a change. Therefore, the protein variant encoded by the gene may have a homology of not less than 80%, in another example not less than 90%, in another example not less than 95%, in another example not less than 98%, and in another example not less than 99%, with respect to the entire amino acid sequence shown in SEQ ID NO. 2, 8, or 10, as long as the protein prior to inactivation is able to function as aminopeptidase or dipeptidase. In this specification, the term "homology" can also refer to "identity".

Homology between two amino acid sequences can be determined using well-known methods, for example, the computer program BLAST 2.0, which calculates three parameters: score, identity, and similarity.

Moreover, the *pepA*, *B*, or *D* gene may be a variant, which hybridizes with the nucleotide sequence shown in SEQ ID NO: 1, 7, or 9, or a probe which can be prepared from the nucleotide sequence, under stringent conditions, provided that it encodes a functional protein prior to inactivation. "Stringent conditions" include those, under which a specific hybrid, for example, a hybrid having homology of not less than 60%, in another example not less than 70%, in another example not less than 80%, in another example not less than 90%, in another example not less than 95%, in another example not less than 98%, and in another example not less than 99% is formed and a non-specific hybrid, for example, a hybrid having homology lower than the above, is not formed. For example, stringent conditions are exemplified by washing one time, in another example two or three times at a salt concentration corresponding to 1x SSC, 0.1% SDS, preferably 0.1x SSC, 0.1% SDS at 60°C. Duration of washing depends on the type of membrane used for blotting and, as a rule, should be what is recommended by the manufacturer. For example, the recommended duration of washing for the HybondTM N+ nylon membrane (Amersham) under stringent conditions is 15 minutes. Washing may be performed 2 to 3 times. The length of the probe may be suitably selected depending on the hybridization conditions, and is usually 100 bp to 1 kbp.

Expression of a gene can be attenuated by introducing a mutation into the gene on the chromosome so that the intracellular activity of the protein encoded by the gene is decreased as compared with an unmodified strain. Mutations which result in attenuation of expression of the gene include the replacement of one base or more to cause an amino acid substitution in the protein encoded by the gene (missense mutation), introduction of a stop codon (nonsense mutation), deletion of one or two bases to cause a frame shift, insertion of a drug-resistance gene, or deletion of a part of the gene or the entire gene (Qiu, Z. and Goodman, M.F., J. Biol. Chem., 272, 8611-8617 (1997); Kwon, D. H. et al, J. Antimicrob. Chemother., 46, 793-796 (2000)). Expression of the *pepA* gene can also be attenuated by modifying an expression regulating sequence such as the promoter, the Shine-Dalgarno (SD) sequence, etc. (WO95/34672, Carrier, T.A. and Keasling, J.D., Biotechnol Prog 15, 58-64 (1999)).

For example, the following methods may be employed to introduce a mutation by gene recombination. A mutant gene encoding a mutant protein with decreased activity can be prepared, and the bacterium to be modified can be transformed with a DNA fragment containing the mutant gene. Then, the native gene on the chromosome is replaced with the mutant gene by homologous recombination, and the resulting strain can be selected. Gene replacement using homologous recombination can be conducted by employing a linear DNA, which is known as "Red-driven integration" (Datsenko, K.A. and Wanner, B.L., Proc. Natl. Acad. Sci. USA, 97, 12, p 6640-6645

(2000)), or by employing a plasmid containing a temperature-sensitive replication origin (U.S. Patent 6,303,383 or JP 05-007491A). Furthermore, site-specific mutation by gene substitution can also be incorporated using homologous recombination such as set forth above using a plasmid which is unable to replicate in the host.

Expression of the gene can also be attenuated by inserting a transposon or an IS factor into the coding region of the gene (U.S. Patent No. 5,175,107), or by conventional methods, such as by mutagenesis with UV irradiation or nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine), site-directed mutagenesis, gene disruption using homologous recombination, or/and insertion-deletion mutagenesis (Yu, D. et al., Proc. Natl. Acad. Sci. USA, 2000, 97:12: 5978-83 and Datsenko, K.A. and Wanner, B.L., Proc. Natl. Acad. Sci. USA, 2000, 97:12: 6640-45), also called "Red-driven integration".

Methods for preparation of plasmid DNA, digestion and ligation of DNA, transformation, selection of oligonucleotides as primers, and the like may be ordinary methods well known to one skilled in the art. These methods are described, for instance, in Sambrook, J., Fritsch, E.F., and Maniatis, T., "Molecular Cloning A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press (1989).

L-amino acid-producing bacteria

Bacteria which are able to produce an L-amino acid such as L-arginine, L-citrulline, or L-lysine, may be employed.

The bacterium in accordance with the presently disclosed subject matter can be obtained by inactivating one or more genes encoding peptidase in a bacterium, which has a native or inherent ability to produce L-amino acid. Alternatively, the bacterium can be obtained by imparting the ability to produce L-amino acid to a bacterium already having an inactivated gene(s) encoding peptidase.

L-arginine producing bacteria

Examples of L-arginine-producing bacteria or parent strains, which can be used to derive L-arginine-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* strain 237 (VKPM B-7925) (U.S. Patent Application 2002/058315 A1) and its derivative strains harboring mutant N-acetylglutamate synthase (Russian Patent Application No. 2001112869), *E. coli* strain 382 (VKPM B-7926) (EP1170358A1), an arginine-producing strain into which the *argA* gene encoding N-acetylglutamate synthetase has been introduced (EP1170361A1), and the like.

Examples of parent strains which can be used to derive L-arginine producing bacteria also include strains in which expression of one or more genes encoding L-arginine biosynthetic enzymes is/are enhanced. Examples of such genes include the genes encoding N-acetylglutamate synthase (*argA*), N-acetylglutamate kinase (*argB*), N-acetylglutamyl phosphate reductase (*argC*), acetylornithine transaminase (*argD*), acetylornithine deacetylase (*argE*), ornithine carbamoyltransferase (*argF/I*), argininosuccinic acid synthetase (*argG*), argininosuccinic acid lyase (*argH*), and carbamoyl phosphate synthetase (*carAB*). The abbreviations in parentheses after the enzyme names represent the gene names.

L-citrulline producing bacteria

Examples of L-citrulline-producing bacteria or parent strains, which can be used to derive L-citrulline-producing bacteria belonging to the genus *Escherichia* include, but are not limited to, *E. coli* strains 333 (VKPM B-8084) and 374 (VKPM B-8086), both harboring mutant feedback-resistant carbamoyl phosphate synthetase (Russian Patent RU2264459 C2), strains *E. coli*, in which α -ketoglutarate synthase activity is increased, and ferredoxin NADP⁺ reductase, pyruvate synthase or α -ketoglutarate dehydrogenase activities are additionally modified (EP 2133417 A1), and strain *P. ananantis* NA1sucAsdhA, in which succinate dehydrogenase and α -ketoglutarate dehydrogenase activities are decreased (US Patent Application No 2009286290), and the like.

As L-citrulline is an intermediate of L-arginine biosynthetic pathway, examples of parent strains, which can be used to derive L-citrulline-producing bacteria, include strains, in which expression of one or more genes encoding an L-arginine biosynthetic enzyme is enhanced. Examples of such genes include, but are not limited to, genes encoding N-acetylglutamate synthase (*argA*), N-acetylglutamate kinase (*argB*), N-acetylglutamyl phosphate reductase (*argC*), acetylornithine transaminase (*argD*), acetylornithine deacetylase (*argE*), ornithine carbamoyltransferase (*argF/I*), and carbamoyl phosphate synthetase (*carAB*), or combinations thereof.

Examples of parent strains which can be used to derive L-citrulline-producing bacteria of the present invention can also include L-arginine-producing strains, in which activity of argininosuccinic acid synthetase (ArgG), which catalyzed conversion of L-citrulline to L-arginine, is reduced compared with parent strain. Activity of argininosuccinic acid synthetase can be decreased by inactivation of the corresponding gene *argG* by conventional methods as described above.

L-lysine-producing bacteria

Examples of L-lysine-producing bacteria or parent strains belonging to the genus *Escherichia* include mutants having resistance to an L-lysine analogue. The L-lysine analogue inhibits growth of bacteria belonging to the genus *Escherichia*, but this inhibition is fully or partially desensitized when L-lysine is present in the medium. Examples of the L-lysine analogue include, but are not limited to, oxalysine, lysine hydroxamate, S-(2-aminoethyl)-L-cysteine (AEC), γ -methyllysine, α -chlorocaprolactam, and so forth. Mutants having resistance to these lysine analogues can be obtained by subjecting bacteria belonging to the genus *Escherichia* to a conventional artificial mutagenesis treatment. Specific examples of bacterial strains useful for producing L-lysine include *Escherichia coli* AJ11442 (FERM BP-1543, NRRL B-12185; see U.S. Patent No. 4,346,170) and *Escherichia coli* VL611. In these microorganisms, feedback inhibition of aspartokinase by L-lysine is desensitized.

The strain WC196 may be used as an L-lysine producing bacterium of *Escherichia coli*. This bacterial strain was bred from the W3110 strain, which was derived from *Escherichia coli* K-12, by replacing the wild type *lysC* gene on the chromosome of the W3110 strain with a mutant *lysC* gene encoding a mutant aspartokinase III desensitized to feedback inhibition by L-lysine in which threonine at position 352 had been replaced with isoleucine, and conferring AEC resistance to the resulting strain (U.S. patent No. 5,661,012). The resulting strain was designated *Escherichia coli* AJ13069 and was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (currently National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository, Tsukuba Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) on December 6, 1994 and received an accession number of FERM P-14690. Then, it was converted to an international deposit under the provisions of the Budapest Treaty on September 29, 1995, and received an accession number of FERM BP-5252 (U.S. Patent No. 5,827,698).

Examples of parent strains which can be used to derive bacteria which are able to produce L-lysine also include strains in which expression of one or more genes encoding an L-lysine biosynthetic enzyme are enhanced. Examples of such genes include, but are not limited to, genes encoding dihydrodipicolinate synthase (*dapA*), aspartokinase (*lysC*), dihydrodipicolinate reductase (*dapB*), diaminopimelate decarboxylase (*lysA*), diaminopimelate dehydrogenase (*ddh*) (U.S. Patent No. 6,040,160), phosphoenolpyruvate carboxylase (*ppc*), aspartate semialdehyde dehydrogenase (*asd*), and aspartase (*aspA*) (EP 1253195 A). In addition, the parent strains may have increased expression of the gene involved in energy efficiency (*cyo*) (EP 1170376 A), the gene encoding nicotinamide nucleotide transhydrogenase (*pntAB*)

(U.S. Patent No. 5,830,716), the *ybjE* gene (WO2005/073390), or combinations thereof.

It is known that wild-type dihydrodipicolinate synthetase derived from *Escherichia coli* is subject to feedback inhibition by L-lysine, while wild-type aspartokinase derived from *Escherichia coli* is subject to suppression and feedback inhibition by L-lysine. Therefore, when the *dapA* and *lysC* genes are used, these genes can be mutant genes coding the enzymes that are not subject to the feedback inhibition by L-lysine.

Examples of DNA encoding a mutant dihydrodipicolinate synthetase desensitized to feedback inhibition by L-lysine include a DNA encoding a protein that has the amino acid sequence of the enzyme in which the histidine at position 118 is replaced by tyrosine. Examples of DNA encoding a mutant aspartokinase desensitized to feedback inhibition by L-lysine include a DNA encoding an AKIII having the amino acid sequence in which the threonine at position 352, the glycine at position 323, and the methionine at position 318 are replaced by isoleucine, asparagine and isoleucine, respectively (for these mutants, see U.S. Patent No. 5,661,012 and U.S. Patent No. 6,040,160). Such mutant DNAs can be obtained by site-specific mutagenesis using PCR or the like.

Wide host-range plasmids RSFD80, pCAB1, and pCABD2 are known as plasmids containing a mutant *dapA* gene encoding a mutant dihydrodipicolinate synthetase and a mutant *lysC* gene encoding a mutant aspartokinase (U.S. Patent No. 6,040,160). *Escherichia coli* JM109 strain transformed with RSFD80 was named AJ12396 (U.S. Patent No. 6,040,160), the strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (currently, International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology) on October 28, 1993 and assigned an accession number of FERM P-13936, and the deposit was then converted to an international deposit under the provisions of Budapest Treaty on November 1, 1994 and assigned an accession number of FERM BP-4859. RSFD80 can be obtained from the AJ12396 strain by a known method.

Examples of parent strains which can be used to derive bacteria that are able to produce L-lysine also include strains having decreased or no activity of an enzyme that catalyzes a reaction that generates a compound other than L-lysine by branching off from the biosynthetic pathway of L-lysine. Examples of such enzymes include homoserine dehydrogenase, lysine decarboxylase (U.S. Patent No. 5,827,698), and the malic enzyme (WO2005/010175). In order to reduce or eliminate the lysine decarboxylase activity, the expression of both the *cadA* gene and *ldcC* gene coding for

lysine decarboxylase can be reduced (International Patent Publication WO2006/038695).

Examples of *cadA*- and *ldcC* genes-disrupted strain include the *Escherichia coli* WC196LC (WC196 Δ cadA Δ ldc) strain. The WC196LC strain, which was designated AJ110692, was deposited at National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary (Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) on October 7, 2008 as an international deposit, and assigned an accession number of FERM BP-11027.

2. Method of the present invention

Exemplary methods include producing an L-amino acid by cultivating the bacterium described herein in a culture medium to produce and secrete L-amino acid, such as L-arginine, L-citrulline, and/or L-lysine into the medium, and collecting L-amino acid from the medium.

The cultivation, collection, and purification of L-amino acid from the medium may be performed by conventional fermentation methods wherein an amino acid is produced using a bacterium.

The medium used for the culture may be either a synthetic or natural medium, so long as the medium includes a carbon source and a nitrogen source and minerals and, if necessary, appropriate amounts of nutrients which the chosen bacterium requires for growth. The carbon source may include various carbohydrates such as glucose and sucrose, and various organic acids. Depending on the mode of assimilation of the chosen microorganism, alcohol, including ethanol and glycerol, may be used. As the nitrogen source, various ammonium salts such as ammonia and ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean-hydrolysate, and digested fermentative microorganism can be used. As minerals, potassium monophosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium chloride, and the like can be used. As vitamins, thiamine, yeast extract, and the like, can be used.

The cultivation can be preferably performed under aerobic conditions, such as by a shaking culture, and by a stirring culture with aeration, at a temperature of 20 to 40 °C, preferably 30 to 38 °C. The pH of the culture is usually between 5 and 9, preferably between 6.5 and 7.2. The pH of the culture can be adjusted with ammonia, calcium carbonate, various acids, various bases, and buffers. Usually, 1 to 5-day cultivation leads to accumulation of L-amino acid in the liquid medium.

After cultivation, solids such as cells can be removed from the liquid medium by centrifugation or membrane filtration, and then L-amino acid can be collected and purified by ion-exchange, concentration, and/or crystallization methods.

Examples

The present invention will be more concretely explained below with reference to the following non-limiting Examples.

Example 1. Construction of a strain with an inactivated *pepA* gene.

1. Deletion of the *pepA* gene.

The *pepA* gene in a bacterial strain was deleted by the method initially developed by Datsenko, K.A. and Wanner, B.L. (Proc. Natl. Acad. Sci. USA, 2000, 97(12), 6640-6645) called "Red-driven integration". According to this procedure, the PCR primers P1 (SEQ ID NO: 3) and P2 (SEQ ID NO: 4), which are homologous to both the region adjacent to the *pepA* gene and the gene which confers antibiotic resistance in the template plasmid, were constructed. The plasmid pMW118-attL-Cm-attR (WO 05/010175) was used as the template in the PCR reaction. Conditions for PCR were as follows: initial DNA denaturation for 30 sec at 94 °C, followed by 25 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, elongation at 72 °C for 1 min 30 sec; and the final elongation for 2 min at 72 °C.

The 1.7 kb PCR product (Fig. 1) was purified from an agarose gel and used for electroporation of the *E. coli* strain MG1655 (ATCC 700926), which contains the plasmid pKD46. The pKD46 plasmid (Datsenko, K.A. and Wanner, B.L., Proc. Natl. Acad. Sci. USA, 2000, 97:12:6640-45) contains a temperature-sensitive replication origin, and includes a 2,154 nucleotide DNA fragment of phage λ (nucleotide positions 31088 to 33241, GenBank accession no. J02459), as well as the genes of the λ Red homologous recombination system (γ , β , exo genes), which are under the control of the arabinose-inducible P_{araB} promoter. The pKD46 plasmid is necessary for integration of the PCR product into the chromosome of the MG1655 strain. The strain MG1655 can be obtained from American Type Culture Collection. (P.O. Box 1549 Manassas, VA 20108, U.S.A.).

Electrocompetent cells were prepared as follows: *E. coli* MG1655/pKD46 cells were grown overnight at 30 °C in LB medium containing ampicillin (100 mg/l), and the culture was diluted 100 times with 5 ml of SOB medium (Sambrook et al, "Molecular Cloning: A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press, 1989) containing ampicillin and L-arabinose (1 mM). The cells were

grown with aeration at 30 °C to an OD₆₀₀ of ≈0.6 and then were made electrocompetent by concentrating 100-fold and washing three times with ice-cold deionized H₂O. Electroporation was performed using 70 µl of cells and ≈100 ng of the PCR product. Cells after electroporation were incubated with 1 ml of SOC medium (Sambrook et al, "Molecular Cloning: A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press, 1989) at 37 °C for 2.5 hours and then were plated onto L-agar containing chloramphenicol (30 µg/ml) and grown at 37 °C to select Cm^R recombinants. Then, to eliminate the pKD46 plasmid, two passages on L-agar with Cm at 42 °C were performed and the colonies were tested for sensitivity to ampicillin.

2. Verification of the *pepA* gene deletion by PCR.

Mutants in which the *pepA* gene had been deleted were marked with a Cm resistance gene and were verified by PCR using the locus-specific primers P3 (SEQ ID NO: 5) and P4 (SEQ ID NO: 6). For this purpose, a freshly isolated colony was suspended in 20 µl water and then 1 µl of the suspension was used for PCR. Conditions for PCR were as follows: initial DNA denaturation for 30 sec at 94 °C; then 30 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and elongation at 72 °C for 2 min; the final elongation for 2 min at 72 °C. The PCR product obtained in the PCR reaction using the cells of the parent *pepA*⁺ strain MG1655 as the template was 1.65 kb in length. The PCR product obtained in the PCR reaction using the cells of the mutant MG1655 Δ*pepA*::*cat* strain as the template was 1.71 kb nucleotides in length (Fig.2).

3. Elimination of Cm resistance gene (*cat* gene) from the chromosome of *E. coli* strain MG1655 Δ*pepA*::*cat*

The Cm resistance gene was eliminated from the chromosome of the *E. coli* MG1655 *pepA*::*cat* strain using the *int-xis* system. For that purpose, *E. coli* strain MG1655 *pepA*::*cat* was transformed with pMWts-Int/Xis plasmid (WO 05/010175). Transformant clones were selected on the LB-medium containing 100 µg/ml of ampicillin. Plates were incubated overnight at 30°C. Transformant clones were cured from the *cat* gene and pMWts-Int/Xis plasmid by spreading the separate colonies at 37°C (at this temperature repressor CIts is partially inactivated and transcription of the *int/xis* genes is derepressed) followed by selection of Cm^SAp^R variants. Elimination of the *cat* gene from the chromosome of the strain was verified by PCR with locus-specific primers P3 (SEQ ID NO: 5) and P4 (SEQ ID NO: 6). Conditions for PCR were as follows: initial DNA denaturation for 30 sec at 94 °C; then 30 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and elongation at 72 °C

for 2 min; the final elongation for 2 min at 72 °C. The PCR product obtained in the reaction with the cells of MG1655 Δ pepA::cat strain as a template, was 1.71 kb in length. The PCR product obtained in the reaction with cells without *cat* gene as a template was 160 bp in length. Thus, the strain with the inactivated *pepA* gene and *cat* gene deleted was obtained.

Example 2. Production of L-arginine by *E. coli* strain 382 Δ pepA.

To test the effect of inactivation of the *pepA* gene on L-arginine production, the DNA fragment from the chromosome of the above-described *E. coli* strain MG1655 Δ pepA was transferred to the L-arginine producing *E. coli* strain 382 by P1 transduction (Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, Plainview, NY) to obtain the *E. coli* 382 Δ pepA strain. The strain 382 has been deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia, 117545 Moscow, 1 Dorozhny proezd, 1) on April 10, 2000 under accession number VKPM B-7926 and then converted to a deposit under the Budapest Treaty on May 18, 2001.

E. coli strains, 382 and 382pepA, were separately cultivated with shaking at 37 °C for 18 hours in 3 ml of nutrient broth, and 0.3 ml of the cultures were inoculated into 2 ml of a fermentation medium in 20 x 200-mm test tubes and cultivated at 32 °C for 48 hours on a rotary shaker.

After the cultivation, the amount of L-arginine which has accumulated in the medium was determined by paper chromatography using the following mobile phase: butanol: acetic acid : water = 4 : 1 : 1 (v/v). A solution of ninhydrin (2%) in acetone was used as a visualizing reagent. A spot containing L-arginine was cut out, the L-arginine was eluted with 0.5% water solution of CdCl₂, and the amount of L-arginine was estimated spectrophotometrically at 540 nm.

The composition of the fermentation medium (g/l) is as follows:

Glucose	48.0
(NH ₄) ₂ SO ₄	35.0
KH ₂ PO ₄	2.0
MgSO ₄ ·7H ₂ O	1.0
Thiamine HCl	0.0002
Yeast extract	1.0
L-isoleucine	0.1
CaCO ₃	5.0

Glucose and magnesium sulfate are sterilized separately. CaCO₃ is dry-heat sterilized at 180 C for 2 hours. The pH is adjusted to 7.0.

The results of test tube fermentations are shown in Table 1. As it can be seen from Table 1, the strain with an inactivated *pepA* gene was able to produce a higher amount of L-arginine as compared with the parent L-arginine producing *E. coli* strain 382.

Table 1

Strain	Amount of L-arginine, g/l
382	8.0 ± 0.2
382Δ <i>pepA</i>	9.4 ± 0.7

Example 3. Production of citrulline by *E. coli* strain 382Δ*pepA*.

To test the effect of inactivation of the *pepA* gene on L-arginine production, the DNA fragment from the chromosome of the above-described *E. coli* strain MG1655 Δ*pepA* can be transferred to *E. coli* citrulline-producing strain 382Δ*argG* by P1 transduction (Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, Plainview, NY) to obtain *E. coli* 382Δ*argG*Δ*pepA* strain. The strain 382Δ*argG* can be obtained by deletion of *argG* gene on the chromosome of 382 strain (VKPM B-7926) by the method initially developed by Datsenko, K.A. and Wanner, B.L. (Proc. Natl. Acad. Sci. USA, 2000, 97(12), 6640-6645) called "Red-driven integration". According to this procedure, the PCR primers homologous to both the region adjacent to the *argG* gene and the gene which confers antibiotic resistance in the template plasmid can be constructed. The plasmid pMW118-attL-Cm-attR (WO 05/010175) can be used as the template in the PCR reaction.

Both *E. coli* strains, 382Δ*argG* and 382Δ*argG*Δ*pepA*, can be separately cultivated with shaking at 37 °C for 18 hours in 3 ml of nutrient broth, and 0.3 ml of the obtained cultures were inoculated into 2 ml of a fermentation medium in 20 x 200-mm test tubes and cultivated at 32 °C for 48 hours on a rotary shaker.

After the cultivation, the amount of citrulline which accumulates in the medium can be determined by paper chromatography using the following mobile phase: butanol : acetic acid : water = 4 : 1 : 1 (v/v). A solution of ninhydrin (2%) in acetone can be used as a visualizing reagent. A spot containing citrulline can be cut out, citrulline can be eluted with 0.5% water solution of CdCl₂, and the amount of citrulline can be estimated spectrophotometrically at 540 nm.

The composition of the fermentation medium (g/l) can be as follows:

Glucose	48.0
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(NH ₄) ₂ SO ₄	35.0
KH ₂ PO ₄	2.0
MgSO ₄ ·7H ₂ O	1.0
Thiamine HCl	0.0002
Yeast extract	1.0
L-isoleucine	0.1
L-arginine	0.1
CaCO ₃	5.0

Glucose and magnesium sulfate are sterilized separately. CaCO₃ is dry-heat sterilized at 180 °C for 2 hours. The pH is adjusted to 7.0.

Example 4: Construction of L-lysine-producing bacterium having decreased amino -peptidase activity

Strains which do not express amino -peptidase A or B or D (WC196Δ*cadA*Δ*ldcC*Δ*pepA*::Km, WC196Δ*cadA*Δ*ldcC*Δ*pepB*::Km and WC196Δ*cadA*Δ*ldcC*Δ*pepD*::Km strains) were constructed from the WC196Δ*cadA*Δ*ldcC* strain.

The WC196Δ*cadA*Δ*ldcC* strain is able to produce L-lysine and was used to derive the object *pep* gene-deficient strain. Each *pep* gene in WC196Δ*cadA*Δ*ldcC* was deleted by the method called "Red-driven integration", in similar manner shown in Example 1.

The *pepA*, *pepB* or *pepD* gene was deleted by using the primers of SEQ ID NOS: 11 and 12 for *pepA*, the primers of SEQ ID NOS: 13 and 14 for *pepB*, the primers of SEQ ID NOS: 15 and 16 for *pepD*, respectively. The WC196Δ*cadA*Δ*ldcC*Δ*pepA*::Km, WC196Δ*cadA*Δ*ldcC*Δ*pepB*::Km and WC196Δ*cadA*Δ*ldcC*Δ*pepD*::Km strains in which each amino -peptidase gene was deleted, were thereby obtained. The *cadA* gene and the *ldcC* gene in the WC196Δ*cadA*Δ*ldcC* strain may also be deleted in a similar manner.

Furthermore, each strain was cultured at 37°C in L medium until the final OD₆₀₀ of the culture became about 0.6. Then, an equal volume of a 40% glycerol solution was added to the culture, the mixture was stirred, and then divided into appropriate volumes and stored at -80°C. These are called glycerol stocks.

Example 5: Evaluation of L-lysine producing ability of the strain which decreased amino -peptidase activity

Each of the glycerol stocks of the strains was thawed, and 100 μL of each was evenly applied to an L plate and cultured at 37°C for 24 hours. The above glycerol

stocks were thawed, and uniformly applied to an L-plate and culture was performed at 37°C for 24 hours. The suspension of the obtained cells was diluted 101 times, OD₆₂₀ value (n) of the diluted suspension was measured, and the cell suspension in a volume corresponding to 50/n was inoculated into 20 mL of a fermentation medium in a 500-mL Sakaguchi flask, and culture was performed at 37°C for 48 hours on a reciprocally shaking culture machine (115 rpm). After the culture, amount of L-lysine accumulated in the medium was measured in a known manner (Biotec Analyzer AS210, SAKURA SEIKI).

The composition of the fermentation medium (g/l) is as follows:

Composition of L-lysine fermentation medium:

Glucose	40
(NH ₄) ₂ SO ₄	24
K ₂ HPO ₄	1.0
MgSO ₄ ·7H ₂ O	1.0
FeSO ₄ ·7H ₂ O	0.01
MnSO ₄ ·5H ₂ O	0.01
Yeast extract	2.0
CaCO ₃ (Japanese Pharmacopoeia)	30
Distilled water	To final volume of 1 L

The medium was adjusted to pH 7.0 with KOH, and autoclaved at 115°C for 10 minutes. Glucose and MgSO₄·7H₂O were separately dry-heat sterilized at 180 C for 2 hours.

The results are shown in Table 2. The yield (%) represents the yield of L-lysine based on glucose. As it can be seen from Table 2, the WC196ΔcadAΔldcCΔpepA, WC196ΔcadAΔldcCΔpepB, WC196ΔcadAΔldcCΔpepD, strains were able to produce a higher amount of L-lysine as compared with the parent L-lysine producing *E. coli* strain WC196ΔcadAΔldcC.

Table 2

CT48	OD620	Lys (g/L)	Glc (g/L)	Yield (%)
WC196 ΔcadA ΔldcC	26.0	1.4	0.0	3.5
WC196 ΔcadA ΔldcC ΔpepA::Km	26.8	1.6	0.0	3.9
WC196 ΔcadA ΔldcC ΔpepB::Km	25.9	1.6	0.0	4.0
WC196 ΔcadA ΔldcC ΔpepD::Km	25.6	1.6	0.0	4.0

While the invention has been described in detail with reference to preferred embodiments thereof, it will be apparent to one skilled in the art that various changes

can be made, and equivalents employed, without departing from the scope of the invention. All the cited references herein are incorporated as a part of this application by reference.

Industrial Applicability

According to the present invention, production of L-arginine, L-citrulline, and L-lysine by a bacterium of *Enterobacteriaceae* family can be improved.

CLAIMS

1. A bacterium of the *Enterobacteriaceae* family having ability to produce L-amino acid, wherein said bacterium has been modified to attenuate expression of one or more genes selected from the group consisting of *pepA*, *pepB*, and *pepD* genes.

2. The bacterium according to claim 1, wherein expression of the gene(s) is/are attenuated by inactivation of the gene(s).

3. The bacterium according to claim 1 or 2, wherein said bacterium belongs to the genus *Escherichia*.

4. The bacterium according to claim 3, wherein said bacterium is *Escherichia coli*.

5. The bacterium according to claim 1 or 2, wherein said bacterium belongs to the genus *Pantoea*.

6. The bacterium according to any one of claims 1 to 5, wherein the L-amino acid is selected from the group consisting of L-arginine, L-citrulline, and L-lysine.

7. The bacterium according to any one of claims 1 to 6, wherein the bacterium has been modified to attenuate expression of *pepA*, *pepB*, and *pepD* genes, and the L-amino acid is L-lysine.

8. A method for producing an L-amino acid comprising:
cultivating the bacterium according to any of claims 1 to 7 in a culture medium,
and
collecting the L-amino acid from the culture medium.

9. The method according to claim 8, wherein said L-amino acid is selected from the group consisting of L-arginine, L-citrulline, and L-lysine.

Fig. 1

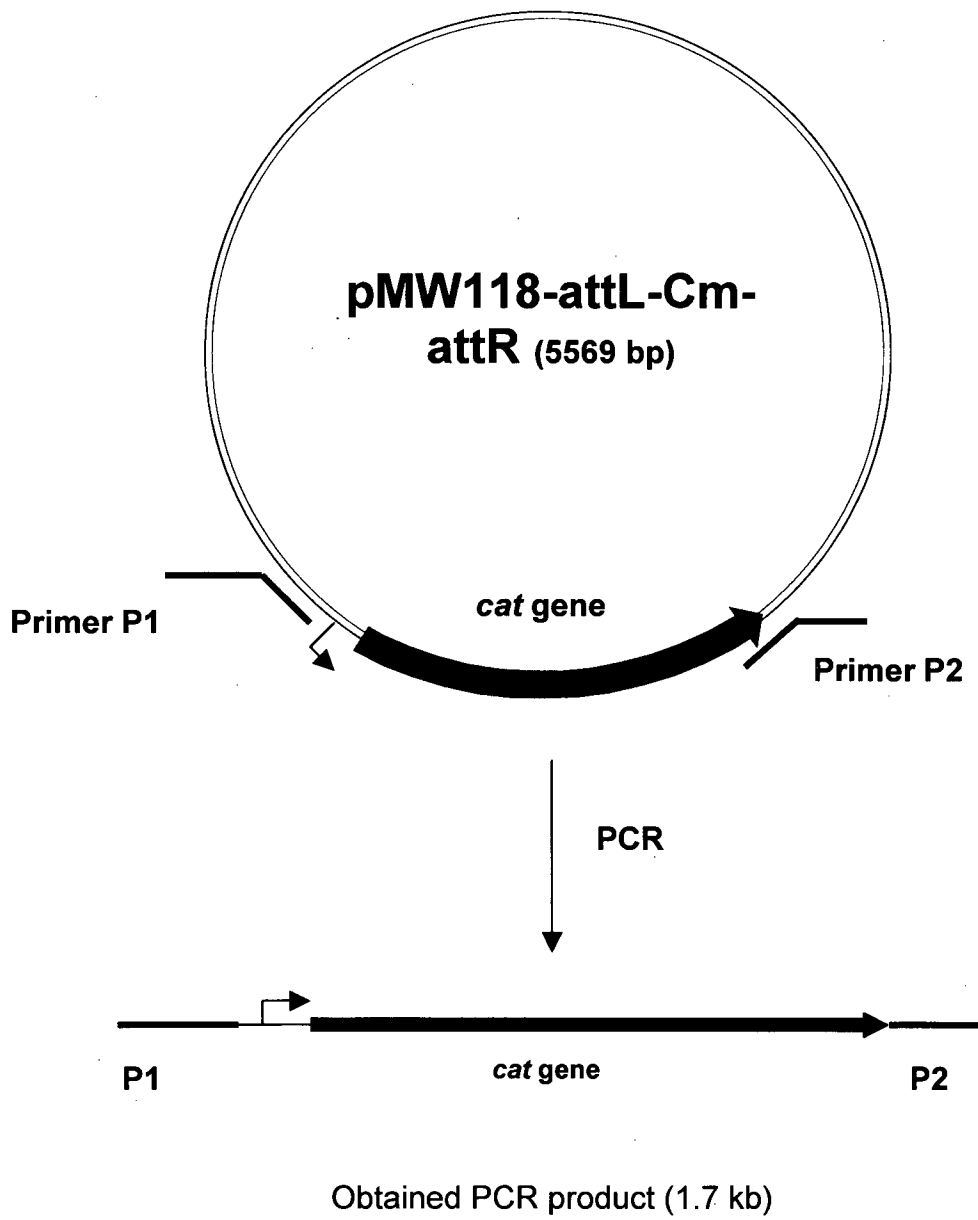
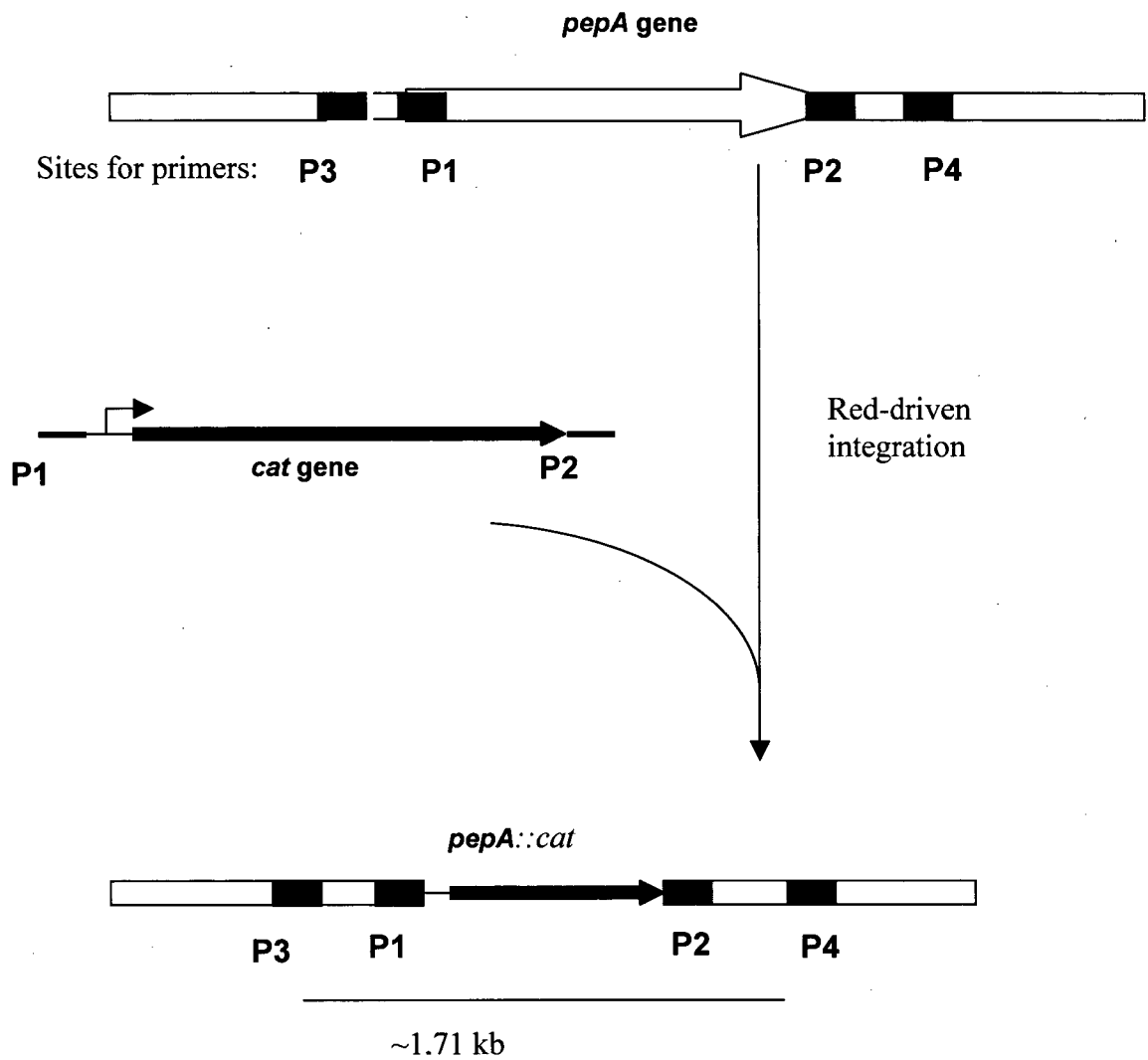


Fig. 2



INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2011/063301

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P13/08 C12P13/10
ADD.

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)
C12P

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

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

EP0-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 870 457 A1 (KYOWA HAKKO KOGYO KK [JP]) 26 December 2007 (2007-12-26) paragraph [0076] - paragraph [0130]; example 1	1-9
X	----- KAZUHIKO TABATA ET AL: "Fermentative Production of L-Alanyl-L-Glutamine by a Metabolically Engineered Escherichia coli Strain Expressing L-Amino Acid .alpha.-Ligase", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 73, no. 20, 1 October 2007 (2007-10-01), pages 6378-6385, XP007912741, ISSN: 0099-2240, DOI: 10.1128/AEM.01249-07 [retrieved on 2007-08-24] page 6378 - page 6384 -----	1-4,7



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

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"&" document member of the same patent family

Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/JP2011/063301

Patent document cited in search report	Publication date	Patent family member(s)	Publication date	
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			WO 2006104186 A1	05-10-2006
			US 2009130708 A1	21-05-2009
