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(54) **Title**: A METHOD FOR PRODUCING AN L-AMINO ACID BELONGING TO THE GLUTAMATE FAMILY, USING A CORYNEFORM BACTERIUM

(57) **Abstract**: The present invention provides a method for producing an L-amino acid belonging to the glutamate family, using a coryneform bacterium which has been modified so that expression of one or more gene(s) of the *NCgl_2067-NCgl_2065* operon in said bacterium is/are attenuated.

DESCRIPTION

A METHOD FOR PRODUCING AN L-AMINO ACID BELONGING TO THE GLUTAMATE FAMILY, USING A CORYNEFORM BACTERIUM

Tecnical Field

The present invention relates to the microbiological industry, and specifically to a method for producing an L-amino acid belonging to the glutamate family using a coryneform bacterium which has been modified so that expression of one or more gene(s) of the *NCgl_2067-NCgl_2065* operon in the bacterium is/are attenuated.

Background Art

Conventionally, L-amino acids are industrially produced by fermentation methods utilizing strains of microorganisms obtained 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 by the resulting L-amino acid (see, for example, WO 95/16042 or US patent Nos. 4,346,170; 5,661,012 and 6,040,160).

Many methods for producing L-glutamine by culturing a coryneform bacterium have been disclosed. For example, EP1229121 A2 discloses a method for producing L-glutamine by culturing a coryneform bacterium which has L-glutamine producing ability and has been modified so that its intracellular glutamine synthetase activity is enhanced, and preferably has been further modified so that its intracellular glutamate dehydrogenase activity is enhanced, in a medium to produce and cause accumulation of L-glutamine in the medium, and collecting the L-glutamine.

It is known that transcription of the *Bacillus subtilis* gene coding of glutamine synthetase (*glnA*) is regulated by the nitrogen source. The *glnA* gene is located in an operon with the *glnR* gene. The *glnR* gene codes for a negative regulator which directly controls expression of the glutamine synthetase gene (*glnA*). Certain mutations in *glnR* gene, such as a large, in-frame deletion and a start codon mutation, lead to high-level constitutivity of the operon; whereas other mutations caused low-level constitutivity (Schreier HJ et al., J Mol Biol. 1989, 210(1):51-63).

Recently, regulation of glutamine and glutamate metabolism by the nitrogen regulatory protein GlnR in *Streptococcus pneumoniae* was analyzed. Using DNA microarray analyses of *S. pneumoniae* D39 wild-type and its isogenic *glnR* mutant which had been grown in nitrogen-rich medium supplemented with glutamine, a list of operons and genes that were up-regulated the most in the *glnR* mutant was determined, and includes the following genes: *glnA* (encoding glutamine synthetase GlnA), *glnPQ* (encoding ABC transporter amino acid-binding protein/permease), and *gdhA* (encoding glutamate dehydrogenase glutamate dehydrogenase). All of these genes have a GlnR operator in their promoter regions. Also the *zwf* gene, encoding the glucose-6-phosphate dehydrogenase and located downstream of, and in the same orientation as *glnPQ,* was up-regulated (Kloosterman et al., J Biol Chem. 2006, 281(35):25097-25109). Currently, there have been no reports about the presence of the putative *glnR* gene in the genomes of coryneform bacteria.

There have been no reports of attenuating expression of one or more genes of the *NCgl_2067-NCgl_2065* operon for the purpose of improving productivity of L-amino acid belonging to the glutamate family.

Disclosure of the Invention

Aspects of the present invention include enhancing the productivity of strains which produce L-amino acids belonging to the glutamate family, and providing a method for producing an L-amino acid belonging to the glutamate family, using these strains.

An open reading frame, (ORF) NCgl_2066, was found in the genome of *Corynebacterium glutamicum* which is homologous to the *glnR* gene from *Streptococcus pneumoniae.* The ORF NCgl_2066 is located in an operon with two other ORFs - NCgl_2067 and NCgl_2065. The correspoding ORFs were named *NCgl_2067, NCgl_2066,* and *NCgl_2065* genes, and the operon was given the name *NCgl_2067-NCgl_2065* operon.

The above aspects were achieved by finding that attenuation of the expression of one or more gene(s) of the *NCgl_2067-NCgl_2065* operon can enhance production of L-amino acids belonging to the glutamate family, such as L-glutamic acid, L-glutamine, L-proline, L-histidine, L-arginine, L-ornithine, and L-citrulline.

The present invention provides a coryneform bacterium having an increased ability to produce amino acids belonging to the glutamate family, such as L-glutamic acid, L-glutamine, L-proline, L-histidine, L-arginine, L-ornithine, and L-citrulline.

It is an aspect of the present invention to provide a coryneform bacterium which is able to produce an L-amino acid belonging to the glutamate family, wherein said

bacterium has been modified so that the expression of one or more gene(s) of the *NCgl_2067-NCgl_2065* operon in said bacterium is attenuated.

It is a further aspect of the present invention to provide the bacterium as described above, wherein said bacterium has been modified to attenuate the expression of the *NCgl_2067* gene.

It is a further aspect of the present invention to provide the bacterium as described above, wherein said expression of the *NCgl_2067* gene is attenuated by inactivation of the *NCgl_2067* gene.

It is a further aspect of the present invention to provide the bacterium as described above, wherein said bacterium has been modified to attenuate the expression of the *NCgl_2066* gene.

It is a further aspect of the present invention to provide the bacterium as described above, wherein said expression of the *NCgl_2066* gene is attenuated by inactivation of the *NCgl_2066* gene.

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

It is a further aspect of the present invention to provide the bacterium as described above, wherein said bacterium is *Brevibacterium flavum.*

It is a further aspect of the present invention to provide the bacterium as described above, wherein said L-amino acid belonging to the glutamate family is selected from the group consisting of L-glutamic acid, L-glutamine, L-proline, L-histidine, L-arginine, L-ornithine and L-citrulline.

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

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

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

It is a further aspect of the present invention to provide the method as described above, wherein said L-amino acid belonging to the glutamate family is selected from the group consisting of L-glutamic acid, L-glutamine, L-proline, L-histidine, L-arginine, L-ornithine, and L-citrulline.

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

The present invention is described in detail below.

Brief Description of the Drawings

Figure 1 shows alignment of the GlnR (denoted as spr0433) protein from *Streptococcus pneumoniae* R6 and putative NCgl_2066 protein from *Corynebacterium glutamicum* ATCC13032.

Figure 2 shows structure of the *NCgl_2067-NCgl_2065* operon from *Corynebacterium glutamicum* ATCC13032

Figure 3 shows structure of the pBS4S plasmid.

Detailed Description of the Preferred Embodiments

The present invention is described in detail below.

1. Bacterium of the present invention

A coryneform bacterium is described which can produce an L-amino acid belonging to the glutamate family, wherein the bacterium has been modified to attenuate expression of one or more gene(s) of the *NCgl_2067-NCgl_2065* operon.

The phrase "bacterium producing an L-amino acid" can mean a bacterium which has an ability to produce and excrete an L-amino acid into a medium, when the bacterium is cultured in the medium.

The phrase "bacterium producing an L-amino acid" as used herein also can mean a bacterium which is able to produce and cause accumulation of an L-amino acid in a culture medium in an amount larger than a wild-type or parental strain ofcoryneform bacterium, for example, bacterial strains listed below, such as *Corynebacterium glutamicum* ATCC 13032, ATCC 31833, *Brevibacterium flavum* ATCC 13826, ATCC 14067, AJ12418 and B-6642, and can mean that the microorganism is able to cause accumulation in a medium of an amount not less than 0.5g/l, or 1.0g/l, of the target L-amino acid.

The phrase "L-amino acid belonging to the glutamate family" can include L-glutamic acid, L-glutamine, L-proline, L-histidine, L-arginine, L-ornithine, L-citrulline, and combinations thereof.

The term "coryneform bacteria" can include conventional coryneform bacterium according to the classification known to a person skilled in the art of microbiology, and also can include bacteria that had been classified into the genus *Brevibacterium*, but are

currently classified into the genus *Corynebacterium* (Liebl W et al., Int J Syst Bacteriol. 1991, 41(2):255-260), as well as the *Brevibacterium* bacteria that are highly related to *Corynebacterium* bacteria. Examples of such coryneform bacterium include the following:

Corynebacterium acetoacidophilum
Corynebacterium acetoglutamicum
Corynebacterium alkanolyticum
Corynebacterium callunae
Corynebacterium glutamicum (*Micrococcus glutamicus*)
Corynebacterium lilium
Corynebacterium melassecola
Corynebacterium thermoaminogenes (Corynebacterium efficiens)
Corynebacterium herculis
Brevibacterium divaricatum
Brevibacterium flavum
Brevibacterium immariophilum
Brevibacterium lactofermentum (Corynebacterium glutamicum)
Brevibacterium roseum
Brevibacterium saccharolyticum
Brevibacterium thiogenitalis
Brevibacterium ammoniagenes
Brevibacterium album
Brevibacterium cerinum
Microbacterium ammoniaphilum,
Microbacterium flavum (*Corynebacterium flavescens*) etc.

Specific examples of the coryneform bacterium include, but are not limited to, strains such as *C. acetoacidophilum* ATCC 13870, *C. acetoglutamicum* ATCC 15806, *C. alkanolyticum* ATCC 21511, *C. callunae* ATCC 15991, *C. glutamicum* ATCC13020, ATCC 13032, ATCC 13060, ATCC 13869, *C. lilium* ATCC 15990, *C. melassecola* ATCC 17965, *C. thermoaminogenes* AJ12340 (FERM BP-1539), *C. herculis* ATCC 13868, *B. divaricatum* ATCC 14020, *B. flavum* ATCC 13826, ATCC 14067, AJ12418 (FERM BP-2205), *B. immariophilum* ATCC 14068, *B. lactofermentum* (*C. glutamicum*) ATCC 13869, *B. roseum* ATCC 13825 *B. saccharolyticum* ATCC 14066, *B. thiogenitalis* ATCC 19240, *B. ammoniagenes* ATCC 6871, ATCC 6872, *B. album* ATCC 15111, *B. cerinum* ATCC 15112, *Microbacterium ammoniaphilum* ATCC 15354, and so forth.

These strains are available from the American Type Culture Collection (ATCC, Address: P.O. Box 1549, Manassas, VA 20108, United States of America). That is, each strain is given a unique registration number which is listed in the catalogue of the ATCC. Strains can be ordered using this registration number. The AJ 12340 strain was deposited at 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 at Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305-5466, Japan) on October 27, 1989 under the provisions of the Budapest Treaty and given an accession number of FERM BP-1539. The AJ12418 strain was deposited at National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry on January 5, 1989 under the provisions of the Budapest Treaty and given an accession number of FERM BP-2205.

The phrase "a bacterium belonging to the genus *Corynebacterium* or *Brevibacterium*" can mean that the bacterium is classified into the genus *Corynebacterium* or *Brevibacterium* according to the classification known to a person skilled in the art of microbiology. Examples of a bacterium belonging to the genus *Corynebacterium* or *Brevibacterium* include, but are not limited to, *Corynebacterium glutamicum* and *Brevibacterium flavum*.

The phrase "bacterium has been modified to attenuate expression of one or more gene(s) of the *NCgl_2067-NCgl_2065* operon" can mean that the bacterium has been modified in such a way that the modified bacterium contains a reduced amount of one or more of the proteins, NCgl_2067, NCgl_2066 and NCgl_2065 as compared with an unmodified bacterium, or the modified bacterium is unable to synthesize the NCgl_2067, NCgl_2066 and/or NCgl_2065 protein(s). The phrase "bacterium has been modified to attenuate expression of one or more gene(s) of the *NCgl_2067-NCgl_2065* operon" also can mean that the bacterium has been modified in such a way that the modified gene(s) encodes mutant NCgl_2067, NCgl_2066 and/or NCgl_2065 protein(s) with decreased activities. Since it is thought that genes *NCgl_2067, NCgl_2066* and *NCgl_2065* are organized as an operon, attenuation of expression of one of the genes can lead to polar effects on expression other genes of the *NCgl_2067-NCgl_2065* operon.

The presence or absence of gene(s) of the *NCgl_2067-NCgl_2065* operon in 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 determined 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

amounts of the proteins encoded by the genes of the *NCgl_2067-NCgl_2065* operon can be measured by well-known methods, including SDS-PAGE followed by an immunoblotting assay (Western blotting analysis), and the like.

The phrase "inactivation of one or more gene(s) of the *NCgl_2067-NCgl_2065* operon" can mean that the modified gene(s) encodes a completely inactive protein(s). It is also possible that the modified DNA region is unable to naturally express the gene due to deletion of a part of or the entire gene, shifting of the reading frame of the gene, introduction of missense/nonsense mutation(s), or modification of an adjacent region of the gene, including sequences controlling gene expression, such as promoter(s), enhancer(s), attenuator(s), ribosome-binding site(s), etc.

The *NCgl_2067* gene (synonyms – *CG2358,* Cgl2147) codes for a hypothetical protein with unknown function. The *NCgl_2067* gene of *C. glutamicum* (nucleotides complement to the nucleotides from 2270870 to 2270256 in the GenBank accession number NC_003450; gi: 58036263) is located between the *NCgl_2066* and the *ileS* genes, on the chromosome of *C. glutamicum* ATCC 13032. The nucleotide sequence of the *NCgl_2067* gene and the amino acid sequence of predicted NCgl_2067 protein encoded by the *NCgl_2067* gene are shown in SEQ ID NO: 1 and SEQ ID NO: 2, respectively.

The *NCgl_2066* gene (synonyms – *CG2357,* Cgl2146) codes for a hypothetical putative transcriptional regulator. The *NCgl_2066* gene of *C. glutamicum* (nucleotides complement to the nucleotides from 2,270,262 to 2,269,258 in the GenBank accession number NC_003450; gi: 58036263) is located between the *NCgl_2067* and *NCgl_2065* gene on the chromosome of *C. glutamicum* ATCC 13032, and both genes are oriented in the same direction as the *NCgl_2066* gene, and these three genes are presumably organized as an operon. The nucleotide sequence of the *NCgl_2066* gene and the amino acid sequence of predicted NCgl_2066 protein encoded by the *NCgl_2066* gene are shown in SEQ ID NO: 3 and SEQ ID NO: 4, respectively.

The *NCgl_2065* gene (synonyms – *CG2356,* Cgl2145) is predicted to encode a DMT family permease. The *NCgl_2065* gene of *C. glutamicum* (nucleotides complement to the nucleotides from 2,269,246 to 2,268,386 in the GenBank accession number NC_003450; gi: 58036263) is located between the *NCgl_2066* and *NCgl_2064* genes on the chromosome of *C. glutamicum* ATCC 13032. The nucleotide sequence of the *NCgl_2066* gene and the amino acid sequence of predicted NCgl_2066 protein encoded by the *NCgl_2066* gene are shown in SEQ ID NO: 5 and SEQ ID NO: 6, respectively.

Since there may be some differences in DNA sequences between the genera or strains of coryneform bacteria, genes of the *NCgl_2067-NCgl_2065* operon to be inactivated on the chromosome are not limited to the genes shown in SEQ ID NO: 1, SEQ

ID NO: 3 and SEQ ID NO: 5, but can include genes homologous to SEQ ID NO: 1, SEQ ID NO: 3, and SEQ ID NO: 5, which encode a variant proteins of the NCgl_2067, NCgl_2066 and NCgl_2065 proteins. 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 product as the NCgl_2067, NCgl_2066 or NCgl_2065 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 can be 1 to 30, in another example 1 to 15, and in another example 1 to 5 in SEQ ID NO: 2, SEQ ID NO: 4, and SEQ ID NO:6. These changes in the variant 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 each gene of the *NCgl_2067-NCgl_2065* operon can have a homology of not less than 80%, in another example not less than 90%, and in another example not less than 95%, in another example not less than 98%, in another example not less than 99%, with respect to the entire amino acid sequence shown in SEQ ID NO: 2, SEQ ID NO: 4, or SEQ ID NO:6, as long as the activity of the NCgl_2067, NCgl_2066 or NCgl_2065 protein prior to inactivation of each gene of the *NCgl_2067-NCgl_2065* operon is maintained.

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, each gene of the *NCgl_2067-NCgl_2065* operon can be a variant which hybridizes under stringent conditions with the nucleotide sequence complementary to the sequence shown in SEQ ID NO: 1, SEQ ID NO: 3, or SEQ ID NO: 5, or a probe which can be prepared from the nucleotide sequence under stringent conditions, provided that it encodes a functional NCgl_2067, NCgl_2066, or NCgl_2065 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%, and in another example not less than 95%, in another example not less than 98% , 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 can be exemplified by washing one time or more, or in another example, two or three times at a salt concentration of 1 × SSC, 0.1% SDS, or in another example, 0.1 × SSC, 0.1% SDS at 60 °C. Duration of washing depends on the type of membrane used for blotting and, as a rule,

can be what is recommended by the manufacturer. For example, the recommended duration of washing for the Hybond™ N+ nylon membrane (Amersham) under stringent conditions is 15 minutes. The washing step can be performed 2 to 3 times. The length of the probe can be suitably selected depending on the hybridization conditions, and is usually 100 bp to 1 kbp.

Expression of gene(s) of the *NCgl_2067-NCgl_2065* operon can be attenuated by introducing a mutation into the gene(s) on the chromosome so that intracellular activity of the protein(s) encoded by the gene(s) are/is decreased as compared with an unmodified strain. Such a mutation on the gene can be 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. 1997, 272:8611-8617; Kwon, D. H. et al., J. Antimicrob. Chemother. 2000, 46:793-796). Expression of genes of the *NCgl_2067-NCgl_2065* operon 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 1999, 15:58-64).

For example, the following methods can be employed to introduce a mutation by gene recombination. A mutant gene encoding a mutant protein having a decreased activity is prepared, and the bacterium to be modified is 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 is selected. Such gene replacement using homologous recombination can be conducted by a method employing a linear DNA, which is known as "Red-driven integration" (Datsenko, K.A. and Wanner, B.L., Proc. Natl. Acad. Sci. USA 2000, 97(12):6640-6645), or by methods employing a plasmid containing a temperature-sensitive replication origin (U.S. Patent 6,303,383 or JP 05-007491A). Furthermore, the incorporation of a site-specific mutation by gene substitution using homologous recombination such as set forth above can also be conducted with a plasmid lacking the ability to replicate in the host.

Expression of the gene can also be attenuated by insertion of 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 a mutagenesis treatment using a UV irradiation or a mutagen such as nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine).

Inactivation of the gene can also be performed by conventional methods, such as a mutagenesis treatment using UV irradiation or a mutagen such as 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".

As L-glutamic acid is a precursor in biosynthesis of L-glutamine, L-proline, L-histidine L-arginine, L-ornithine, and L-citrulline, increasing production of L-glutamic acid results in increased production of a part or all of these amino acids.

Methods for preparation of plasmid DNA, digestion and ligation of DNA, transformation, selection of an oligonucleotide as a primer, 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

As a bacterium in accordance with the presently disclosed subject matter which is modified to attenuate expression of one or more gene(s) of the *NCgl_2067-NCgl_2065* operon, bacteria which are able to produce L-amino acids belonging to the glutamate family are described.

The bacterium in accordance with the presently disclosed subject matter can be obtained by attenuating expression of one or more genes of the *NCgl_2067-NCgl_2065* operon in a bacterium which inherently has the ability to produce L-amino acids. Alternatively, the bacterium can be obtained by imparting the ability to produce L-amino acids to a bacterium already having the attenuated expression of one or more genes of the *NCgl_2067-NCgl_2065* operon.

L-glutamic acid-producing bacteria

Examples of parent strains which can be used to derive L-glutamic acid-producing bacteria can include, but are not limited to, strains belonging to coryneform bacteria, such as strains *C. glutamicum* ATCC 13032, ATCC 31833, *C. acetoacidophilum* ATCC 13870, *C. herculis* ATCC 13868, *C. lilium* ATCC 15990, *B. divaricatum* ATCC 14020, *B. flavum* ATCC 14067, *B. imaliophilum* ATCC 14068, *B. lactofermentum* ATCC 13869, *B. thiogenitalis* ATCC 19240, and so forth.

Examples of parent strains which can be used to derive L-glutamic acid-producing bacteria also include strains in which expression of one or more genes encoding an L-glutamic acid enzyme are enhanced. Examples of such genes include genes encoding glutamate dehydrogenase, glutamine synthetase, glutamate synthetase, isocitrate

dehydrogenase, aconitate hydratase, citrate synthase, phosphoenolpyruvate carboxylase, pyruvate carboxylase, pyruvate dehydrogenase, pyruvate kinase, phosphoenolpyruvate synthase, enolase, phosphoglyceromutase, phosphoglycerate kinase, glyceraldehyde-3-phophate dehydrogenase, triose phosphate isomerase, fructose bisphosphate aldolase, phosphofructokinase, and glucose phosphate isomerase.

Methods of enhancing gene expression can include increasing the gene copy number by introducing a gene into a vector that is able to function in a coryneform bacterium. Gene expression can also be enhanced by introducing multiple copies of the gene into a bacterial chromosome by, for example, homologous recombination, Mu integration, or the like. The copy number of a gene can also be increased by introducing multiple copies of the gene into the chromosomal DNA of the bacterium. To introduce multiple copies of the gene into a bacterial chromosome, homologous recombination can be carried out using multiple copies of a sequence as targets in the chromosomal DNA. Gene expression can also be enhanced by placing the objective DNA under the control of a potent promoter.

Alternatively, the effect of a promoter can be enhanced by, for example, introducing a mutation into the promoter to increase the transcription level of a gene located downstream of the promoter. Furthermore, it is known that the substitution of several nucleotides in the spacer region between ribosome binding site (RBS) and the start codon, especially the sequences immediately upstream of the start codon, can profoundly affect the mRNA translatability. For example, a 20-fold range in the expression levels was found, depending on the nature of the three nucleotides preceding the start codon (Gold et al., Annu. Rev. Microbiol. 1981, 35:365-403; Hui et al., EMBO J. 1984, 3:623-629). Moreover, it is also possible to introduce a nucleotide substitution into a promoter region of a gene on the bacterial chromosome, which results in a stronger promoter function. The alteration of the expression control sequence can be performed, for example, in the same manner as the gene substitution using a temperature-sensitive plasmid, as disclosed in WO 00/18935 and JP 1-215280 A.

Examples of microorganisms modified so that expression of the citrate synthetase gene, the phosphoenolpyruvate carboxylase gene, and/or the glutamate dehydrogenase gene is/are enhanced can include those microorganisms disclosed in the following: JP2001-333769A (EP1078989A), JP2000-106869A (EP955368A), JP2000-189169A(EP952221A), and JP2001-333769A (EP1078989A). Examples of a method of imparting L-glutamic acid-producing ability to coryneform bacterium can include increasing expression of the *fasR* gene and increasing of sensitivity to a surfactant (WO2007024010 A1). Examples of an L-glutamic acid-producing coryneform bacterium

can include a strain which has been modified to enhance expression of the *yggB* gene (JP2007-097573 A).

Other examples of parent strains which can be used to derive L-glutamic acid-producing bacteria include strains in which expression of one or more genes encoding enzymes that catalyze synthesis of a compound other than L-glutamic acid, but which direct away from the L-glutamic acid biosynthesis pathway, are attenuated. Examples of such genes include genes encoding isocitrate lyase, α-ketoglutarate dehydrogenase, phosphotransacetylase, acetate kinase, acetohydroxy acid synthase, acetolactate synthase, formate acetyltransferase, lactate dehydrogenase, and glutamate decarboxylase. For attenuation of expression levels of such genes, the methods described above may be used.

Examples of L-glutamic acid-producing coryneform bacterium can include a strain in which the α-ketoglutarate dehydrogenase activity is decreased (JP7-834672A and JP06-237779), strain with inactive *gluX* gene (US 2010279363 A1), a strain with attenuated expression of *sucC* and/or *sucD* genes (EP1103611 A1), and so forth.

Specific examples of strains having an L-glutamic acid-producing ability can also include strains which are resistant to benzopirone or naphtoquinone (JP56-1889A), monofluoroacetic acid (JP 50-113209A), to adenine and thymine (JP57-065198), to α-ketomalonic acid (JP57-2689A), to guanidine (JP56-35981A), to daunomicin (JP58-158192A), and stains which are sensitive to penicillin (JP04-88994A).

Specific examples of L-glutamic acid-producing coryneform bacterium can also include the following strains: *B. flavum* AB949 (FERM BP-2632) (JP 50- 113209A), AJl1355 (FERM P-5007) (JP56-1889A), AJl1217 (FERM P-4318) (JP57-2689A), AJl1564 (FERM P-5472) (JP56-140895A), AJ11439 (FERM P-5136) (JP56-35981A), *B. lactofermentum* AJl1426 (FERM P5123) (JP56-048890A), AJ11796 (FERM P6402) (JP58-158192A), *C. glutamicum* AJl1628 (FERM P-5736) (JP 57-065198A), AJ11440 (FERM P5137) (JP56-048890A), H7684 (FERM BP-3004; JP04-88994A), AJl1355 (FERM P-5020) (JP56-1889A), AJ11218 (FERM P-4319) (JP57-2689A).

L-glutamine-producing bacteria

Examples of parent strains which can be used to derive L-glutamine-producing bacteria can include, but are not limited to, strains belonging to coryneform bacteria, such as strains *B. flavum* AJ11573 (FERM P-5492) (JP56-161495A) AJ11576 (FERM BP-10381) (JP56-161495A) AJ12212 (FERM P-8123) (JP61-202694A), AJ12418 (FERM BP-2205) (JP02-186994A), DH18 (FERM P-11116) (JP03-232497A), *C. glutamicum* AJ11574 (FERM P-5493) (JP56-151495A), *C. melassecola* DH344 (FERM P-11117) (JP3-232497A).

Examples of parent strains which can be used to derive L-glutamine-producing bacteria can also include strains in which expression of one or more genes encoding enzyme(s) of L-glutamine biosynthesis pathway are enhanced. Examples of such genes include genes encoding glutamine synthetase and glutamic acid dehydrogenase (US7262035 B2, EP1229121 B1), and the like.

Other examples of parent strains which can be used to derive L-glutamine-producing bacteria can also include strains in which expression of one or more genes encoding enzyme(s) that catalyze synthesis of compound other than L-glutamine, but which are directed away from the L-glutamine biosynthesis pathway, are attenuated. For attenuation of expression levels of such genes, the methods described above may be used. As a parent strain, an L-glutamine-producing strain with decreased glutaminase activity (EP1424397 B1), or a strain in which the *gluABCD* operon system for glutamine transport has been down-regulated or deleted (WO2008026698 A1) can be used, and the like.

Specific examples of strains having an L-glutamic acid-producing ability can also include strains which are resistant to an L-amino acid analog. Examples of parent strains can include strains resistant to 6-diazo-5-oxo-norleucine (JP-A-3-232497), to purine analogs and/or methionine sulfoxide (JP-A-61-202694), to α-ketomalonic acid (JP-A-56-151495), and so forth.

L-histidine-producing bacteria

Examples of parent strains which can be used to derive L-histidine-producing bacteria include, but are not limited to, strains belonging to coryneform bacteria, such as strains *C. glutamicum* FERM BP-485 and FERM BP-486 (U.S. Patent No. 4,495,283), *C. acetoacidophilum* AJ 1562 and *Microbacterium flavum* (*C. flavescens*) ATCC 10340 (JP 47002549 B), *C. glutamicum* AJ 12092 (FERM P-7273) and AJ 12426 (FERM BP-2213), *B. flavum* AJ 3420 (FERM P-2316) and *B. flavum* AJ 12425 (FERM BP-2212) (U.S. Patent. No. 5,294,547), *C. glutamicum* (*B. flavum*) ATCC 21604 (VKPM B-1080) (U.S. Patent No. 3,713,977), *C. glutamicum* ("Micrococcus glutamicus"); ATCC 13761; (NCIB 10334, VKPM B-4382) (U.S. Patent No. 3,220,929), *C. glutamicum* ATCC 21339 (VKPM B-1003) (U.S. Patent No. 3,676,301).

Specific examples of strains having an L-histidine-producing ability include strains belonging to coryneform bacteria, such as *B. flavum* FERM-P 2317, *B. lactofermentum* FERM-P 1565 (AJ 3386) *C. glutamicum* ATCC 14297 *B. lactofermentum* ATCC 21086 *C. acetoacidophilum* ATCC 21407, *B. flavum* ATCC 21406 (AJ 3225), and so forth.

Specific examples of strains having an L-histidine-producing ability include strains belonging to genus *Brevibacterium*, such as a strain *B. flavum* AJ11846 which is resistant

to thiamin antagonists (2-thiazolalanine, triazolecarboxamid, and cobalamine) (JP 02018838 B), and *B. falvum* AJ 3579 strain which requires threonine, proline, shikimic acid, xanthine, or guanine and is resistant to 2-thiazolalanine.(JP 51024594).

L-proline producing bacteria

Examples of parent strains which can be used to derive L-proline-producing bacteria can include, but are not limited to, strains belonging to coryneform bacteria, such as strains *B. lactofermentum* AJ 11225 (FERM P-4370) (Japanese Patent Application Laid-Open No. 60-87788), *B. flavum* AJ 11512 (FERM P-5332), AJ 11513 (FERM P-5333), AJ 11514 (FERM P-5334), *C. glutamicum* AJ 11522 (FERM P-5342), AJ 11523 (FERM P-5343) (see Japanese Patent Publication No. 62-36679), and so forth.

Examples of parent strains which can be used to derive L-proline producing bacteria also include strains in which expression of one or more genes encoding an L-proline biosynthetic enzyme are enhanced. Examples of an L-proline-biosynthetic enzyme include glutamate kinase, γ-glutamyl phosphate reductase, and pyroline-5-carboxylate reductase (Ankri S et al., J Bacteriol. 1996, 178(15):4412-4419.). Also, L-proline producing bacteria can be used which have decreased activity of an enzyme that catalyzes a reaction which is directed away from the L-proline biosynthetic pathway and results in the production of other compounds. For example, L-proline-producing ability may be imparted by decreasing ornithine-aminotransferase activity (J Bacteriol. 1996 Aug; 178(15):4412-9).

L-arginine-producing bacteria

Examples of parent strains which can be used to derive L-arginine-producing bacteria can include, but are not limited to, strains belonging to coryneform bacteria, such as strains *C. glutamicum* AJ12092 and *B. flavum* AJ11169 (EP 378223 B1, JP 2817155 B2, US 5284757 A), *B. flavum* AJ 12144 (FERM P-7642) and *C. glutamicum* AJ 12145 (FERM P-7643) (Japanese Patent Publication No. 5-27388), *B. flavum* ATCC 21493 and *C. glutamicum* ATCC 21659; (Japanese Patent Application Laid-Open No. 5-3793), *B. flavum* FERM-P 4948, *C. glutamicum* FERM-P 7274 (AJ 12093), *B. flavum* NRRL 12235 (AJ 11337) *C. acetoglutamicum* NRRL 12239 (AJ 11342).

It is known that an L-arginine-producing ability can also be efficiently enhanced by using a mutant N-acetylglutamate kinase which is resistant to feedback inhibition by L-arginine (WO 2006035831 A1).

Specific examples of strains having an L-arginine-producing ability include strains belonging to the genus *Brevibacterium* or *Corynebacterium,* and which are resistant to

arginine hydroxamate, arginine antagonists, argininol, sulfamides, cysteine or its analogs (EP 336387 B1, JP 2578468 B2, US 5034319 A), aliphatic guanidine or aliphatic chain derivative guanidine (JP 2817155 B2, EP 0378223 B1), and so forth. The productivity of L-arginine strains of coryneform bacterium can be also improved by imparting resistance to sulfa drugs or argininol, or resistance to chemicals such as 8-azaguanine, alpha-amino-beta-hydroxyvaleric acid, etc.

Examples of parent strains which can be used to derive L-arginine producing bacteria also include strains auxotrophic for amino acids such as L-histidine, L-proline, L-threonine, L-tryptophan, L-lysine, etc. Examples of parent strains which can be used to derive L-arginine producing bacteria also include strains that are known as citrulline or arginine auxotrophs (Japanese Patent Publication No. 43-8712), variants of arginine auxotroph (Japanese Patent Application Laid-Open No. 53-24096), variants belonging to the genus *Corynebacterium* having a resistance to 2-thiazole-alanine, sulfaguanidine or 2-fluoropyruvic acid (Japanese Patent Application Laid-Open No. 61-119194) and so forth.

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 an L-arginine biosynthetic enzyme are enhanced. Examples of such genes include genes encoding N-acetylglutamyl phosphate reductase (*argC*), ornithine acetyl transferase (*argJ*), N-acetylglutamate kinase (*argB*), acetylornithine transaminase (*argD*), ornithine carbamoyl transferase (*argF*), argininosuccinic acid synthetase (*argG*), argininosuccinic acid lyase (*argH*), and carbamoyl phosphate synthetase (*carAB*).

L-arginine producing bacterium can be improved by inactivation of the arginine repressor encoded by *argR* gene. Methods for inactivation of *argR* gene are described above.

L-ornithine producing bacteria

Examples of parent strains which can be used to derive L-ornithine-producing bacteria include, but are not limited to, strains belonging to coryneform bacteria, such as strains *B. lactofermentum* FERM BP-2344, FERM-P 5936 (AJ 11678) and *C. glutamicum* FERM BP-2345 (JP 2817185 (B2), EP 393708 B1), *C. glutamicum* FERM-P 5644 (AJ 11589), ATCC 13232, and the like.

As L-ornithine is an intermediate of L-arginine biosynthetic pathway, examples of parent strains, which can be used to derive L-ornithine-producing bacteria include strains in which expression of one or more genes encoding an L-arginine biosynthetic enzyme is enhanced. L-ornithine producing bacterium can be easily obtained from any arginine producing bacterium, by inactivation of ornithine carbamoyltransferase encoded by both

argF and *argI* genes. Methods for inactivation of ornithine carbamoyltransferase are described above.

L-citrulline producing bacteria

Examples of parent strains which can be used to derive L-citrulline-producing bacteria include, but are not limited to, strains belonging to coryneform bacteria, strains *C. glutamicum* FERM-P 5643 (AJ 11588), *B. flavum* FERM-P 1645 (AJ 3408), and so forth.

As L-citrulline is an intermediate in the L-arginine biosynthetic pathway, examples of parent strains, which can be used to derive L-citrulline producing bacterium can be easily obtained from any L-arginine producing bacterium, for example, by inactivation of argininosuccinate synthase encoded by *argG* gene.

2. Method of the present invention

In a method for producing an L-amino acid of the presently disclosed subject matter, the bacterium as described herein is cultured in a culture medium to produce and excrete the L-amino acid into the medium, and the L-amino acid is collected from the medium.

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

The medium used for 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 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 performed under aerobic conditions, such as a shaking culture, and a stirring culture with aeration, at a temperature of 20 to 40°C, or 30 to 38°C. The pH of the culture is usually between 5 and 9, or 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, a 1 to 5-day cultivation leads to accumulation of the target 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 the 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 attenuated expression of *NCgl_2067* gene

1-1. Deletion of the *NCgl_2067* gene

A strain in which the *NCgl_2067* gene is deleted was constructed by using the non-replicative plasmid pSB4S (see Fig.3; the construction of the plasmid is described in US patent No. 7,794,989). The in-frame deletion of the *NCgl_2067* gene was constructed. For this purpose, overlap extension PCR was conducted. First, a DNA fragment was obtained by PCR with primers NC2067F (SEQ ID NO: 7) and NC2067R1 (SEQ ID NO: 8) and chromosomal DNA of *B. flavum* B-6642 (described in patent of Russian Federation RU 2084520 C2) as the template. The strain *B. flavum* B-6642 was deposited as an international deposit at the Russian National Collection of Industrial Microorganisms (VKPM) (1 Dorozhny proezd., 1 Moscow 117545, Russia) on June 28, 1993 under the accession number of VKPM B-6642 and then converted to a deposit under the Budapest Treaty on March 5, 2012.

Second, a DNA fragment was obtained in PCR with oligonucleotides NC2067F1 (SEQ ID NO: 9) and NC2067R (SEQ ID NO: 10) and chromosomal DNA of *B. flavum* B-6642 as the template. After that, the two DNA fragments described above were used as the template in the first round of PCR with primers NC2067-SmF (SEQ ID NO: 11) and NC2067-SmR (SEQ ID NO: 12). Conditions for all PCRs were as follows: denaturation step for 30 sec at 94°C; profile for 25 cycles: 30 sec at 94°C, 30 sec at 58°C, 60 sec at 72°C; final step: 2 min at 72°C.

After that, the obtained PCR product was purified and treated with endonuclease *Sma*I and then ligated into a *Sma*I-cleaved plasmid pBS4S to obtain pBS4SΔ2067.

Electrocompetent cells were prepared as follows: *B. flavum* B-6642 was grown overnight at 30 °C in CM2GxYE medium (peptone – 10g/l, yeast extract – 20g/l, NaCl – 5g/l, glucose – 5g/l, pH is adjusted to 7.0), and then the culture was diluted about 20 times with fresh CM2GxYE medium. The cells were grown with aeration at 30°C to an OD_{600} of ≈0.6 and ampicillin was added (10 μg/ml) and cultivated for 1 hour. The obtained cells

were washed three times with ice-cold deionized H_2O. Electroporation was performed using 50 μl of cells and ≈5μg of the plasmid pBS4SΔ2067 with the following parameters: 2.0 kV, 0,1 cm electrode gap. Cells after electroporation were incubated with 1 ml CM2GxYE medium at 30°C for 2 hours with shaking and then were plated onto 2LA-agar (peptone – 10g/l, yeast extract – 10g/l, NaCl – 5g/l, agar – 20g/l, pH is adjusted to 7.0) containing kanamycin (25 μg/ml) and grown at 30°C to select Km^R recombinants for 48 h. The obtained integrants were also unable to grow on 2LA medium containing 10% sucrose.

1-2. Verification of the *NCgl_2067* gene deletion by PCR

The mutants in which the *NCgl_2067* gene was deleted were verified by PCR. Locus-specific primers P1 (SEQ ID NO: 13) and P2 (SEQ ID NO: 14) were used in PCR for the verification. The PCR product obtained in the reaction with the cells of the parental strain *B. flavum* B-6642 as the template, was ~1.35 kbp in length. The PCR product obtained in the reaction with the cells of mutant strain as the template was ~0.78 kbp in length. Deletion of *NCgl_2067* gene was also confirmed by the sequencing of the corresponding chromosomal DNA fragment. The mutant strain was given the name *B. flavum* B-6642ΔNCgl_2067.

Example 2. Construction of a strain with attenuated expression of *NCgl_2066* gene

2-1. Deletion of the *NCgl_2066* gene

A strain in which the *NCgl_2066* gene was deleted was constructed as the same manner as described in Example 1. The in-frame deletion of the *NCgl_2066* gene was constructed. For this purpose, overlap extension PCR was conducted. First, a DNA fragment was obtained in PCR with primers NC2066F (SEQ ID NO: 15) and NC2066R1 (SEQ ID NO: 16) and chromosomal DNA of *B. flavum* B-6642 as the template. Second, a DNA fragment was obtained in PCR with oligonucleotides NC2066F1 (SEQ ID NO: 17) and NC2066R (SEQ ID NO: 18) and chromosomal DNA of *B. flavum* B-6642 as the template. After that, the two DNA fragments described above were used as the template in the first round of PCR with primers NC2066-SmF (SEQ ID NO: 19) and NC2066-SmR (SEQ ID NO: 20). After that, the obtained PCR product was purified and treated with endonuclease *Sma*I, and then ligated into *Sma*I-cleaved plasmid pBS4S to obtain pBS4SΔ2066.

Electrocompetent cells were prepared as follows: *B. flavum* B-6642 was grown overnight at 30 °C in CM2GxYE medium (peptone – 10g/l, yeast extract – 20g/l, NaCl – 5g/l, glucose – 5g/l, pH is adjusted to 7.0), and then the culture was diluted about 20 times with fresh CM2GxYE medium. The cells were grown with aeration at 30°C to an OD_{600}

of ≈0.6 and ampicillin was added (10 μg/ml) and cultivated for 1 hour. The obtained cells were washed three times with ice-cold deionized H_2O. Electroporation was performed using 50 μl of cells and ≈5 μg of the plasmid pBS4SΔ2066 with the following parameters: 2.0 kV, 0.1 cm electrode gap. Cells after electroporation were incubated with 1 ml CM2GxYE medium at 30°C for 2 hours with shaking and then were plated onto 2LA-agar (peptone – 10g/l, yeast extract – 10g/l, NaCl – 5g/l, agar – 20g/l, pH is adjusted to 7.0) containing kanamycin (25 μg/ml) and grown at 30°C to select Km^R recombinants for 48 h. The obtained integrants were also unable to grow on 2LA medium containing 10% sucrose.

2-2. Verification of the *NCgl_2066* gene deletion by PCR

The mutants in which the *NCgl_2066* gene was deleted were verified by PCR. Locus-specific primers NC2066F (SEQ ID NO: 15) and NC2066R (SEQ ID NO: 18) were used in PCR for the verification. The PCR product obtained in the reaction with the cells of parental strain *B. flavum* B-6642 as the template, was ~2.12 kbp in length. The PCR product obtained in the reaction with the cells of mutant strain as the template was ~1.15 kbp in length. Deletion of the *NCgl_2066* gene was also confirmed by the sequencing of the corresponding chromosomal DNA fragment. The mutant strain was given the name *B. flavum* B-6642ΔNCgl_2066.

Example 3. Production of L-glutamic acid and L-glutamine by *B. flavum* strain B-6642ΔNCgl_2067

B. flavum strains, B-6642ΔNCgl_2067, and B-6642, were separately grown for 18-24 hours at 30°C on 2LA-agar plates. Then, one loop of the cells was transferred into test tubes containing 2ml of fermentation medium. The fermentation medium contains $(NH_4)_2SO_4$ (70g/l), KH_2PO_4 (2,5g/l), thiamine (350mg/l), biotin (100mg/l), $MgSO_4\,7H_2O$ (0.4g/l), Mameno (soybean protein hydrolysate, 5.7mg/l), sucrose (120g/l), and $CaCO_3$ (50g/l). The pH is adjusted to 7.

Cultivation was carried out at 30°C for 48 hours with shaking. After the cultivation, the amount of L-glutamic acid (Glu) and L-glutamine (Gln)which are produced was determined by TLC (thin-layer chromatography) using the following mobile phase: butanol : acetic acid : water = 4 : 1 : 1 (v/v). A solution of ninhydrin (1%) in acetone was used as a visualizing reagent. The spots containing L-glutamic acid and L-glutamine were cut out, and L-glutamic acid and L-glutamine were eluted with 0.5% water solution of $CdCl_2$. The amounts were estimated spectrophotometrically at 540 nm. The results of two independent test tube fermentations are shown in Table 1. As follows from Table 1, strain B-6642ΔNCgl_2067 with attenuated expression of *NCgl_2067* gene produced higher

amounts of L- glutamic acid and L-glutamine, as compared with the parent strain B-6642.

Table 1

Strain	Gln,g/l	Glu,g/l
B-6642	3.9±0.1	4.2±0.2
B-6642ΔNCgl_2067	4.4±0.3	4.6±0.1

Example 4. Production of L-glutamic acid and L-glutamine by *B. flavum* strain B-6642Δ NCgl_2066

B. flavum strains, B-6642ΔNCgl_2066 and B-6642, were separately grown for 18-24 hours at 30°C on 2LA-agar plates. Then, one loop of the cells was transferred into test tubes containing 2ml of fermentation medium. The fermentation medium contains $(NH_4)_2SO_4$ (70g/l), KH_2PO_4 (2,5g/l), thiamine (350mg/l), biotin (100mg/l), $MgSO_4$ $7H_2O$(0.4g/l), Mameno (5.7mg/l), sucrose (120g/l), and $CaCO_3$ (50g/l). The pH is adjusted to 7.

Cultivation was carried out at 30°C for 48 hours with shaking. After the cultivation, the amount of L-glutamic acid and L-glutamine which is produced was determined by TLC (thin-layer chromatography) using the following mobile phase: butanol : acetic acid : water = 4 : 1 : 1 (v/v). A solution of ninhydrin (1%) in acetone was used as a visualizing reagent. The spots containing L-glutamic acid and L-glutamine were cut out, L-glutamic acid and L-glutamine were eluted with 0.5% water solution of $CdCl_2$, and the amounts were estimated spectrophotometrically at 540 nm.

The results of two independent test tube fermentations are shown in Table 2. As follows from Table 2, strain B-6642ΔNCgl_2066 with attenuated expression of *NCgl_2066* gene produced higher amounts of L-glutamic acid and L-glutamine, as compared with the parent strain B-6642.

Table 2

Strain	Gln,g/l	Glu,g/l
B-6642	4.8±0.1	3.9±0.1
B-6642ΔNCgl_2066	5.8±0.3	4.3±0.1

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, an L-amino acid belonging to the glutamate family is efficently produced by a coryneform bacterium.

CLAIMS

1. A coryneform bacterium which is able to produce an L-amino acid belonging to the glutamate family, wherein said bacterium has been modified to attenuate the expression of one or more gene(s) of the *NCgl_2067-NCgl_2065* operon.

2. The bacterium according to claim 1, wherein said bacterium has been modified to attenuate the expression of the *NCgl_2067* gene.

3. The bacterium according to claim 2, wherein said expression of the *NCgl_2067* gene is attenuated by inactivation of the *NCgl_2067* gene.

4. The bacterium according to claim 1, wherein said bacterium has been modified to attenuate the expression of the *NCgl_2066* gene.

5. The bacterium according to claim 4, wherein said expression of the *NCgl_2066* gene is attenuated by inactivation of the *NCgl_2066* gene.

6. The bacterium according to claim 1, wherein said bacterium belongs to the genus *Corynebacterium* or *Brevibacterium.*

7. The bacterium according to claim 6, wherein said bacterium is *Brevibacterium flavum.*

8. The bacterium according to any of claims 1 to 7, wherein said L-amino acid is selected from the group consisting of L-glutamic acid, L-glutamine, L-proline, L-histidine, L-arginine, L-ornithine, and L-citrulline.

9. The bacterium according to claim 8, wherein said L-amino acid is selected from the group consisting of L-glutamic acid and L-glutamine.

10. A method for producing an L-amino acid belonging to the glutamate family comprising:

- cultivating the bacterium according to any of claims 1 to 9 in a medium, and
- collecting said L-amino acid from the medium.

11. The method according to claim 10, wherein said L-amino acid is selected from the group consisting of L-glutamic acid, L-glutamine, L-proline, L-histidine, L-arginine, L-ornithine, and L-citrulline.

12. The method according to claim 11, wherein said L-amino acid is selected from the group consisting of L-glutamic acid and L-glutamine.

Fig. 1

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spr0443    15   IGSVMKLTDLSARQIRYYEDQELIKPDRNEGNRRMYSLNDMDRLLEIKDYISEGYNIA--  72
                IG V KL+ +SAR +R+YE    L++P ++     R YS  D+ R+  I+   S G ++
NCgl2066    3   IGEVSKLSGVSARMLRHYEKLGLVEPKQSTAGYREYSEGDVRRIFHIEGLRSLGLSLKQV  62

spr0443    73   --AIKKKYAEREAKSKKAVSQTEVRRALHNELL  103
                  A++    + +A   + +++T  R ++  ELL
NCgl2066   63   GDALEDPDFDPQAVISEMIAETSARISMERELL  95
```

Fig. 2

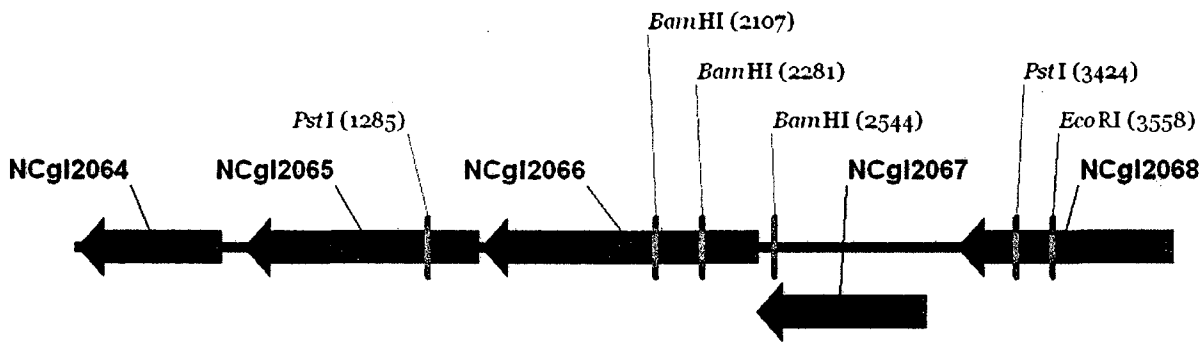
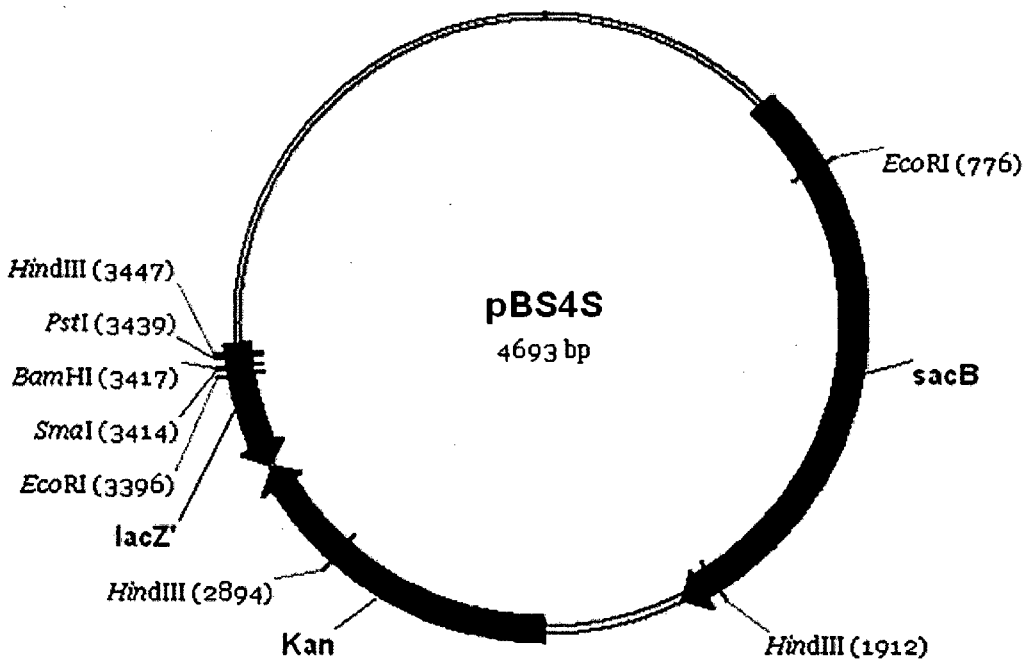


Fig. 3


pBS4S
4693 bp
EcoRI (776)
sacB
HindIII (1912)
Kan
HindIII (2894)
lacZ'
EcoRI (3396)
SmaI (3414)
BamHI (3417)
PstI (3439)
HindIII (3447)

INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2012/061625

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/21 C07K14/34 C12P13/10 C12P13/14 C12P13/24
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)
C12N C07K 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 practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, FSTA, 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	WO 2006/138689 A2 (MICROBIA INC [US]; MADDEN KEVIN T [US]; WALBRIDGE MICHAEL J [US]; YORG) 28 December 2006 (2006-12-28)	1-9
A	page 95, line 7 - line 17; claims 1-26; sequences 199,277 page 8, line 1 - line 6 ----- -/--	10-12

[X] Further documents are listed in the continuation of Box C.

[X] 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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"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 August 2012	21/08/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Devijver, Kristof

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	the whole document	10-12
A	EP 2 107 128 A2 (KYOWA HAKKO BIO CO LTD [JP]) 7 October 2009 (2009-10-07) claims 1-9; sequences 2351,5851,2349,5849,2348,5848,	1-12
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A	KLOOSTERMAN TOMAS G ET AL: "Regulation of glutamine and glutamate metabolism by GlnR and GlnA in Streptococcus pneumoniae", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 281, no. 35, September 2006 (2006-09) , pages 25097-25109, XP002681501, ISSN: 0021-9258 cited in the application the whole document	1-12
A	EP 1 229 121 A2 (AJINOMOTO KK [JP]) 7 August 2002 (2002-08-07) cited in the application claims 1-9	1-12

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Information on patent family members

International application No
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