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(54) **Process for producing L-amino acids**

Verfahren zur Herstellung von L-Aminosäuren

Procédé de preparation de L-acides aminés

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**Description**

## Technical Field

5 **[0001]** The present invention relates to a process for producing an L-amino acid using a microorganism having the capability of producing the L-amino acid, and having the L-amino acid transport activity higher than that of the parent strain wherein the L-amino acid is selected from the group consisting of L-serine, L-glutamine, L-cysteine, L-phenylalanine and L-threonine. More specifically, the present invention relates to a process for producing said L-amino acids wherein the productivity of said L-amino acid is increased by constructing a microorganism whose activity of transporting  
10 said L-amino acids from inside of the cell to outside of the cell is higher than that of the parent strain, allowing the microorganism to produce said L-amino acids, and efficiently exporting the produced L-amino acid from inside of the cell of the microorganism to outside of the cell wherein the L-amino acid is selected from the group consisting of L-serine, L-glutamine, L-cysteine, L-phenylalanine and L-threonine.

## 15 Background Art

**[0002]** Production of an amino acid by utilizing a microorganism is known as amino acid fermentation, and is traditionally widely conducted in the field of applied microbiology. In amino acid fermentation, in the final step thereof, amino acid transport activity, that is, how to facilitate the efflux of the resulting amino acid to the outside of the cell of the bacterium  
20 is an important process that influences amino acid productivity; various attempts have been made to date to increase the efficiency of efflux to the outside of the cell.

**[0003]** Usually, active transport using bioenergy is necessary to transport an amino acid in the cell of a microorganism to outside of the cell. Proteins that export intracellular amino acids to outside of the cell (efflux proteins) have been identified; it is known that amino acid productivity can be also conferred or enhanced by enhancing the expression of  
25 the proteins. For example, a process for producing L-lysine using a strain of a microorganism of the genus *Corynebacterium* wherein the expression of the L-lysine, L-arginine efflux gene (*lysE*) (see non-patent document 1) is enhanced (see patent document 1); a process for producing L-cysteine, L-cystine, N-acetylserine or thiazoline derivative using a strain of a microorganism of the genus *Escherichia* wherein the expression of the efflux gene (*rhtA*) of L-threonine, L-homoserine (see patent document 2), and L-cysteine, L-cystine, N-acetylserine or thiazoline derivative efflux gene  
30 (*ydeD/eamA*) (see non-patent document 3) are enhanced (patent document 2); a process for producing L-amino acids, including L-lysine, using a strain of a microorganism of the genus *Escherichia* wherein the expression of the L-lysine efflux gene (*ybjE*) involved in L-lysine resistance is enhanced (patent document 3) are known.

**[0004]** However, no report is available on an efflux protein for L-serine and L-glutamine, and a process for producing the amino acids wherein the activity of the protein is enhanced.

35 **[0005]** By the way, the *Escherichia coli* *norM* gene is known to be an efflux pump gene related to quinolone resistance (non-patent document 4). The *emrD* gene is reported as an SDS transport gene (non-patent document 5). While *rarD* is predicted to be a drug transport gene, none of them is known to have an amino acid efflux activity (non-patent document 6). Meanwhile, the *eamA(ydeD)* gene is reported as a gene having efflux activity for L-cysteine, L-cystine, N-acetylserine or thiazoline derivative (non-patent document 3).  
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[Prior art documents]

[patent documents]

45 **[0006]**

[patent document 1] WO97/23597

[patent document 2] JP-A-11-56381

[patent document 3] JP-A-2005-237379  
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[non-patent documents]

**[0007]**

55 [non-patent document 1] *Mol. Microbiol.*, 22, 815-826(1996)

[non-patent document 2] *Res. Microbiol.*, 154, 123-135(2003)

[non-patent document 3] *Mol. Microbiol.*, 36, 1101-1112(2000)

[non-patent document 4] *J. Antimicrob. Chemother.*, 51, 545-56 (2003)

[non-patent document 5] J. Bacteriol., 183, 5803-5812 (2001)

[non-patent document 6] Science, 308, 1321-1323 (2005)

[Summary of the Invention]

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Problems to Be Solved by the Invention

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**[0008]** A problem to be solved by the present invention is to provide an efficient process for producing an L-amino acid by allowing a microorganism whose L-amino acid transport activity is higher than that of the parent strain to produce the L-amino acid. More specifically, the problem is to provide a novel manufacturing process of high productivity for the five neutral amino acids, including L-serine and L-glutamine, for which no manufacturing process based on enhanced L-amino acid transport activity has been available to date, by enhancing the efflux protein.

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Means of Solving the Problems

**[0009]** The present invention provides the embodiments as disclosed in the claims. The present invention provides:

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(i) A process for producing an L-amino acid which comprises culturing a microorganism in a medium wherein the microorganism has higher L-amino acid transport activity when compared to the parent strain wherein the microorganism expresses a protein described in any one of (1) to (3),

(1) A protein comprising the amino acid sequence shown in SEQ ID NO:6;

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(2) A protein consisting of the amino acid sequence resulting from deletion, substitution or addition of 1 to 20 amino acids in the amino acid sequence shown in SEQ ID NO:6, and having L-amino acid transport activity;

(3) A protein consisting of an amino acid sequence having 80% or more homology to the amino acid sequence shown in SEQ ID NO:6, and having L-amino acid transport activity;

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and producing and accumulating the L-amino acid in the medium, and collecting the L-amino acid from the medium, wherein the L-amino acid is selected from the group consisting of L-serine, L-glutamine, L-cysteine, L-phenylalanine and L-threonine.

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(ii) The process for producing the L-amino acid according to item (i) above, wherein the microorganism is transformed with the DNA described in any one of (1) to (3) below, or wherein the expression of the gene that encodes for the protein is enhanced in the microorganism by modifying the expression regulatory sequence of the DNA described in any one of (1) to (3) below:

(1) A DNA that encodes the protein described in any one of (1) to (3) in item (i) above;

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(2) A DNA comprising the nucleotide sequence shown in SEQ ID NO:5;

(3) A DNA that hybridizes under stringent conditions with the DNA consisting of the nucleotide sequence complementary to the nucleotide sequence shown in SEQ ID NO:5, and encodes for the protein having L-amino acid transport activity.

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Effect of the Invention

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**[0010]** The manufacturing process of the present invention is a manufacturing process that is highly productive in producing L-serine, L-glutamine, L-cysteine, L-phenylalanine or L-threonine.

**[0011]** The manufacturing process of the present invention is a method of efficiently producing L-serine and L-glutamine using a microorganism by enhancing the activity of a protein having the activity of transporting an L-amino acid in the cell body of the microorganism to outside of the cell body. Also provided is a novel manufacturing process for L-cysteine, L-threonine and L-phenylalanine comprising enhancing the L-amino acid transport activity in the same way.

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**[0012]** The inventor of this invention found that the publicly known transport gene *rarD* of *Escherichia coli* has the function of transporting an amino acid to outside of the cell, and also found that these transport genes can be advantageously utilized for producing L-serine or L-glutamine, or L-cysteine, L-threonine, or L-phenylalanine.

**[0013]** It is also disclosed herein that *eamA*, which had been known to have L-amino acid transport activity, is also

responsible for L-serine transport, and devised a process for producing L-serine by utilizing this.

**[0014]** According to the method of the present invention, enhancing the activity of the aforementioned amino acid transport gene *rarD* makes it possible to remarkably improve the production of L-amino acids selected from the group consisting of L-serine, L-glutamine, L-cysteine, L-phenylalanine and L-threonine by selective active transport of the produced L-amino acid to outside of the cell. Additionally, the microorganism used to produce any of said L-amino acids does not depend on the type of outer membrane (presence or absence of cell wall, capsule and mucus layer), whether it is Gram-positive or Gram-negative. Preferably, the manufacturing process of the present invention is a manufacturing process of high versatility that can be used both for Gram-positive bacteria such as genus *Corynebacterium*, genus *Bacillus*, and genus *Streptomyces*, and for Gram-negative bacteria such as genus *Escherichia*, genus *Serratia*, and genus *Pseudomonas*.

**[0015]** The manufacturing process of the present invention dramatically improves the production efficiency for L-serine and L-glutamine compared with conventional processes. L-serine, in particular, is an amino acid that plays an important role in living organisms despite its identity as a non-essential amino acid, and is of high utility as a raw material for amino acid mixtures in the field of pharmaceuticals and the field of cosmetics. L-glutamine is an amino acid that acts to keep normal the functions of the stomach, intestines and muscles in the body, and serves as a raw material for anti-alcoholism compositions. If a highly productive manufacturing process for these L-amino acids is established to enable their industrial mass-production, its industrial applicability would be very high.

**[0016]** For L-cysteine, L-threonine and L-phenylalanine as well, the manufacturing process of the present invention has enabled more economic production. L-cysteine is an amino acid that is highly valuable in the cosmetic industry as a raw material for cosmetics because of its whitening effect. L-threonine and L-phenylalanine are both essential amino acids; L-threonine, as an ingredient of amino acid infusions and health foods, and L-phenylalanine, as a raw material for the low-calorie sweetener Aspartame (methyl ester of aspartylphenylalanine, 200 times as sweet as sugar), are useful amino acids whose productivity is expected to be improved by the manufacturing process of the present invention.

[Modes for carrying out the Invention]

#### 1. Microorganisms used in the manufacturing process of the present invention

Microorganisms whose L-amino acid transport activity is higher than that of the parent strain

**[0017]** Microorganisms whose activity of a protein having L-amino acid transport activity is higher than that of the parent strain are (a) microorganisms obtained by modifying a DNA that encodes for a protein having L-amino acid transport activity on the chromosomal DNA of the parent strain, wherein the microorganisms are i) a microorganism whose specific activity of the protein has improved compared with the parent strain, and ii) a microorganism whose production amount of a protein having L-amino acid transport activity has improved compared with the parent strain, and (b) microorganisms obtained by transforming the parent strain with a DNA that encodes for a protein having L-amino acid transport activity. As a parent strain mentioned herein, whether a wild type strain or a mutant strain can be used, and is the original strain which is the subject of modification or transformation. A wild type strain refers to the strain having the phenotype that is most frequently observed in a wild population. The parent strain includes, when the microorganism is *Escherichia coli*, for example, the wild type strains *E. coli* K-12 strain, B strain, B/r strain, and W strain, or strains that are mutants thereof; the mutant strains include *E. coli* XL1-Blue, *E. coli* XL2-Blue, *E. coli* DH1, *E. coli* MC1000, *E. coli* ATCC12435, *E. coli* W1485, *E. coli* JM109, *E. coli* HB101, *E. coli* No.49, *E. coli* W3110, *E. coli* NY49, *E. coli* MP347, *E. coli* NM522, *E. coli* BL21, *E. coli* ME8415 and *E. coli* ATCC9637.

**[0018]** Other proteins disclosed herein having L-amino acid transport activity include the proteins described in any one of (1) to (3) below:

- (1) proteins comprising the amino acid sequence shown in any one of SEQ ID NOS:2, 4, and 8;
- (2) proteins consisting of the amino acid sequence resulting from deletion, substitution or addition of one or more amino acids in the amino acid sequence shown in any one of SEQ ID NOS:2, 4, and 8, and having L-amino acid transport activity; and
- (3) proteins consisting of the amino acid sequence having 80% or more homology to the amino acid sequence shown in any one of SEQ ID NOS:2, 4, and 8, and having L-amino acid transport activity.

**[0019]** Here, the DNA sequences of SEQ ID NOS:1, 3, and 7 encode for the aforementioned *norM* gene, *emrD* gene, *rarD* gene and *eamA* gene, respectively, in *Escherichia coli*; the amino acid sequences shown in the SEQ ID NOS:2, 4, and 8 represent *norM* protein, *emrD* protein and *eamA* protein, respectively, encoded by the aforementioned genes.

**[0020]** A protein of the present invention, as defined in claim 1, consisting of the amino acid sequence resulting from deletion, substitution or addition of one to 20 amino acid residues, and having L-amino acid transport activity in the above

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can be acquired by introducing a site-directed mutation into, for example, a DNA that encodes for the protein consisting of the amino acid sequence shown in SEQ ID NO: 6, using one of the site-directed mutagenesis methods described in Molecular Cloning, A Laboratory Manual, Third Edition; Cold Spring Harbor Laboratory Press (1989) (hereinafter, abbreviated as Molecular Cloning, Third Edition), Current Protocols in Molecular Biology, John Wiley & Sons (1987-1997) (hereinafter, abbreviated as Current Protocols in Molecular Biology), Nucleic Acids Research, 10, 6487 (1982), Proc. Natl. Acad. Sci. USA, 79, 6409(1982), Gene, 34, 315 (1985), Nucleic Acids Research, 13, 4431 (1985), Proc. Natl. Acad. Sci. USA, 82, 488 (1985),

**[0021]** The number of amino acid residues deleted, substituted or added is a number that can be deleted, substituted or added by an obvious method such as the above-described site-directed mutation methods, and is 1 to 20.

**[0022]** Deletion, substitution or addition of one to 20 amino acids in the amino acid sequence shown in SEQ ID NO: 6 may be such that one to 20 amino acid residues are deleted, substituted or added at an optionally chosen position in the same sequence.

**[0023]** Positions where an amino acid residue can be deleted or added include, for example, 10 amino acid residues on the N-terminal side and C-terminal side of the amino acid sequence shown in SEQ ID NO:6.

**[0024]** A deletion, substitution or addition may occur concurrently; it does not matter whether the amino acid substituted or added is of the natural type or the non-natural type. Natural type amino acids include L-alanine, L-asparagine, L-aspartic acid, L-arginine, L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine and L-cysteine.

**[0025]** Examples of mutually replaceable amino acids are shown below. The amino acids included in the same group are mutually replaceable.

Group A: leucine, isoleucine, norleucine, valine, norvaline, alanine, 2-aminobutanoic acid, methionine, O-methylserine, t-butylglycine, t-butylalanine, cyclohexylalanine

Group B: aspartic acid, glutamic acid, isoaspartic acid, isoglutamic acid, 2-aminoadipic acid, 2-aminosuberic acid

Group C: asparagine, glutamine

Group D: lysine, arginine, ornithine, 2,4-diaminobutanoic acid, 2,3-diaminopropionic acid

Group E: proline, 3-hydroxyproline, 4-hydroxyproline

Group F: serine, threonine, homoserine

Group G: phenylalanine, tyrosine

**[0026]** Proteins having L-amino acid transport activity include a protein consisting of the amino acid sequence having 80% or more, 90% or more, 95% or more, 97% or more, 98% or more, 99% or more homology, to the amino acid sequence shown in SEQ ID NO: 6, and having L-amino acid transport activity.

**[0027]** Amino acid sequence and nucleotide sequence homologies can be determined using the algorithm BLAST of Karlin and Altschul [Proc. Natl. Acad. Sci. USA, 90, 5873(1993)] or FASTA [Methods Enzymol., 183, 63 (1990)]. Based on this algorithm BLAST, programs called BLASTN and BLASTX have been developed [J. Mol. Biol., 215, 403(1990)]. When nucleotide sequences are analyzed with BLASTN on the basis of BLAST, parameters are set to, for example, score=100 and wordlength=12. When amino acid sequences are analyzed with BLASTX on the basis of BLAST, parameters are set to, for example, score=50 and wordlength=3. When the BLAST and Gapped BLAST programs are used, the default parameters of the respective programs are used. The specific ways of these analytical methods are well known.

**[0028]** Whether the protein consisting of the amino acid sequence resulting from deletion, substitution or addition of one to 20 amino acid residues in the amino acid sequence shown in SEQ ID NO: 6, and having L-amino acid transport activity can be confirmed by, for example, a method wherein a transformant that expresses the protein whose activity is to be confirmed is prepared using a DNA recombination technique, and labeled L-amino acid and inside-out membrane vesicles that can be prepared from the transformant [J. Biol. Chem., 277, 49841 (2002)] are used [J. Biol. Chem., 280, 32254 (2005)].

**[0029]** Whether the protein consisting of an amino acid sequence resulting from deletion, substitution or addition of one to 20 amino acid residues in the amino acid sequence shown in SEQ ID NO: 6, and having L-amino acid transport activity can also be confirmed by, for example, transforming the parent strain with a DNA that encodes for the protein whose activity is to be confirmed to prepare a transformant wherein the protein activity is higher than that of the parent strain, and comparing the amounts of L-amino acid produced and accumulated in the culture broths of the parent strain or the transformant.

**[0030]** As disclosed herein, the microorganisms above (a-i) obtained by modifying a DNA that encodes for the protein having L-amino acid transport activity on the chromosomal DNA of the parent strain, wherein the specific activity of the protein has been improved compared with the parent strain, include a microorganism having a mutated protein whose L-amino acid transport activity has improved compared with the parent strain because it has a protein having the amino acid sequence resulting from substitution of 1 or more amino acids, preferably 1 to 10 amino acids, more preferably 1

to 5 amino acids, still more preferably 1 to 3 amino acids, in the amino acid sequence of the protein possessed by the parent strain.

**[0031]** As disclosed herein, the microorganisms above (a)-ii) obtained by modifying a DNA that encodes for the protein having L-amino acid transport activity on the chromosomal DNA of the parent strain, wherein the amount produced of the protein having L-amino acid transport activity has been improved compared with the parent strain, include a microorganism wherein the amount of the protein produced has been improved compared with the amount of the protein having L-amino acid transport activity produced by the parent strain because it has a promoter region wherein 1 base or more, preferably 1 to 10 bases, more preferably 1 to 5

**[0032]** bases, still more preferably 1 to 3 bases, are substituted in the nucleotide sequence of the transcription regulatory region or promoter region of the gene that encodes for the protein, present on the chromosomal DNA of the parent strain.

**[0033]** The microorganisms above (b) obtained by transforming a parent strain with the DNA that encodes for the protein having L-amino acid transport activity include microorganisms obtained by transforming the parent strain using:

[4] DNA that encodes for the protein described in any one of

[1] to [3] above;

[5] DNA having the nucleotide sequence shown in SEQ ID NO: 5 according to the present invention; or

[6] a DNA according to the present invention that hybridizes with the DNA consisting of the nucleotide sequence complementary to the nucleotide sequence shown in SEQ ID NO:5 under stringent conditions, and encodes for the protein having L-amino acid transport activity.

**[0034]** Such microorganisms include i) a microorganism having an extraneous DNA that encodes for the protein having L-amino acid transport activity on the chromosomal DNA, and ii) a microorganism having the same outside of the chromosome. Specifically, the microorganism i) is a microorganism having on the chromosome one or two or more newly introduced DNAs when the parent strain does not carry the DNA that encodes for the protein having L-amino acid transport activity, or a microorganism having on the chromosomal DNA two or more DNAs that encode for the protein having L-amino transport activity, including newly introduced DNA, when the parent strain originally carries the DNA that encodes for the protein having L-amino acid transport activity. The microorganism ii) is a microorganism having the DNA that encodes for the protein having L-amino acid transport activity on a plasmid DNA.

**[0035]** Herein, "L-amino acid transport activity" refers to the activity of effluxing an L-amino acid from inside of a cell to outside of the cell.

**[0036]** To "hybridize" as mentioned above means that a DNA hybridizes with another DNA having a particular nucleotide sequence or a portion of the DNA. Therefore, the DNA having a particular nucleotide sequence or a portion thereof can be used as a probe for Northern or Southern blot analysis, and can be also used as an oligonucleotide primer for PCR analysis. DNAs used as probes include DNAs of at least 100 bases or more, preferably 200 bases or more, more preferably 500 bases or more; DNAs used as primers include DNAs of at least 10 bases or more, preferably 15 bases or more.

**[0037]** Methods of DNA hybridization experiments are well known; for example, those skilled in the art are able to determine hybridization conditions according to the description of this application. The hybridization can be performed under conditions described in Molecular Cloning, 2nd edition and 3rd edition (2001), Methods for General and Molecular Bacteriology, ASM Press (1994), Immunology methods manual, Academic press (Molecular), and many other standard textbooks.

**[0038]** The above-described "stringent conditions" are preferably the conditions of incubating a DNA-immobilizing filter and a probe DNA in a solution containing 50% formamide, 5×SSC (750mmol/l sodium chloride, 75mmol/l sodium citrate), 50mmol/l sodium phosphate (pH 7.6), 5×Denhardt's solution, 10% dextran sulfate, and 20μg/l denatured salmon sperm DNA, at 42°C overnight, followed by washing the filter in, for example, a 0.2×SSC solution at about 65°C, but lower stringent conditions can be used. The stringent conditions can be changed by adjusting the formamide concentration (as the formamide concentration is lowered, the stringency decreases), and changing the salt concentration and temperature conditions. Low stringent conditions include, for example, the conditions of incubation in a solution containing 6×SSCE (20×SSCE comprises 3mol/l sodium chloride, 0.2mol/l sodium dihydrogen phosphate, 0.02mol/l EDTA, pH 7.4), 0.5% SDS, 30% formamide, and 100μg/l denatured salmon sperm DNA, at 37°C overnight, followed by washing using a 1×SSC, 0.1% SDS solution at 50°C. Still lower stringent conditions include the conditions of performing hybridization under the above-described low stringent conditions using a solution at a high salt concentration (for example, 5×SSC), followed by washing.

**[0039]** The above-described various conditions can also be set by adding or changing a blocking reagent used to suppress the background of the hybridization experiment. The addition of the blocking reagent may be accompanied by a change in the hybridization conditions to adapt the conditions.

**[0040]** DNAs that can be hybridized under the above-described stringent conditions include, for example, a DNA having at least 90% or more, preferably 95% or more, more preferably 97% or more, still more preferably 98% or more,

particularly preferably 99% or more homology, to the DNA consisting of the nucleotide sequence shown in SEQ ID NO: 5 as calculated on the basis of the above-described parameters using the above-described BLAST and/or FASTA.

## 2. Preparation of microorganisms used in the present invention

(1) Preparation of microorganism wherein the activity of the protein having L-amino acid transport activity is higher than that of the parent strain

**[0041]** Among the microorganisms wherein the activity of the protein having L-amino acid transport activity is higher than that of the parent strain, a microorganism wherein the specific activity is higher than that of the protein having L-amino acid transport activity of the parent strain can be acquired by introducing a mutation into a DNA that encodes for the protein having L-amino acid transport activity by subjecting the DNA to a mutation treatment using a mutagen in vitro or error-prone PCR, then replacing the mutated DNA with the DNA before introduction of the mutation, that encodes for the protein having L-amino acid transport activity, present on the chromosomal DNA of the parent strain, using a publicly known method [Proc. Natl. Acad. Sci. USA., 97, 6640 (2000)] to prepare a modified strain that expresses the mutated DNA, and comparing the L-amino acid transport activities of the parent strain and the modified strain by the above-described method.

**[0042]** Among the microorganisms wherein the activity of the protein having L-amino acid transport activity is higher than that of the parent strain, a microorganism wherein the amount of the protein produced has been improved compared with the amount produced of the parent strain can be identified by a method wherein a mutation is introduced into a DNA having the transcription regulatory region and promoter region of the gene that encodes for the protein having L-amino acid transport activity, possessed by the parent strain, for example, a nucleotide sequence 200 bp, preferably 100 bp upstream of the initiation codon of the protein, by subjecting the DNA to a mutation treatment in vitro or error-prone PCR, after which the mutated DNA is replaced with the transcription regulatory region and promoter region of the gene that encodes for the protein having L-amino acid transport activity, present on the chromosomal DNA of the parent strain, before introduction of the mutation, using a publicly known method [Proc. Natl. Acad. Sci. USA., 97, 6640 (2000)] to prepare a modified strain having the mutated transcription regulatory region or promoter region, and the amounts transcribed of the genes that encode for the proteins having L-amino acid transport activity of the parent strain and the modified strain by RT-PCR or Northern hybridization, or a method wherein the amounts produced of the proteins having L-amino acid transport activity of the parent strain and the modified strain are compared by SDS-PAGE.

**[0043]** By replacing the promoter region of the gene that encodes for the protein having L-amino acid transport activity of the parent strain with a publicly known potent promoter sequence, it is also possible to obtain a microorganism wherein the amount produced of the protein having L-amino acid transport activity has been improved compared with the parent strain.

**[0044]** Such promoters include promoters derived from *Escherichia coli* and phage, that function in *E. coli*, such as the  $P_{trp}$  promoter ( $P_{trp}$ ), the  $P_{lac}$  promoter ( $P_{lac}$ ), the  $P_L$  promoter, the  $P_R$  promoter, and the  $P_{SE}$  promoter, the SPO1 promoter, the SPO2 promoter and the  $penP$  promoter. Also included are promoters comprising two serially connected  $P_{trp}$  units, artificially built promoters such as the  $tac$  promoter, the  $lacT7$  promoter, and the  $let I$  promoter.

**[0045]** Furthermore, the  $xylA$  promoter [Appl. Microbiol. Biotechnol., 35, 594-599 (1991)] for expression in a microorganism belonging to the genus *Bacillus*, the P54-6 promoter [Appl. Microbiol. Biotechnol., 53, 674-679 (2000)] for expression in a microorganism belonging to the genus *Corynebacterium* can also be used.

**[0046]** How to acquire a DNA that encodes for the protein having L-amino acid transport activity, and how to prepare a microorganism by transforming a parent strain with the DNA are described in detail.

### (a) Acquisition of DNA that encodes for the protein having L-amino acid transport activity

**[0047]** A DNA that encodes for the protein having L-amino acid transport activity can be acquired by, for example, Southern hybridization of a chromosomal DNA library of a microorganism such as *E. coli* using a probe DNA that can be designed on the basis of the nucleotide sequence of a DNA that encodes for the protein having the amino acid sequence shown in SEQ ID:6 or PCR with the chromosomal DNA of a microorganism, preferably *E. coli*, as the template, using a primer DNA that can be designed on the basis of the nucleotide sequence [PCR Protocols, Academic Press (1990)].

**[0048]** A DNA that encodes for the protein having L-amino acid transport activity can also be acquired from the chromosomal DNA or cDNA library of a microorganism having the nucleotide sequence of a DNA that encodes for the protein having the amino acid sequence shown in SEQ ID NO:6 by the above-described method, on the basis of the nucleotide sequence obtained by searching various gene sequence databases for a sequence having 80% or more, preferably 90% or more, more preferably 95% or more, still more preferably 97% or more, particularly preferably 98% or more, most preferably 99% or more homology, to the nucleotide sequence.



**[0049]** By integrating the DNA acquired, as it is or after being cleaved with an appropriate restriction enzyme, into a vector by a conventional method, and introducing the recombinant DNA obtained into a host cell, then performing an analysis using a commonly used nucleotide sequence analytical method, for example, the dideoxy method [Proc. Natl. Acad. Sci., USA, 74, 5463 (1977)] or a nucleotide sequence analyzer such as the 3700 DNA analyzer (manufactured by Applied Biosystems Company), the nucleotide sequence of the DNA can be determined.

**[0050]** The vector is exemplified by pBluescriptII KS(+) (manufactured by Stratagene Company), pDIRECT [Nucleic Acids Res., 18, 6069 (1990)], pCR-Script Amp SK(+) (manufactured by Stratagene Company), pT7Blue (manufactured by Novagen Company), pCR II (manufactured by Invitrogen Company) and pCR-TRAP (manufactured by GenHunter Company).

**[0051]** Host cells include microorganisms belonging to the genus *Escherichia*. Microorganisms belonging to the genus *Escherichia* include, for example, *E. coli* XL1-Blue, *E. coli* XL2-Blue, *E. coli* DH1, *E. coli* MC1000, *E. coli* ATCC 12435, *E. coli* W1485, *E. coli* JM109, *E. coli* HB101, *E. coli* No.49, *E. coli* W3110, *E. coli* NY49, *E. coli* MP347, *E. coli* NM522, *E. coli* BL21 and *E. coli* ME8415.

**[0052]** Any method of introducing a DNA into the host-cell can be used to introduce the recombinant DNA; examples include a method using the calcium ion [Proc. Natl. Acad. Sci., USA, 69, 2110 (1972)], the protoplast method (JP-A-SHO-63-248394) and the electroporation method [Nucleic Acids Res., 16, 6127 (1988)].

**[0053]** If the determination of the nucleotide sequence shows the DNA acquired to be a partial-length DNA, the partial-length DNA may be subjected to Southern hybridization to a chromosomal DNA library using a probe to acquire a full-length DNA.

**[0054]** Furthermore, the desired DNA can be also prepared by chemical synthesis on the basis of the nucleotide sequence of the determined DNA, using the model 8905 DNA synthesizer manufactured by Perceptive Biosystems Company.

**[0055]** DNAs that can be acquired as described above include, for example, a DNA that encodes for the protein having the amino acid sequence shown in SEQ ID NO: 6, and a DNA having the nucleotide sequence shown in SEQ ID NO:5.

(b) Acquisition of a microorganism transformed with a plasmid vector that expresses a protein having L-amino acid transport activity

**[0056]** On the basis of the DNA that encodes for the protein having L-amino acid transport activity, obtained by the method above(a), a DNA fragment of appropriate length comprising the portion that encodes for the protein having L-amino acid transport activity is prepared as necessary. By replacing a nucleotide in the nucleotide sequence of the portion that encodes for the protein having L-amino acid transport activity so as to be a most suitable codon for the expression in the host cell, a transformant wherein the amount of the protein has been improved can be acquired.

**[0057]** A recombinant DNA is prepared by inserting the DNA fragment downstream of the promoter of an appropriate expression vector.

**[0058]** By introducing the recombinant DNA into a host cell suitable for the expression vector, a transformant wherein the activity of a protein having L-amino acid transport activity has been improved compared with the host cell, that is, the parent strain, can be obtained.

**[0059]** Useful expression vectors include an expression vector that is capable of self-replication in the above-described host cell or can be integrated into the chromosome, and that comprises a promoter at a position where a DNA that encodes for the protein having L-amino acid transport activity can be transcribed.

**[0060]** When using a prokaryote as the host cell, the recombinant DNA having a DNA that encodes for the protein having L-amino acid transport activity is preferably a recombinant DNA that is capable of self-replication in the prokaryote, and is configured with a promoter, a ribosome-binding sequence, the DNA that encodes for the protein having L-amino acid transport activity, and a transcription termination sequence. A gene that controls the promoter may be contained.

**[0061]** Examples of expression vectors include pColdI (manufactured by Takara Bio Company), pCDF-1b, pRSF-1b (both manufactured by Novagen Company), pMAL-c2x (manufactured by New England Biolabs Company), pGEX-4T-1 (manufactured by GE Healthcare Bioscience Company), pTrcHis (manufactured by Invitrogen Company), pSE280 (manufactured by Invitrogen Company), pGEMEX-1 (manufactured by Promega Company), pQE-30 (manufactured by QIAGEN Company), pET-3 (manufactured by Novagen Company), pKYP10 (JP-A-SHO-58-110600), pKYP200 [Agric. Biol. Chem., 48, 669 (1984)], pLSA1 [Agric. Biol. Chem., 53, 277 (1989)], pGEL1 [Proc. Natl. Acad. Sci., USA, 82, 4306 (1985)], pBluescriptII SK(+), pBluescript II KS(-) (manufactured by Stratagene Company), pTrs30 [prepared from *Escherichia coli* JM109/pTrs30 (FERM BP-5407)], pTrs32 [prepared from *Escherichia coli* JM109/pTrs32 (FERM BP-5408)], pPAC31 (WO98/12343), pUC19 [Gene, 33, 103 (1985)], pSTV28 (manufactured by Takara Bio Company), pUC118 (manufactured by Takara Bio Company) and pPA1 (JP-A-SHO-63-233798).

**[0062]** The promoter may be any one that functions in host cells such as of *E. coli*. Examples include promoters derived from *E. coli* or phage, such as the *trp* promoter ( $P_{trp}$ ), the *lac* promoter ( $P_{lac}$ ), the  $P_L$  promoter, the  $P_R$  promoter, and the  $P_{SE}$  promoter, as well as the SPO1 promoter and the SP02 promoter, the *penP* promoter. Promoters comprising

two serially connected  $P_{trp}$  units, artificially designed and modified promoters such as the tac promoter, the lacT7 promoter, and the let I promoter, can also be used.

**[0063]** Furthermore, the xylA promoter for expression in a microorganism belonging to the genus *Bacillus* [Appl. Microbiol. Biotechnol., 35, 594-599 (1991)] and the P54-6 promoter for expression in a microorganism belonging to the

genus *Corynebacterium* [Appl. Microbiol. Biotechnol., 53, 674-679 (2000)] can also be used.

**[0064]** It is preferable to use a plasmid wherein the distance between the Shine-Dalgarno sequence, which is a ribosome-binding sequence, and the initiation codon is adjusted to an appropriate distance (for example, 6 to 18 bases).

**[0065]** In the recombinant DNA prepared by inserting a DNA that encodes for the protein having L-amino acid transport activity to an expression vector, a transcription termination sequence is not always necessary; however, it is preferable that a transcription termination sequence is arranged immediately downstream of the structural gene.

**[0066]** Such recombinant DNAs include, for example, pSnorM, pSemrD, pSrarD and pSeamA described below.

**[0067]** Hosts for the recombinant DNA include prokaryotes, more preferably bacteria.

**[0068]** Examples of the prokaryote include microorganisms belonging to the genus *Escherichia*, the genus *Serratia*, the genus *Bacillus*, the genus *Brevibacterium*, the genus *Corynebacterium*, the genus *Microbacterium*, the genus *Pseudomonas*, the genus *Agrobacterium*, the genus *Alicyclobacillus*, the genus *Anabaena*, the genus *Anacystis*, the genus *Arthrobacter*, the genus *Azotobacter*, the genus *Chromatium*, the genus *Erwinia*, the genus *Methylobacterium*, the genus *Phormidium*, the genus *Rhodobacter*, the genus *Rhodopseudomonas*, the genus *Rhodospirillum*, the genus *Scenedesmus*, the genus *Streptomyces*, the genus *Synechococcus* and the genus *Zymomonas*, for example, *Escherichia coli*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus licheniformis*, *Bacillus pumilus*, *Brevibacterium ammoniagenes*, *Brevibacterium immariophilum*, *Brevibacterium saccharolyticum*, *Brevibacterium flavum*, *Brevibacterium lactofermentum*, *Corynebacterium glutamicum*, *Corynebacterium acetoacidophilum*, *Microbacterium ammoniophilum*, *Serratia ficaria*, *Serratia fonticola*, *Serratia liquefaciens*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Agrobacterium radiobacter*, *Agrobacterium rhizogenes*, *Agrobacterium rubi*, *Anabaena cylindrical*, *Anabaena doliolum*, *Anabaena flos-aquae*, *Arthrobacter citreus*, *Arthrobacter globiformis*, *Arthrobacter hydrocarboglutamicus*, *Arthrobacter mysorens*, *Arthrobacter nicotianae*, *Arthrobacter paraffineus*, *Arthrobacter protophormiae*, *Arthrobacter roseoparaffinus*, *Arthrobacter sulfureus*, *Arthrobacter ureafaciens*, *Chromatium buderi*, *Chromatium tepidum*, *Chromatium vinosum*, *Chromatium warmingii*, *Chromatium fluviatile*, *Erwinia uredovora*, *Erwinia carotovora*, *Erwinia ananas*, *Erwinia herbicola*, *Erwinia punctata*, *Erwinia terreus*, *Methylobacterium rhodesianum*, *Methylobacterium extorquens*, *Phormidium* sp. ATCC29409, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodopseudomonas blastica*, *Rhodopseudomonas marina*, *Rhodopseudomonas palustris*, *Rhodospirillum rubrum*, *Rhodospirillum salexigens*, *Rhodospirillum salinarum*, *Streptomyces ambofaciens*, *Streptomyces aureofaciens*, *Streptomyces aureus*, *Streptomyces fungicidicus*, *Streptomyces griseochromogenes*, *Streptomyces griseus*, *Streptomyces lividans*, *Streptomyces olivogriseus*, *Streptomyces rameus*, *Streptomyces tanashiensis*, *Streptomyces vinaceus* and *Zymomonas mobilis*.

**[0069]** Examples of preferable prokaryote include bacteria belonging to the genus *Escherichia*, the genus *Serratia*, the genus *Bacillus*, the genus *Brevibacterium*, the genus *Corynebacterium*, the genus *Pseudomonas* or the genus *Streptomyces*, for example, the above-mentioned species belonging to the genus *Escherichia*, the genus *Serratia*, the genus *Bacillus*, the genus *Brevibacterium*, the genus *Corynebacterium*, the genus *Pseudomonas* or the genus *Streptomyces*. Examples of more preferable bacteria include *Escherichia coli*, *Corynebacterium glutamicum*, *Corynebacterium ammoniagenes*, *Corynebacterium lactofermentum*, *Corynebacterium flavum*, *Corynebacterium efficiens*, *Bacillus subtilis*, *Bacillus megaterium*, *Serratia marcescens*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Streptomyces coelicolor* and *Streptomyces lividans*, and particularly preferred is *Escherichia coli*.

(c) Acquisition of a microorganism wherein a DNA that encodes for the protein having L-amino acid transport activity is integrated in the chromosomal DNA

**[0070]** By integrating a DNA that encodes for the protein having L-amino acid transport activity, obtained by the method above (a), into an anywhere position in the chromosomal DNA, it is also possible to acquire a microorganism wherein the activity of the protein having L-amino acid transport activity is higher than that of the parent strain.

**[0071]** Methods for integrating a DNA that encodes for the protein having L-amino acid transport activity into an anywhere position in the chromosomal DNA of a microorganism include methods based on homologous recombination; when using *E. coli* as the host, that is, the parent strain, the method described in Proc. Natl. Acad. Sci. USA., 97, 6640 (2000) can be mentioned.

(2) Preparation of a microorganism having the capability of producing an L-amino acid

**[0072]** The microorganism having the capability of producing an L-amino acid, used in the process for producing L-amino acid of the present invention may be any microorganism having the capability. When a strain isolated from nature

itself has the capability, the microorganism may be the strain as it is; in case of a modified or transformed mutant strain, it may be a microorganism to which the capability of producing a desired L-amino acid is conferred artificially by a publicly known method.

**[0073]** The publicly known method is exemplified by:

- (a) methods wherein at least one mechanism behind the control of amino acid biosynthesis is mitigated or cancelled,
  - (b) methods wherein the expression of at least one enzyme involved in amino acid biosynthesis is enhanced,
  - (c) methods wherein the number of copies of at least one enzyme gene involved in amino acid biosynthesis is amplified,
  - (d) methods wherein at least one metabolic pathway that branches from the biosynthesis pathway for an amino acid to a metabolite other than the amino acid is weakened or blocked, and
  - (e) methods wherein a cell strain whose resistance to amino acid analogues is higher than that of the wild-type strain is selected;
- the above-described publicly known methods can be used alone or in combination.

**[0074]** Methods above (a) are described in, for example, *Agric. Biol. Chem.*, 43, 105-111(1979), *J. Bacteriol.*, 110, 761-763(1972) and *Appl. Microbiol. Biotechnol.*, 39, 318-323(1993); methods (b) above are described in, for example, *Agric. Biol. Chem.*, 43, 105-111(1979) and *J. Bacteriol.*, 110, 761-763(1972); methods (c) above are described in, for example, *Appl. Microbiol. Biotechnol.*, 39, 318-323(1993) and *Agric. Biol. Chem.*, 39, 371-377(1987); methods (d) above are described in, for example, *Appl. Environ. Microbiol.*, 38, 181-190(1979) and *Agric. Biol. Chem.*, 42, 1773-1778(1978); methods (e) above are described in, for example, *Agric. Biol. Chem.*, 36, 1675-1684(1972), *Agric. Biol. Chem.*, 41, 109-116(1977), *Agric. Biol. Chem.*, 37, 2013-2023(1973) and *Agric. Biol. Chem.*, 51, 2089-2094(1987). With reference to the above-described documents, microorganisms having the capability of producing various amino acids can be prepared.

**[0075]** Furthermore, regarding how to prepare a microorganism having the capability of producing an amino acid in any one of (a) to (e) above or a combination thereof, many examples are described in *Biotechnology 2nd ed.*, Vol.6, *Products of Primary Metabolism* (VCH Verlagsgesellschaft mbH, Weinheim, 1996), section 14a, 14b, *Advances in Biochemical Engineering/Biotechnology*, 79, 1-35 (2003), and *Aminosan Hakko*, Japan Scientific Societies Press, Hiroshi Aida et al. (1986); in addition to the above, many reports are available on specific methods of preparing a microorganism having the capability of producing an amino acid, including JP-A-2003-164297, *Agric. Biol. Chem.*, 39, 153-160 (1975), *Agric. Biol. Chem.*, 39, 1149-1153(1975), JP-A-SHO-58-13599, *J. Gen. Appl. Microbiol.*, 4, 272-283(1958), JP-A-AHO-63-94985, *Agric. Biol. Chem.*, 37, 2013-2023(1973), pamphlet for International Patent Application Publication 97/15673, JP-A-SHO-56-18596 and JP-A-SHO-56-144092, JP-T-2003-511086; with reference to the above-described documents, a microorganism having the capability of producing one or more kinds of amino acids can be prepared.

**[0076]** Microorganisms having the capability of producing an amino acid, that can be prepared by one of the above-described method include, for example, microorganisms that lack the *sdaA* gene, the *sdaB* gene, the *sdaC* gene and the *glyA* gene, which possess L-serine degradation and uptake activities, and that exhibit enhanced expression of the *serA* gene deregulated to L-serine, as L-serine producers; microorganisms that lack the *glnE* gene as L-glutamine producers; microorganisms that carry the *cysE* gene deregulated to L-cysteine, for example, as L-cysteine producers; microorganisms that express the *pheA* gene deregulated to L-phenylalanine and/or the *aroF* gene deregulated to tyrosine as L-phenylalanine producers, and microorganisms to which  $\alpha$ -amino- $\beta$ -hydroxyvaleric acid (AHV) resistance and L-isoleucine, L-methionine and L-proline auxotrophy are conferred as L-threonine producers bacteria.

**[0077]** The above-described microorganism that produces and accumulates an amino acid may be any microorganism to which one of the methods (a) to (e) above is applicable, or any microorganism having the above-described genetic characters, and is preferably a prokaryote, more preferably a bacterium. Hosts for the recombinant DNA include prokaryotes, more preferably bacteria.

**[0078]** Specific examples of microorganisms that produce an amino acid include the *Escherichia coli* ATCC9637/*sdaABCglyA/pSserA*fbr2 strain, which lacks L-serine degrading enzymes (*sdaA*, *sdaB*, *glyA*) and the uptake system (*sdaC*), and which carries an expression plasmid for the *serA* gene deregulated to L-serine, as an L-serine producer; *Escherichia coli* JGLE1 and *Escherichia coli* JGLBE1, which are described in the pamphlet for International Patent Application Publication 06/001379 or the pamphlet for US Patent Application Publication 2005-0287626, as L-glutamine producers; the *Escherichia coli* ATCC9637/*sdaABCcysE256/pScysE*fbr1 strain, which lacks L-serine degrading enzymes (*sdaA*, *sdaB*) and the uptake system (*sdaC*), which has the *cysE* gene on the chromosomal DNA replaced with the *cysE* gene deregulated to L-cysteine, and which carries an expression plasmid for the *cysE* gene deregulated to L-cysteine, as an L-cysteine producer; the *Escherichia coli* NM522/*pPheA*fbraroFfbr strain, which carries an expression plasmid for the *pheA* gene deregulated to L-phenylalanine and the *aroF* gene deregulated to L-tyrosine, as L-phenylalanine producers; ATCC21148, ATCC21277 and ATCC21650 as L-threonine producers.

**[0079]** Furthermore, specific examples of microorganisms having the capability of producing an amino acid include

FERM BP-5807 and ATCC13032 as L-glutamic acid producers; FERM P-4806 and ATCC14751 as L-glutamine producers; FERM P-5084 and ATCC13286 as L-lysine producers; FERM P-5479, VKPM B-2175 and ATCC21608 as L-methionine producers; FERM BP-3757 and ATCC14310 as L-isoleucine producers; ATCC13005 and ATCC19561 as L-valine producers; FERM BP-4704 and ATCC21302 as L-leucine producers; FERM BP-4121 and ATCC15108 as L-alanine producers; ATCC21523 and FERM BP-6576 as L-serine producers; FERM BP-2807 and ATCC19224 as L-proline producers; FERM P-5616 and ATCC21831 as L-arginine producers; ATCC13232 as an L-ornithine producer; FERM BP-6674 and ATCC21607 as L-histidine producers; DSM10118, DSM10121, DSM10123 and FERM BP-1777 as L-tryptophan producers; ATCC13281 and ATCC21669 as L-phenylalanine producers; ATCC21652 as an L-tyrosine producer; W3110/pHC34 (described in JP-T-2003-511086) as an L-cysteine producer; Escherichia coli SOLR/pRH71 described in WO96/27669 as an L-4-hydroxyproline producer; FERM BP-5026 and FERMBP-5409 as L-3-hydroxyproline producers, and FERM P-5643 and FERM P-1645 as L-citrullin producers.

**[0080]** The microbial strains shown by the FERM numbers above can be obtained from the independent administrative corporation International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology (Japan); the microbial strains shown by the ATCC numbers from the American Type Culture Collection (US); the microbial strains shown by the VKPM numbers from the Russian National Collection of Industrial Microorganisms (Russia); and the microbial strains shown by the DSM numbers from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Germany).

### 3. Manufacturing process for L-amino acid of the present invention

**[0081]** A culture of a microorganism that can be prepared by the method described in above item 2 can be acquired by culturing the microorganism using a natural medium or synthetic medium that comprises a carbon source, a nitrogen source and minerals that can be utilized by the microorganism, and that enables efficient cultivation of the transformant.

**[0082]** The carbon source may be any one that can be utilized by the organism; carbohydrates such as glucose, fructose, sucrose, molasses containing them, and starch or starch hydrolysates, organic acids such as acetic acid and propionic acid, alcohols such as ethanol and propanol can be used.

**[0083]** Useful nitrogen sources include ammonia, ammonium salts of inorganic acids or organic acids such as ammonium chloride, ammonium sulfate, ammonium acetate, and ammonium phosphate, other nitrogen-containing compounds, as well as peptone, meat extract, yeast extract, corn steep liquor, casein hydrolysates, soy cake and soy cake hydrolysates, various fermented cell bodies, and digests thereof.

**[0084]** Useful inorganic salts include primary potassium phosphate, secondary potassium phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate and calcium carbonate.

**[0085]** The cultivation is normally performed under aerobic conditions such as shaking culture or deep spinner culture. Cultivation temperature is preferably 15 to 40°C; cultivation time is normally 5 hours to 7 days. During the cultivation, pH is kept at 3.0 to 9.0. Adjustments of the pH are achieved using an inorganic or organic acid, an alkali solution, urea, calcium carbonate and/or ammonia.

**[0086]** Antibiotics such as ampicillin and tetracycline may be added to the medium during the cultivation as necessary.

**[0087]** When culturing a microorganism transformed with an expression vector using an inducible promoter, an inducer may be added to the medium as necessary. For example, when culturing a microorganism transformed with an expression vector using the lac promoter, isopropyl-β-D-thiogalactopyranoside may be added; when culturing a microorganism transformed with an expression vector using the trp promoter, indolacrylic acid may be added to the medium.

**[0088]** The transformant of a microorganism having the capability of producing an L-amino acid, and expressing a protein having L-amino acid transport activity, constructed as described above, is cultured in a medium to produce an L-amino. The L-amino acid produced is efficiently transported from inside of the cell body into the medium by the L-amino acid transport activity possessed by the transformant, and accumulates in the medium. Therefore, by collecting the L-amino from the culture, the desired L-amino can be efficiently produced.

**[0089]** Collection of the L-amino accumulated in the aqueous medium or culture can be achieved by an ordinary method using activated charcoal or ion exchange resin or by organic solvent extraction, crystallization, thin-layer chromatography or high performance liquid chromatography.

**[0090]** Amino acid producing strains were prepared by the methods described below.

#### [1] Construction an expression plasmid for deregulated serA gene

**[0091]** PCR was performed with the chromosomal DNA of the Escherichia coli W3110 strain as the template, using synthetic DNAs consisting of the nucleotide sequences shown in SEQ ID NOS:18 and 19, respectively, as a primer set. The PCR was carried out by preparing 50μL of a reaction liquid comprising 0.1μg of the chromosomal DNA as the template, 0.3μmol/L of each primer, 1 unit of KOD-plus-DNA polymerase (manufactured by Toyobo), 5 μL of ×10 buffer

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solution for KOD-plus- DNA polymerase (manufactured by Toyobo), 100 $\mu$ mol/L MgSO<sub>4</sub>, and 200 $\mu$ mol/L of each dNTP (dATP, dGTP, dCTP and dTTP), and repeating the step of treatment at 94°C for 15 seconds, at 55°C for 30 seconds, and at 68°C for 2 minutes 30 times.

5 [0092] The amplified DNA fragment obtained by the PCR was digested with BglIII and HindIII, and pTrs30 with BamHI and HindIII, after which the two DNAs were ligated together using a ligation kit (manufactured by Takara Bio Company), and the Escherichia coli DH5 $\alpha$  strain was transformed using the ligation product DNA. By the method described above, a plasmid DNA wherein the serA gene was joined downstream of the trp promoter was acquired, and this was named as pTrs30-serA.

10 [0093] PCR was performed with pTrs30-serA as the template, using synthetic DNAs consisting of the nucleotide sequences shown in SEQ ID NOS:20 and 21, respectively, whose 5' terminus was modified with a phosphate group, as a primer set.

[0094] The PCR reaction was carried out with the same conditions and reaction liquid composition as the above but using 0.01 $\mu$ g of pTrs30-serA DNA as the template.

15 [0095] After the PCR reaction, amplification of an about 5.8kb DNA fragment was confirmed; the amplified DNA fragment was purified according to a conventional method.

[0096] The linear DNA fragments amplified above were ligated together into a circular using a ligation kit (manufactured by Takara Bio Company); the Escherichia coli DH5 $\alpha$  strain was transformed using the circular DNA. With ampicillin resistance as an index, a transformant was selected; plasmid DNA was extracted from the transformant obtained.

20 [0097] Thus, a plasmid DNA having a structure wherein the serA gene deregulated to L-serine, resulting from replacement of the 294th glycine in the amino acid sequence shown in SEQ ID NO:17 by L-valine, was inserted downstream of the trp promoter of pTrs30, was prepared, and this was named as pSserAfr1.

[0098] Furthermore, PCR was performed with pSserAfr1 as the template using synthetic DNAs consisting of the nucleotide sequences shown in SEQ ID NOS:22 and 23, respectively, whose 5' terminus was modified with a phosphate group, as a primer set.

25 [0099] The PCR reaction liquid composition and reaction conditions were the same as those described above.

[0100] The amplified linear DNA fragments were ligated together into a circular DNA, and the Escherichia coli DH5 $\alpha$  strain was transformed using the circular DNA. Plasmid DNA was extracted from the transformant obtained.

30 [0101] Thus the plasmid DNA having the structure wherein the serA gene deregulated to L-serine, resulting from replacement of the 294th glycine in the amino acid sequence shown in SEQ ID NO:17 by L-valine, and the 364th L-asparagine by L-alanine, was inserted downstream of the trp promoter of pTrs30, was prepared. this was named as pSserAfr2.

### [2] Preparation of microorganisms lacking the sdaA, sdaB, sdaC and glyA genes

35 [0102] Deletion of a particular gene on the chromosomal DNA of Escherichia coli was performed according to a method utilizing a homologous recombination system of lambda phage [Proc. Natl. Acad. Sci. USA., 97, 6641-6645(2000)]. The plasmids pKD46, pKD3 and pCP20 described below were used after being extracted by a publicly known method from Escherichia coli strains carrying the plasmids, obtained from the Escherichia coli Genetic Stock Center (Yale University, US).

#### 40 (1) Cloning of drug resistance gene fragments for gene deletion

45 [0103] PCR was performed using DNAs consisting of the nucleotide sequences shown in SEQ ID NOS:24 and 25, and 26 and 27, respectively, as a primer set for amplifying a DNA fragment for deleting the sdaA gene, DNAs consisting of the nucleotide sequences shown in SEQ ID NOS:28 and 29, and 30 and 31, respectively, as a primer set for amplifying a DNA fragment for deleting the sdaC-sdaB gene, and DNAs consisting of the nucleotide sequences shown in SEQ ID NOS:32 and 33, and 34 and 35, respectively, as a primer set for amplifying a DNA fragment for deleting the glyA gene, with the chromosomal DNA of the Escherichia coli ATCC9637 strain as the template. The PCR was carried out by repeating the step of treatment at 94°C for 1 minute, at 55°C for 2 minutes, and at 72°C for 1 minute 30 times using 50 40 $\mu$ L of a reaction liquid comprising 0.1 $\mu$ g of the chromosomal DNA, 0.5 $\mu$ mol/L of each primer, 2.5 units of Pfu DNA polymerase, 4 $\mu$ L of  $\times 10$  buffer solution for Pfu DNA polymerase, and 200 $\mu$ mol/L of each deoxyNTP.

[0104] By the PCR, desired homologous sequence fragments of upstream and downstream regions for deleting each of the sdaA, sdaB, sdaC and glyA genes (referred to as upstream DNA fragment and downstream DNA fragment, respectively) were acquired.

55 [0105] Next, by a crossover PCR method [J. Bacteriol., 179, 6228-6237 (1997)] with the upstream DNA fragment and downstream DNA fragment of each of the above-described genes, and HindIII-cleaved pKD3 as the template, using a synthetic DNA consisting of the nucleotide sequences shown in SEQ ID NOS24 and 27, respectively, as a primer set for the DNA fragment for deleting the sdaA gene, or using synthetic DNAs consisting of the nucleotide sequences shown

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in SEQ ID NOS:28 and 31, respectively, as a primer set for the DNA fragment for deleting the *sdaC-sdaB* gene, or using a synthetic DNA consisting of the nucleotide sequences shown in SEQ ID NOS:32 and 35, respectively, as a primer set for the DNA fragment for deleting the *glyA* gene, a DNA fragment comprising the chloramphenicol resistance gene portion of pKD3 inserted into the center, and the three DNA fragments joined together (DNA fragments for deleting the *sdaA*, *sdaB*, *sdaC* and *glyA* genes) was acquired.

### (2) Preparation of *Escherichia coli* lacking the *sdaA* gene

**[0106]** The *Escherichia coli* ATCC9637 strain was transformed with pKD46; the transformant obtained was named as *Escherichia coli* ATCC9637/pKD46.

**[0107]** The DNA fragment for deleting the *sdaA* gene, acquired by method above, was introduced by the electroporation into *Escherichia coli* ATCC9637/pKD46 obtained by cultivation in the presence of 10mmol/L L-arabinose and 50 $\mu$ g/ml ampicillin; with chloramphenicol resistance as the index, a transformant wherein the DNA fragment was integrated on the chromosomal DNA of *Escherichia coli* ATCC9637/pKD46 by homologous recombination (the transformant was named as *Escherichia coli* ATCC9637/pKD46 *sdaA::cat*) was selected.

**[0108]** *Escherichia coli* ATCC9637/pKD46 *sdaA::cat* was inoculated to a LB agar medium [LB medium [10g/l Bactotrypton (manufactured by Difco Company), 5g/l yeast extract (manufactured by Difco Company), 5g/l sodium chloride] supplemented with 1.5% agar] containing 25mg/L chloramphenicol, and cultured at 42°C for 14 hours, after which single colony was isolated. Each colony obtained was replicated to a LB agar medium containing 25mg/L chloramphenicol and a LB agar medium containing 100mg/l ampicillin, and cultured at 37°C; with chloramphenicol resistance and ampicillin susceptibility as the index, a strain deprived of pKD46 (*Escherichia coli* ATCC9637 *sdaA::cat*) was selected.

**[0109]** Next, *Escherichia coli* ATCC9637 *sdaA::cat* was transformed with pCP20 to acquire a strain carrying pCP20 (*Escherichia coli* ATCC9637/pCP20 *sdaA::cat*).

**[0110]** *Escherichia coli* ATCC9637/pCP20 *sdaA::cat* was inoculated to a drug-free LB agar medium, and cultured at 42°C for 14 hours, after which single colony was isolated. Each colony obtained was replicated to a drug-free LB agar medium, a LB agar medium containing 25mg/L chloramphenicol, and a LB agar medium containing 100mg/L ampicillin, and cultured at 30°C; several strains exhibiting chloramphenicol susceptibility and ampicillin susceptibility were selected.

**[0111]** The chromosomal DNA was prepared from each of the strains selected above; PCR was performed using DNAs designed on the basis of the nucleotide sequences of DNAs located outside of the *sdaA* gene on the chromosomal DNA as a primer set, with the chromosomal DNA as the template. The PCR was carried out by repeating the step consisting of treatment at 94°C for 1 minute, at 55°C for 2 minutes, and at 72°C for 3 minutes 30 times using 40 $\mu$ L of a reaction liquid comprising 0.1g of the chromosomal DNA, 0.5 $\mu$ mol/L of each primer, 2.5 units of Pfu DNA polymerase, 4 $\mu$ L of  $\times 10$  buffer solution for Pfu DNA polymerase, and 200 $\mu$ mol/L of each deoxyNTP.

**[0112]** A strain confirmed to lack the *sdaA* gene from the chromosomal DNA by the above-described PCR was named as the *Escherichia coli* ATCC9637*sdaA* strain.

### (3) Preparation of *Escherichia coli* multiply lacking the *sdaA*, *sdaB*, *sdaC* and *glyA* genes

**[0113]** By repeating the method performed in (2) on the ATCC9637*sdaA* strain obtained in (2), using the chloramphenicol resistance gene fragment for deleting the *sdaC-sdaB* or *glyA* gene, acquired in (1), a strain further lacking the *sdaC*, *sdaB* and *glyA* genes was prepared.

**[0114]** Acquisition of a strain lacking the genes by the above-described method was confirmed by preparing the chromosomal DNA from each selected strain in the same manner as (2), and performing PCR using DNAs designed on the basis of the nucleotide sequences of DNAs located outside of the *sdaC-sdaB* or *glyA* gene on the chromosomal DNA as a primer set, with the chromosomal DNA as the template.

**[0115]** The strain identified as a strain multiply lacking the *sdaA*, *sdaC-sdaB* and *glyA* genes above was named as the *Escherichia coli* ATCC9637*sdaABCglyA* strain.

### [3] Construction of *Escherichia coli*-derived expression plasmid for deregulated *pheA* gene and deregulated *aroF* gene

#### (1) Building an expression plasmid for deregulated *pheA* gene

**[0116]** A deregulated *pheA* gene was acquired from the plasmid pE *pheA* 22 (JP-A-SHO-61-260892), which expresses the *pheA* gene deregulated to phenylalanine, obtained by introduction of a phenylalanine analogue resistance mutation, and a deregulated *aroF* gene was acquired from the plasmid pE *aroF* 18 (JP-A-SHO-62-65691), which expresses the *aroF* gene deregulated to tyrosine, obtained by introduction of a tyrosine resistance mutation; an expression plasmid was constructed by the method described below.

**[0117]** PCR was performed using synthetic DNAs having the nucleotide sequences shown in SEQ ID NOS:36 and

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SEQ ID NO:37, respectively, as a primer set, with the plasmid pE pheA 22 as the template. The PCR was carried out by preparing 40 $\mu$ L of a reaction liquid comprising 10ng of plasmid DNA, 0.5 $\mu$ mol/L of each primer, 2.5 units of Pfu DNA polymerase, 4 $\mu$ L of  $\times$ 10 buffer solution for Pfu DNA polymerase, and 200 $\mu$ mol/L of each dNTP, and repeating the step consisting of treatment at 94 $^{\circ}$ C for 1 minute, at 55 $^{\circ}$ C for 2 minutes, and at 72 $^{\circ}$ C for 3 minutes 30 times.

5 **[0118]** A 1/10 volume of the reaction liquid was subjected to agarose gel electrophoresis, and amplification of an about 1.1 kb fragment corresponding to a pheA gene fragment was confirmed, after which TE-saturated phenol/chloroform in a volume equal to that of the remaining reaction liquid was added, and they were mixed. The mixed liquid was centrifuged, after which a 2-fold volume of cold ethanol was added to, and mixed with, the upper layer obtained, and the mixture was allowed to stand at -80 $^{\circ}$ C for 30 minutes. The solution was centrifuged, and the precipitate of DNA obtained was dissolved in 20 $\mu$ L of TE.

10 **[0119]** Using 5 $\mu$ L of the solution, the amplified DNA was cleaved with the restriction enzymes ClaI and BamHI; DNA fragments were separated by agarose gel electrophoresis, after which a 1.1 kb DNA fragment comprising the pheA gene was recovered using the Gene Cleaning II kit.

15 **[0120]** A 0.2 $\mu$ g of the expression vector pTrs30 comprising the trp promoter was cleaved with the restriction enzymes ClaI and BamHI, after which DNA fragments were separated by agarose gel electrophoresis, and a 4.6 kb DNA fragment was recovered in the same manner as the above.

**[0121]** The 1.1 kb DNA fragment comprising the pheA gene and the 4.6 kb DNA fragment, obtained above, were ligated by a reaction at 16 $^{\circ}$ C for 16 hours using a ligation kit.

20 **[0122]** The Escherichia coli NM522 strain was transformed using the reaction liquid by a method using the calcium ion, after which the transformant was applied over a LB agar medium supplemented with 50 $\mu$ g/ml ampicillin, and cultured at 30 $^{\circ}$ C overnight.

25 **[0123]** A plasmid was extracted from a colony of transformants that had grown according to a publicly known method; an expression plasmid for the deregulated pheA gene was confirmed to be acquired by restriction enzyme digestion, and the plasmid was named as pPHEA1.

### (2) Construction of expression plasmid for deregulated pheA gene and deregulated aroF gene

30 **[0124]** PCR was performed using synthetic DNAs having the nucleotide sequences shown in SEQ ID NOS:38 and SEQ ID NO:39, respectively, as a primer set, with the plasmid pE aroF 18 as the template. The PCR was carried out using the same reaction liquid composition and reaction conditions as above (1).

35 **[0125]** A 1/10 volume of the reaction liquid was subjected to agarose gel electrophoresis, and amplification of an about 1.1 kb fragment corresponding to an aroF gene fragment was confirmed, after which TE-saturated phenol/chloroform in a volume equal to that of the remaining reaction liquid was added, and they were mixed. The mixed liquid was centrifuged, after which a 2-fold volume of cold ethanol was added to, and mixed with, the upper layer obtained, and the mixture was allowed to stand at -80 $^{\circ}$ C for 30 minutes. The solution was centrifuged, and the precipitate of DNA obtained was dissolved in 20 $\mu$ L of TE.

40 **[0126]** Using 5 $\mu$ L of the solution, the amplified DNA was cleaved with the restriction enzymes BglII and BamHI, and DNA fragments were separated by agarose gel electrophoresis, after which a 1.1 kb DNA fragment comprising the deregulated aroF gene was recovered using the Gene Clean II kit.

45 **[0127]** Next, 0.2 $\mu$ g of the expression plasmid pPHEA1 for deregulated pheA gene obtained in above(1) was cleaved with the restriction enzyme BamHI, after which DNA fragments were separated by agarose gel electrophoresis, and a 5.7 kb DNA fragment was recovered in the same manner as the above. Terminal dephosphorylation of the 5.7 kb DNA fragment was performed by alkaline phosphatase treatment at 60 $^{\circ}$ C for 30 minutes. TE-saturated phenol/chloroform in a volume equal to that of the reaction liquid was added, and they were mixed; the mixture was centrifuged, after which a 2-fold volume of cold ethanol was added to, and mixed with, the upper layer obtained, and the mixture was allowed to stand at -80 $^{\circ}$ C for 30 minutes. The solution was centrifuged, and the precipitate of DNA obtained was dissolved in 20 $\mu$ L of TE.

50 **[0128]** The 1.1kb DNA fragment comprising the deregulated aroF gene and the alkaline phosphatase-treated 5.7 kb DNA fragment, obtained above, were ligated together by a reaction at 16 $^{\circ}$ C for 16 hours using a ligation kit.

**[0129]** The Escherichia coli NM522 strain was transformed using the reaction liquid by a method using the calcium ion, after which the reaction liquid was applied to an LB agar medium containing 50 $\mu$ g/mL ampicillin, and cultured at 30 $^{\circ}$ C overnight.

55 **[0130]** A plasmid was extracted from a colony of transformants that had grown, according to a publicly known method; an expression plasmid for a deregulated aroF gene and a deregulated pheA gene wherein the deregulated aroF gene was inserted in the orthodox orientation with respect to the deregulated pheA gene was confirmed to be acquired by restriction enzyme digestion, and the plasmid was named as pBpheA<sub>f</sub>braroF<sub>f</sub>br.

[4] Preparation of a microorganism lacking the serine decomposition and uptake system *sdaA*, *sdaB*, and *sdaC* genes, and having a deregulated *cysE* gene replacing on the chromosome

(1) Cloning of a drug resistance gene fragment for deleting the *cysE* gene and a gene fragment for replacing a deregulated *cysE* gene

[0131] PCR was performed in the same manner as in [2](1) and using synthetic DNAs consisting of the nucleotide sequences shown in SEQ ID NOS:40 and 41, and by SEQ ID NOS:42 and 43, respectively, as a primer set for amplifying a DNA fragment for deleting the *cysE* gene, with the chromosomal DNA of the *Escherichia coli* W3110 strain as the template. By the PCR, desired homologous sequence fragments of upstream and downstream regions for deleting the *cysE* gene (referred to as upstream DNA fragment and downstream DNA fragment, respectively) were acquired.

[0132] PCR was performed in the same manner as the above using synthetic DNAs consisting of the nucleotide sequences shown in SEQ ID NOS:40 and 44, respectively, as well as synthetic DNAs consisting of the nucleotide sequences shown in SEQ ID NOS:43 and 45, respectively, each as a primer set for amplifying a DNA fragment for replacing the deregulated *cysE* gene, with the chromosomal DNA of the *Escherichia coli* W3110 strain as the template, to acquire homologous sequence fragments of upstream and downstream regions for replacing deregulated *cysE* (referred to as replacement upstream DNA fragment and replacement downstream DNA fragment, respectively).

[0133] Next, to acquire a DNA fragment for deleting the *cysE* gene, crossover PCR was performed with the upstream DNA fragment and downstream fragment for deleting the *cysE* gene, obtained above, and HindIII-cleaved pKD3 as the template, with DNAs consisting of the nucleotide sequences shown in SEQ ID NOS:40 and 43, respectively, as a primer set, to acquire a DNA fragment wherein the chloramphenicol resistance gene portion of pKD3 was inserted into the central portion, and the three DNA fragments are joined.

[0134] To acquire a DNA fragment for replacing a deregulated *cysE* gene, crossover PCR was performed with the above-described replacement upstream DNA fragment and replacement downstream DNA fragment as the template, using DNAs consisting of the nucleotide sequences shown in SEQ ID NOS:40 and 43, respectively, as a primer set; thereby, a DNA fragment wherein the two DNA fragments comprising a desensitization mutation were joined on the *cysE* gene was acquired.

(2) Preparation of *Escherichia coli* having a replacing deregulated *cysE* gene

[0135] The *Escherichia coli* ATCC9637*sdaABC* strain lacking the *sdaA*, *sdaB*, and *sdaC* genes, obtained in [2] (3), was transformed with pKD46; the transformant obtained was named as *Escherichia coli* ATCC9637*sdaABC/pKD46*.

[0136] In the same manner as [2] (2), transformants wherein the DNA fragment for deleting the *cysE* gene of above (1) was integrated on the chromosomal DNA of *Escherichia coli* ATCC9637*sdaABC/pKD46* by homologous recombination (*Escherichia coli* ATCC9637*sdaABC/pKD46 cysE::cat*) were selected, after which strains deprived of the chloramphenicol resistance gene was selected.

[0137] The chromosomal DNA was prepared from each of the strains selected above, and PCR was performed in the same manner as [2] (2) using DNAs designed on the basis of the nucleotide sequences of the DNA located outside of the *cysE* gene on the chromosomal DNA as a primer set.

[0138] The strain that provided a short amplified fragment not containing the *cysE* gene in the above-described PCR was selected as a strain lacking the *cysE* gene, and was named as the *Escherichia coli* ATCC9637*sdaABCcysE1* strain.

[0139] Next, the deregulated *cysE* gene was replaced on the chromosome.

[0140] The above-described *Escherichia coli* ATCC9637*sdaABCcysE1* strain was transformed with pKD46; the transformant obtained was named as *Escherichia coli* ATCC9637*sdaABCcysE1/pKD46*.

[0141] In the same manner as [2] (2), transformants wherein the DNA fragment for replacing the deregulated *cysE* gene of above (1) was integrated on the chromosomal DNA of *Escherichia coli* ATCC9637*sdaABCcysE1/pKD46* by homologous recombination were selected on the basis of growth on the M9 + glucose minimal agar medium [6g/L disodium hydrogen phosphate, 3g/L potassium dihydrogen phosphate, 0.5g/L sodium chloride, 1g/L ammonium chloride, 2g/L glucose, 1mM magnesium sulfate heptahydrate, 0.1mM calcium chloride dihydrate, 10mg/l vitamin B<sub>1</sub>, and agar 15g/L, of which glucose, magnesium sulfate, calcium chloride, and vitamin B<sub>1</sub> were separately sterilized and added].

[0142] The chromosomal DNA was prepared from each of the strains selected above, and PCR was performed in the same manner as [2] (2) using DNAs designed on the basis of the nucleotide sequences of the DNA located outside of the *cysE* gene on the chromosomal DNA as a primer set.

[0143] As a DNA fragment comprising the *cysE* gene was amplified in the above-described PCR, acquirement of a strain having replacement with the deregulated *cysE* gene was confirmed, and this was named the *Escherichia coli* ATCC9637*sdaABCcysE256* strain.



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[5] Construction of Escherichia coli-derived expression plasmid for deregulated cysE gene

[0144] The Escherichia coli ATCC9637sdaABCcysE256 strain obtained in [4] was inoculated to a LB medium and subjected to standing culture at 30°C overnight. After the cultivation, the chromosomal DNA of the microorganism was isolated and purified by the method using saturated phenol described in the Current Protocols in Molecular Biology.

[0145] With the same conditions and reaction liquid composition as those in [1], PCR was performed using synthetic DNAs consisting of the nucleotide sequences shown in SEQ ID NOS:46 and 47, respectively, as a primer set, and using the above-described chromosomal DNA as the template.

[0146] The amplified DNA fragment obtained by the above-described PCR and pTrs30 were digested with HindIII and BamHI, respectively, after which the two DNAs were ligated together using a ligation kit (manufactured by Takara Bio Company), and the Escherichia coli DH5 $\alpha$  strain was transformed using the ligation product DNA. Plasmid DNA was extracted from the transformant obtained.

[0147] An expression vector wherein the deregulated cysE gene was joined downstream of the trp promoter was built by the method described above, and was named as pScysEfbr1.

Examples

[0148] Examples are given below.

### Example 1

(1) Construction of an expression plasmid for the norM gene

[0149] PCR was performed with the chromosomal DNA of the Escherichia coli W3110 strain as the template, using synthetic DNAs consisting of the nucleotide sequences shown in SEQ ID NOS:9 and 10, respectively, as a primer set.

[0150] The PCR was carried out by preparing 50  $\mu$ L of a reaction liquid comprising 0.1  $\mu$ g of the chromosomal DNA as the template, 0.3  $\mu$ mol/L of each primer, 1 unit of KOD-plus-DNA polymerase (manufactured by Toyobo), 5  $\mu$ L of  $\times$ 10 buffer solution for KOD-plus-DNA polymerase (manufactured by Toyobo), 100  $\mu$ mol/L MgSO<sub>4</sub>, and 200  $\mu$ mol/L of each dNTP(dATP, dGTP, dCTP and dTTP), and repeating the step of treatment at 94°C for 15 seconds, at 55°C for 30 seconds, and at 68°C for minutes 30 times.

[0151] Amplification of an about 1.4kb DNA fragment was confirmed, and the DNA fragment was purified according to a conventional method.

[0152] The DNA fragment and the expression vector pTrs30 [can be prepared from Escherichia coli JM109/pTrs30 (FERM BP-5407)] were cleaved with HindIII and BamHI, respectively; DNA fragments were separated by agarose electrophoresis, after which each of the restriction enzyme digested DNA fragments was recovered using the GENE-CLEAN II kit (manufactured by BIO 101 Company).

[0153] The about 1.4kb DNA fragment and pTrs30 restriction enzyme digested fragment obtained by the recovery were ligated together using a ligation kit (manufactured by Takara Bio Company).

[0154] The Escherichia coli DH5 $\alpha$  strain (manufactured by Toyobo) was transformed using the DNA after the ligation; a transformant was selected with ampicillin resistance as an index.

[0155] A plasmid was extracted from the transformant selected, according to a publicly known method, and the structure thereof was analyzed using restriction enzymes; it was confirmed that the plasmid obtained had a structure wherein the norM gene consisting of the nucleotide sequence shown in SEQ ID NO:1 was inserted downstream of the trp promoter of the expression vector pTrs30. The plasmid was named as pTrs30-norM.

[0156] The plasmid pTrs30-norM and the expression vector pSTV29 (manufactured by Takara Bio Company) were cleaved with EcoRI and BamHI, respectively, and fragments were ligated together in the same manner as the above to yield a plasmid DNA having a structure wherein the trp promoter and the norM gene were inserted into pSTV29. The plasmid obtained was named pSnorM.

(2) Construction of an emrD gene expression plasmid

[0157] With the same reaction liquid composition and reaction conditions as (1), PCR was performed with the chromosomal DNA of the Escherichia coli W3110 strain as the template, using synthetic DNAs consisting of the nucleotide sequences shown in SEQ ID NOS:11 and 12, respectively, as a primer set.

[0158] The amplified DNA fragment obtained by the PCR and pTrs30 were digested with HindIII and SacI, respectively, after which a plasmid DNA having a structure wherein the emrD gene consisting of the nucleotide sequence shown in SEQ ID NO:3 was inserted downstream of the trp promoter of pTrs30 was acquired in the same manner as (1). This plasmid DNA obtained was named as pTrs30-emrD.

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**[0159]** The pTrs30-emrD and pSTV29 obtained above were digested with EcoRI and SacI, respectively, after which a plasmid DNA wherein the trp promoter and emrD gene were ligated together to pSTV29 was prepared in the same manner as (1). The plasmid DNA obtained was named as pSemrD.

### 5 (3) Construction of an rarD gene expression plasmid

**[0160]** With the same reaction liquid composition and reaction conditions as (1), PCR was performed with the chromosomal DNA of the Escherichia coli W3110 strain as the template, using synthetic DNAs consisting of the nucleotide sequences shown in SEQ ID NOS:13 and 14, respectively, as a primer set.

10 **[0161]** The amplified DNA fragment obtained by the PCR and pTrs30 were digested with HindIII and BamHI, respectively, after which a plasmid DNA having a structure wherein the rarD gene consisting of the nucleotide sequence shown in SEQ ID NO:5 was inserted downstream of the trp promoter of pTrs30 was prepared in the same manner as (1), and this was named as pTrs30-rarD.

15 **[0162]** The pTrs30-rarD and pSTV29 obtained above were digested with EcoRI and BamHI, respectively, after which a plasmid DNA wherein the trp promoter and the rarD gene were ligated to pSTV29 was constructed in the same manner as (1), and this was named as pSrarD.

### (4) Construction of an eamA gene expression plasmid

20 **[0163]** With the same reaction liquid composition and reaction conditions as (1), PCR was performed with the chromosomal DNA of the Escherichia coli W3110 strain as the template, using synthetic DNAs consisting of the nucleotide sequences shown in SEQ ID NOS:15 and 16, respectively, as a primer set.

25 **[0164]** The amplified DNA fragment obtained by the PCR and pTrs30 were digested with HindIII and BamHI, respectively, after which a plasmid having a structure wherein the eamA gene consisting of the nucleotide sequence shown in SEQ ID NO:7 was inserted downstream of the trp promoter of pTrs30 was prepared in the same manner as (1), and this was named as pTrs30-eamA.

30 **[0165]** The pTrs30-eamA and pSTV29 obtained above were digested with EcoRI and BamHI, respectively, after which a plasmid DNA having a structure wherein the trp promoter and the eamA gene were inserted into pSTV29 was prepared in the same manner as (1), and this was named as pSeamA.

### Example 2. Production of L-serine (L-Ser)

35 **[0166]** The ATCC9637sdaABCglyA strain obtained in Preparation of Amino Acid Producers [2] was transformed with the pSserAfbr2 obtained in Preparation of Amino Acid Producers [1] to acquire Escherichia coli ATCC9637sdaABCglyA/pSserAfbr2, which has the capability of producing a protein possessing L-serine biosynthesis intermediate (3-phospho-hydroxy-pyruvate) synthetase activity.

40 **[0167]** Next, Escherichia coli-ATCC9637sdaABCglyA/pSserAfbr2 was transformed with each of pSnorM, pSemrD, pSrarD, pSeamA and pSTV29 obtained in Preparation of Amino Acid Producers [1] to acquire transformants, which were named as Escherichia coli ATCC9637sdaABCglyA/pSserAfbr2/pSnorM, ATCC9637sdaABCglyA/pSserAfbr2/pSemrD, ATCC9637sdaABCglyA/pSserAfbr2/pSrarD, ATCC9637sdaABCglyA/pSserAfbr2/pSeamA and ATCC9637sdaABCglyA/pSserAfbr2/pSTV29, respectively.

45 **[0168]** The transformant obtained above was inoculated to a large test tube containing 5ml of the medium A [10g/L Trypton (Difco), 5g/L Yeast extract (Difco), 5g/L sodium chloride, 1g/L potassium dihydrogen phosphate, 3g/L dipotassium hydrogen phosphate] supplemented with 100 $\mu$ g/ml ampicillin and 20 $\mu$ g/ml chloramphenicol, and cultured at 30°C for 16 hours.

50 **[0169]** The culture broth was inoculated at 10% to a test tube containing 5ml of the medium B [0.72g/L Yeast extract, 14.4g/L ammonium sulfate, 1.8g/L magnesium sulfate heptahydrate, 72mg/L calcium chloride, 100 $\mu$ g/L vitamin B<sub>1</sub>, 21.6mg/L iron sulfate heptahydrate, 7.2mg/L manganese sulfate, 1.4mg/L copper sulfate, 3.6mg/L zinc sulfate, 1.4mg/L nickel chloride, 1.4mg/L cobalt chloride, 21.6mg/L calcium pantothenate, 14.4mg/L nicotinic acid, 36mg/L thiamine, 14.4mg/L pyridoxine hydrochloride, 72mg/L glycine, 21g/L calcium carbonate, 48g/L glucose, 0.56g/L potassium dihydrogen phosphate, 2.88g/L dipotassium hydrogen phosphate, 0.6g/L disodium hydrogen phosphate; pH not adjusted; glucose, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and disodium hydrogen phosphate were added after being separately boiled] supplemented with 100 $\mu$ g/ml ampicillin and 20 $\mu$ g/ml chloramphenicol, and cultured at 30°C for 24 hours, after which the culture broth was centrifuged, and a culture supernatant was acquired.

55 **[0170]** The products in the culture supernatant were analyzed using HPLC. The results are shown in Table 1.

Table 1

E. coli strain	OD <sub>660</sub>	L-Ser (mg/L)
ATCC9637sdaABCglyA/pSserAfr2/pSTV29	6.1	10.6
ATCC9637sdaABCglyA/pSserAfr2/pSnorM	5.8	14.4
ATCC9637sdaABCglyA/pSserAfr2/pSemrD	7.1	12.3
ATCC9637sdaABCglyA/pSserAfr2/pSrarD	6.2	23.8
ATCC9637sdaABCglyA/pSserAfr2/pSeamA	6.7	14.4

**[0171]** As shown in Table 1, each expression plasmid carrying the norM gene (shown in SEQ ID NO:1; hereinafter denoted in SEQ ID NO alone), the emrD gene (SEQ ID NO:3), the rarD gene (SEQ ID NO:5), or the eamA gene (SEQ ID NO:7) was introduced to increase the amount of expression of norM protein (SEQ ID NO:2), emerD protein (SEQ ID NO:4), rarD protein (SEQ ID NO:6) or eamA protein (SEQ ID NO:8), respectively; as a result, in all cases, the amount of L-serine accumulated in the medium increased.

#### Example 3. Production of L-glutamine (L-Gln)

**[0172]** The JGLE1 strain (pamphlet for International Patent Application Publication 06/001380, pamphlet for US Patent Application Publication 2008-0038786), which is a publicly known L-glutamine producer, was transformed with each of pSnorM, pSrarD and pSTV29 obtained in Preparation of Amino Acid Producers [1]; the transformants obtained were named as Escherichia coli JGLE1/pSnorM, JGLE1/pSrarD and JGLE1/pSTV29, respectively.

**[0173]** Each transformant obtained above was inoculated to a large test tube containing 8ml of a LB medium supplemented with 20 $\mu$ g/ml chloramphenicol, and cultured at 30°C for 16 hours.

**[0174]** The culture broth was inoculated at 1% to a test tube containing 8ml of the medium C [16g/L dipotassium hydrogen phosphate, 14g/L potassium dihydrogen phosphate, 2g/L ammonium sulfate, 1g/L citric acid (anhydrous), 1g/L casamino acid (manufactured by Difco Company), 10g/L glucose, 10mg/L vitamin B<sub>1</sub>, 2g/L magnesium sulfate heptahydrate, 10mg/L manganese sulfate pentahydrate, 50mg/L iron sulfate heptahydrate, 100mg/L L-proline; adjusted to pH 7.2 with 10mol/L sodium hydroxide; glucose, vitamin B<sub>1</sub>, magnesium sulfate heptahydrate, manganese sulfate pentahydrate, iron sulfate heptahydrate, and L-proline were added separately after being boiled] supplemented with 20 $\mu$ g/ml chloramphenicol, and cultured at 30°C for 24 hours, after which the culture broth was centrifuged, and a culture supernatant was acquired.

**[0175]** The products in the culture supernatant were analyzed using HPLC. The results are shown in Table 2.

Table 2

E. coli strain	OD <sub>660</sub>	L-Gln (mg/L)
JGLE1/pSTV29	2.6	122.2
JGLE1/pSnorM	2.2	141.0
JGLE1/pSrarD	2.1	497.4

**[0176]** As shown in Table 2, an expression plasmid carrying the each of the norM gene (SEQ ID NO:1) or the rarD gene (SEQ ID NO:5) was introduced to increase the amount of expression of norM protein (SEQ ID NO:2) or rarD protein (SEQ ID NO:6), respectively; as a result, in both the cases, the amount of L-glutamine accumulated in the medium increased.

#### Example 4. Production of L-cysteine (L-Cys)

**[0177]** The Escherichia coli ATCC9637sdaABCcysE256 strain obtained in Preparation of Amino Acid Producers [4], which carries an expression plasmid for the cysE gene deregulated to L-cysteine, wherein L-serine degrading enzymes (sdaA, sdaB) and the uptake system (sdaC) were lacked, and the cysE gene on the chromosomal DNA was replaced by a deregulated cysE gene, was transformed with pScysEfbr1 obtained in Preparation of Amino Acid Producers [5] to acquire Escherichia coli ATCC9637sdaABCcysE256/pScysEfbr1, a microbial strain having the capability of producing L-cysteine biosynthesis intermediate (O-acetyl-L-serine) synthetase.

**[0178]** Next, Escherichia coli ATCC9637sdaABCcysE256/pScysEfbr was transformed with pSrarD, pSeamA and pSTV29 obtained in Example 1; the transformants obtained were named as Escherichia coli

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ATCC9637sdaABCcysE256/pScysEfbr1/pSrarD, *Escherichia coli* ATCC9637sdaABCcysE256/pScysEfbr1/pSeamA and *Escherichia coli* ATCC9637sdaABCcysE256/pScysEfbr1/pSTV29, respectively.

[0179] Each transformant obtained above was inoculated to a large test tube containing 5ml of the same medium A as in Example 2 supplemented with 100 $\mu$ g/ml ampicillin and 20 $\mu$ g/ml chloramphenicol, and cultured at 30°C for 16 hours.

[0180] The culture broth was inoculated at 10% to a test tube containing 5ml of the medium D [the same composition as the medium B used in Example 2 except that glycine was not contained, and that 2g/L thiosulfuric acid was contained] supplemented with 100 $\mu$ g/ml ampicillin and 20 $\mu$ g/ml chloramphenicol, and cultured at 30°C for 24 hours, after which the culture broth was centrifuged, and a culture supernatant was acquired.

[0181] The products in the culture supernatant were analyzed using HPLC. The results are shown in Table 3.

Table 3

E. coli strain	OD <sub>660</sub>	L-Cys(mg/L)
ATCC9637sdaABCcysE256/pScysEfbr1/pSTV29	63.0	73.4
ATCC9637sdaABCcysE256/pScysEfbr1/pSrarD	34.6	134.3
ATCC9637sdaABCcysE256/pScysEfbr1/pSeamA	21.0	355.6

[0182] As shown in Table 3, an expression plasmid carrying each of the rarD gene (SEQ ID NO:5) or the eamA gene (SEQ ID NO:7) was introduced to increase the amount expressed of rarD protein (SEQ ID NO:6) or eamA protein (SEQ ID NO:8), respectively; as a result, in both the cases, the amount of L-cysteine accumulated in the medium increased.

### Example 5. Production of L-threonine (L-Thr)

[0183] The ATCC21277 strain [USP 3,580,810], an *Escherichia coli* strain that has been reported to produce L-threonine, was transformed with the pSeamA and pSTV29 obtained in Example 1; the transformants obtained were named as *Escherichia coli* ATCC21277/pSeamA and *Escherichia coli* ATCC21277/pSTV29, respectively.

[0184] Each transformant obtained above was inoculated to a large test tube containing 5ml of the same medium A as in Example 2, supplemented with 20 $\mu$ g/ml chloramphenicol, and cultured at 30°C for 16 hours.

[0185] The culture broth was inoculated at 10% to a test tube containing 5ml of the medium E [the same composition as the medium B used in Example 2 except that glycine and yeast extract were not contained, and that 5g/L casamino acid was contained] supplemented with 20 $\mu$ g/ml chloramphenicol, and cultured at 30°C for 24 hours, after which the culture broth was centrifuged, and a culture supernatant was acquired.

[0186] The products in the culture supernatant were analyzed using HPLC. The results are shown in Table 4.

[Table 4]

E. coli strain	OD <sub>660</sub>	L-Thr(mg/L)
ATCC21277/pSTV29	12.1	63.0
ATCC21277/pSeamA	7.5	110.6

[0187] As shown in Table 4, an expression plasmid carrying the norM gene (SEQ ID NO:1) was introduced to intensify the amount of expression of norM protein (SEQ ID NO:2); as a result, the amount of L-threonine accumulated in the medium increased.

### Example 6. Production of L-phenylalanine (L-Phe)

[0188] The NM522 strain was transformed with the expression plasmid pBpheAfbraroFfbr wherein a deregulated aroF gene and a deregulated pheA gene were inserted in the forward orientation, prepared in [3] (2), to acquire *Escherichia coli* NM522/pBpheAfbraroFfbr, a transformant that produces L-phenylalanine synthetase.

[0189] Next, *Escherichia coli* NM522/pBpheAfbraroFfbr was transformed with pSemrD, pSrarD and pTV29 obtained in Example 1; the transformants obtained were named as *Escherichia coli* NM522/pBpheAfbraroFfbr/pSemrD, NM522/pBpheAfbraroFfbr/pSrarD and NM522/pBpheAfbraroFfbr/pSTV29, respectively.

[0190] Each transformant obtained above was inoculated to a large test tube containing 5ml of the same medium A as in Example 2 supplemented with 100 $\mu$ g/ml ampicillin and 20 $\mu$ g/ml chloramphenicol, and cultured at 30°C for 16 hours.

[0191] The culture broth was inoculated at 10% to a test tube containing 5ml of the medium F [the same composition as the medium B used in Example 2 except that glycine was not contained] supplemented with 100 $\mu$ g/ml ampicillin and 20 $\mu$ g/ml chloramphenicol, and cultured at 30°C for 24 hours, after which the culture broth was centrifuged, and a culture

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supernatant was acquired.

**[0192]** The products in the culture supernatant were analyzed using HPLC. The results are shown in Table 5.

[Table 5]

E. coli strain	OD <sub>660</sub>	L-Phe(mg/L)
NM522/pBpheAfbraroFfbr/pSTV29	18.8	85.9
NM522/pBpheAfbraroFfbr/pSemrD	19.2	132.3
NM522/pBpheAfbraroFfbr/pSrarD	11.6	103.0

**[0193]** As shown in Table 5, an expression plasmid carrying each of the emrD gene (SEQ ID NO:3) or the rarD gene (SEQ ID NO:5) was introduced to increase the amount of expression of emerD protein (SEQ ID NO:4) or rarD protein (SEQ ID NO:6), respectively; as a result, in both the cases, the amount of L-phenylalanine accumulated in the medium increased.

### Industrial Applicability

**[0194]** If a highly productive process for producing a L-amino acids is established to enable their industrial mass-production by the manufacturing process of the present invention, its industrial applicability would be very high. For example, L-serine is of high value for utilization as a raw material for amino acid mixtures in the field of pharmaceuticals and the field of cosmetics; L-glutamine serves as a raw material for anti-alcoholism compositions.

**[0195]** L-cysteine is an amino acid that is very highly valued in the cosmetic industry; L-threonine and L-phenylalanine are useful as an ingredient for amino acid infusions and health foods, and as a raw material for the low-calorie sweetener Aspartame, respectively.

[Sequence Listing Free Text]

### **[0196]**

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Thr Asn Ile Glu Phe His Lys Gly Ala Leu Asp Asp Glu Gln Leu Lys  
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35 **Claims**

1. A process for producing an L-amino acid which comprises culturing a microorganism in a medium wherein the microorganism has higher L-amino acid transport activity when compared to the parent strain wherein the microorganism expresses a protein described in any one of (1) to (3),

40

- (1) A protein comprising the amino acid sequence shown in SEQ ID NO:6;
- (2) A protein consisting of the amino acid sequence resulting from deletion, substitution or addition of 1 to 20 amino acids in the amino acid sequence shown in SEQ ID NO:6, and having L-amino acid transport activity;
- (3) A protein consisting of an amino acid sequence having 80% or more homology to the amino acid sequence shown in SEQ ID NO:6, and having L-amino acid transport activity;

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and producing and accumulating the L-amino acid in the medium, and collecting the L-amino acid from the medium, wherein the L-amino acid is selected from the group consisting of L-serine, L-glutamine, L-cysteine, L-phenylalanine and L-threonine.

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2. The process for producing the L-amino acid according to claim 1, wherein the microorganism is transformed with the DNA described in any one of (1) to (3) below, or wherein the expression of the gene that encodes for the protein is enhanced in the microorganism by modifying the expression regulatory sequence of the DNA described in any one of (1) to (3) below:

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- (1) A DNA that encodes the protein described in any one of (1) to (3) in claim 1;
- (2) A DNA comprising the nucleotide sequence shown in SEQ ID NO:5;
- (3) A DNA that hybridizes under stringent conditions with the DNA consisting of the nucleotide sequence com-

plementary to the nucleotide sequence shown in SEQ ID NO:5, and encodes for the protein having L-amino acid transport activity.

- 5 3. The process for producing the L-amino acid according to claim 1 or 2, wherein the microorganism belongs to the genus Escherichia, the genus Corynebacterium, the genus Bacillus, the genus Serratia, the genus Pseudomonas or the genus Streptomyces.

### 10 Patentansprüche

1. Verfahren zur Herstellung einer L-Aminosäure, umfassend das Züchten eines Mikroorganismus in einem Medium, wobei der Mikroorganismus eine höhere L-Aminosäure-Transportaktivität hat verglichen mit dem Elternstamm, wobei der Mikroorganismus ein Protein exprimiert, das in einem von (1) bis (3) beschrieben ist,

- 15 (1) Protein, umfassend die in SEQ ID NO:6 dargestellte Aminosäuresequenz;  
(2) Protein, das aus einer Aminosäuresequenz besteht, die durch Deletion, Austausch oder Hinzufügen von 1 bis 20 Aminosäuren in der in SEQ ID NO:6 dargestellten Aminosäuresequenz entsteht und L-Aminosäure-Transportaktivität hat;  
20 (3) Protein, das aus der Aminosäuresequenz besteht, die 80% oder mehr Homologie mit der in SEQ ID NO:6 dargestellten Aminosäuresequenz hat und L-Aminosäure-Transportaktivität hat;

und das Herstellen und Akkumulieren der L-Aminosäure in dem Medium und das Gewinnen der L-Aminosäure aus dem Medium, wobei die L-Aminosäure ausgewählt ist aus der Gruppe bestehend aus L-Serin, L-Glutamin, L-Cystein, L-Phenylalanin und L-Threonin.

- 25 2. Verfahren zur Herstellung der L-Aminosäure nach Anspruch 1, wobei der Mikroorganismus mit der unten in einem von (1) bis (3) beschriebenen DNA transformiert ist, oder wobei in dem Mikroorganismus die Expression des Gens, das das Protein codiert, durch Modifikation der expressionsregulatorischen Sequenz der unten in einem von (1) bis (3) beschriebenen DNA verstärkt ist:

- 30 (1) DNA, die das in einem von (1) bis (3) in Anspruch 1 beschriebene Protein codiert;  
(2) DNA, umfassend die in SEQ ID NO:5 dargestellte Nucleotidsequenz;  
(3) DNA, die unter stringenten Bedingungen mit der DNA hybridisiert, die aus der Nucleotidsequenz besteht, die komplementär ist zu der in SEQ ID NO:5 dargestellten Nucleotidsequenz, und die das Protein codiert, das  
35 L-Aminosäure-Transportaktivität hat.

3. Verfahren zur Herstellung der L-Aminosäure nach Anspruch 1 oder 2, wobei der Mikroorganismus der Gattung Escherichia, der Gattung Corynebakterium, der Gattung Bacillus, der Gattung Serratia, der Gattung Pseudomonas oder der Gattung Streptomyces angehört.

### 40 Revendications

- 45 1. Procédé de production d'un acide L-aminé qui comprend la culture d'un micro-organisme dans un milieu dans lequel le micro-organisme a une activité de transport d'acide L-aminé supérieure comparativement à la souche parente, le micro-organisme exprimant une protéine décrite dans l'un quelconque des points (1) à (3),

- (1) une protéine comprenant la séquence d'acides aminés représentée dans SEQ ID No: 6 ;  
50 (2) une protéine constituée par la séquence d'acides aminés résultant de la délétion, de la substitution ou de l'addition de 1 à 20 acides aminés par rapport à la séquence d'acides aminés représentée dans SEQ ID No: 6, et ayant une activité de transport d'acide L-aminé ;  
(3) une protéine constituée par une séquence d'acides aminés présentant une homologie de 80 % ou plus avec la séquence d'acides aminés représentée dans SEQ ID No: 6, et ayant une activité de transport d'acide L-aminé ;

55 et la production et l'accumulation de l'acide L-aminé dans le milieu, puis la collecte de l'acide L-aminé à partir du milieu, l'acide L-aminé étant choisi dans le groupe constitué par la L-sérine, la L-glutamine, la L-cystéine, la L-phénylalanine et la L-thréonine.

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2. Procédé de production de l'acide L-aminé selon la revendication 1, dans lequel le micro-organisme est transformé avec l'ADN décrit dans l'un quelconque des points (1) à (3) ci-dessous, ou dans lequel l'expression du gène qui code pour la protéine est améliorée chez le micro-organisme par modification de la séquence de régulation de l'expression de l'ADN décrit dans l'un quelconque des points (1) à (3) ci-dessous :

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(1) un ADN qui code pour la protéine décrite dans l'un quelconque des points (1) à (3) de la revendication 1 ;

(2) un ADN comprenant la séquence de nucléotides représentée dans SEQ ID No: 5 ;

(3) un ADN qui s'hybride dans des conditions stringentes avec l'ADN constitué par la séquence de nucléotides complémentaire de la séquence de nucléotides représentée dans SEQ ID No: 5, et code pour la protéine ayant

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l'activité de transport d'acide L-aminé.

3. Procédé de production de l'acide L-aminé selon la revendication 1 ou 2, dans lequel le micro-organisme appartient au genre *Escherichia*, au genre *Corynebacterium*, au genre *Bacillus*, au genre *Serratia*, au genre *Pseudomonas* ou au genre *Streptomyces*.

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