

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
26 January 2012 (26.01.2012)

(10) International Publication Number
WO 2012/011595 A1

PCT

(51) International Patent Classification:

C12P 13/10 (2006.01) C12N 9/78 (2006.01)
C12R 1/01 (2006.01) C12N 9/80 (2006.01)
C12N 9/10 (2006.01) C12N 1/20 (2006.01)
C12N 9/02 (2006.01)

(21) International Application Number:

PCT/JP2011/066786

(22) International Filing Date:

15 July 2011 (15.07.2011)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

2010130304 21 July 2010 (21.07.2010) RU

(71) Applicant (for all designated States except US): **AJINOMOTO CO., INC.** [JP/JP]; 15-1, Kyobashi 1-chome, Chuo-ku, Tokyo, 1048315 (JP).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **LEONOVA, Tatyana Viktorovna** [RU/RU]; bldg. 5, #852, 81, ul. Svobody, Moscow, 123481 (RU). **VOROSHILOVA, Elvira Borisovna** [RU/RU]; bldg. 403, #217, 4, Severnoe Chertanovo, Moscow, 117648 (RU). **GUSYATINER, Mikhail Markovich** [RU/RU]; bldg. 403, #217, 4, Severnoe Chertanovo, Moscow, 117648 (RU).

(74) Agents: **KAWAGUCHI, Yoshiyuki** et al.; Acropolis 21 Building 6th floor, 4-10, Higashi Nihonbashi 3-chome, Chuo-ku, Tokyo, 1030004 (JP).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description (Rules 13bis.4(d)(i) and 48.2(a)(viii))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: A METHOD FOR PRODUCING AN L-AMINO ACID USING A BACTERIUM OF THE ENTEROBACTERIACEAE FAMILY HAVING ATTENUATED EXPRESSION OF THE *astCADBE* OPERON

(57) Abstract: The present invention provides a method for producing an L-amino acid using a bacterium of the *Enterobacteriaceae* family, particularly a bacterium belonging to genus *Escherichia* or *Pantoea*, which has been modified to attenuate expression of the *astCADBE* operon.



WO 2012/011595 A1

DESCRIPTION

A METHOD FOR PRODUCING AN L-AMINO ACID USING A BACTERIUM OF THE *ENTEROBACTERIACEAE* FAMILY HAVING ATTENUATED EXPRESSION OF THE *astCADBE* OPERON

Technical Field

The present invention relates to the microbiological industry, and specifically to a method for producing an L-amino acid using a bacterium of the *Enterobacteriaceae* family which has been modified to attenuate expression of the *astCADBE* operon.

Background Art

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

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

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

A search for an ammonia-producing pathway of arginine catabolism in *Escherichia coli* led to discovery of the ammonia-producing arginine succinyltransferase (AST) pathway in *E. coli* and identification of the *astCADBE* operon. This operon codes for the five enzymes of the AST pathway. The gene for succinylornithine transaminase was found based on the identity of the protein sequence and the deduced gene product. This gene was designated as *astC*, and it appears to be the first gene of the operon. Homology searches of the second, third, and fifth ORFs of this operon suggest that they code for AST, succinylglutamic semialdehyde

dehydrogenase, and succinylglutamate desuccinylase, the first, fourth, and fifth enzymes of the AST pathway, respectively. A homology search did not reveal a function for the fourth ORF, but disruption of this ORF specifically eliminated the activity of the second enzyme of the pathway, succinylarginine dihydrolase. The observation that each gene overlaps with the one that follows it is consistent with an operon organization and suggests that translation of a polycistronic mRNA may require just one ribosome. Disruption of the first gene impairs synthesis of all the AST enzymes, a finding which is also consistent with an operon structure. Finally, these observations, together with the fact that cells with the plasmid, which contains this putative operon, have elevated levels of all five enzymes, provide further evidence for an *astCADBE* operon.

Mutants deficient in the AST pathway fail to utilize arginine, which shows that the AST pathway is necessary for catabolism of arginine as the sole nitrogen source during aerobic exponential growth. The AST pathway also appears to contribute to ornithine and aspartate degradation. Limiting nitrogen induced this pathway in both *E. coli* and *Klebsiella aerogenes*, but the mechanisms of activation clearly differed in these two organisms. The regulation of the AST pathway under conditions other than limiting nitrogen suggests additional functions. The AST pathway is induced by growth in broth. The AST pathway also appears to be induced upon entry into stationary-phase growth. The function of the AST pathway under such conditions is not clear. Unlike all other organisms that contain the AST pathway, *E. coli* does not degrade arginine as a carbon source. *E. coli* may fail to utilize arginine as a carbon source because the *ast* genes may lack an appropriately controlled promoter or because of inadequate transport during carbon-limited growth (Schneider BL, et. al., J Bacteriol.;180(16):4278-86(1998)).

But currently, there have been no reports of inactivating the *astCADBE* operon for the purpose of producing L-amino acids.

Disclosure of the Invention

Aspects of the present invention include enhancing the productivity of L-amino acid-producing strains and providing a method for producing an L-amino acid using these strains.

The above aspects were achieved by finding that attenuating expression of the gene(s) of the *astCADBE* operon can enhance production of L-amino acids, such as L-threonine, L-lysine, L-cysteine, L-methionine, L-leucine, L-isoleucine, L-valine, L-histidine, glycine, L-serine, L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-

glutamic acid, L-proline, L-arginine, L-citrulline, L-ornithine, L-phenylalanine, L-tyrosine, and L-tryptophan.

The present invention provides a bacterium of the *Enterobacteriaceae* family which has an increased ability to produce an L-amino acid, such as L-threonine, L-lysine, L-cysteine, L-methionine, L-leucine, L-isoleucine, L-valine, L-histidine, glycine, L-serine, L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-proline, L-arginine, L-citrulline, L-ornithine, L-phenylalanine, L-tyrosine, and L-tryptophan.

It is an aspect of the present invention to provide an L-amino acid-producing bacterium of the *Enterobacteriaceae* family, wherein said bacterium has been modified to attenuate expression of one or more genes of the *astCADBE* operon.

It is a further aspect of the present invention to provide the bacterium as described above, wherein expression of the genes is attenuated by inactivation of the *astCADBE* operon.

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

It is a further object of the present invention to provide the bacterium as described above, wherein said bacterium is *Escherichia coli*.

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

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

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

It is a further aspect of the present invention to provide the bacterium as described above, wherein said non-aromatic L-amino acid is selected from the group consisting of L-threonine, L-lysine, L-cysteine, L-methionine, L-leucine, L-isoleucine, L-valine, L-histidine, glycine, L-serine, L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-proline, L-arginine, L-citrulline and L-ornithine.

It is a further aspect of the present invention to provide the bacterium as described above, wherein said L-amino acid is L-arginine.

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

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

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

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

It is a further aspect of the present invention to provide the method as described above, wherein said non-aromatic L-amino acid is selected from the group consisting of L-threonine, L-lysine, L-cysteine, L-methionine, L-leucine, L-isoleucine, L-valine, L-histidine, glycine, L-serine, L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-proline, L-arginine, L-citrulline and L-ornithine.

It is a further aspect of the present invention to provide the method as described above, wherein said L-amino acid is L-arginine.

The present invention is described in detail below.

Description of Embodiments

1. Bacterium

The bacterium in accordance with the presently disclosed subject matter is an L-amino acid-producing bacterium of the *Enterobacteriaceae* family, wherein the bacterium has been modified to attenuate expression of one or more genes of the *astCADBE* operon.

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

The term “L-amino acid-producing bacterium” also can mean a bacterium which is able to produce and cause accumulation of an L-amino acid in a culture medium in an amount larger than a wild-type or parental strain of a bacterium of the *Enterobacteriaceae* family, for example *E. coli*, such as *E. coli* K-12, and can mean that the microorganism is able to cause accumulation in a medium of an amount not less than 0.5 g/L, in another example not less than 1.0 g/L, of the target L-amino acid. The bacterium can produce one kind of L-amino acid or mixture of two or more kinds of L-amino acids.

The term “L-amino acid” includes, for example, L-alanine, L-arginine, L-citrulline, L-ornithine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine.

The term “aromatic L-amino acid” includes, for example, L-phenylalanine, L-tyrosine, and L-tryptophan. The term “non-aromatic L-amino acid” includes, for example, L-threonine, L-lysine, L-cysteine, L-methionine, L-leucine, L-isoleucine, L-valine, L-histidine, glycine, L-serine, L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-proline, L-arginine, L-citrulline and L-ornithine. L-threonine, L-lysine, L-cysteine, L-leucine, L-histidine, L-glutamic acid, L-phenylalanine, L-tryptophan, L-proline, and L-arginine are particular examples.

The *Enterobacteriaceae* family includes bacteria belonging to the genera *Escherichia*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Pantoea*, *Photobacterium*, *Providencia*, *Salmonella*, *Serratia*, *Shigella*, *Morganella*, *Yersinia*, etc. Specifically, those classified into the *Enterobacteriaceae* according to the taxonomy used by the NCBI (National Center for Biotechnology Information) database (<http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=543>) can be used. A bacterium belonging to the genus *Escherichia* or *Pantoea* are particular examples.

The phrase “a bacterium belonging to the genus *Escherichia*” can mean that the bacterium is classified into the genus *Escherichia* according to the classification known to a person skilled in the art of microbiology. Examples of a bacterium belonging to the genus *Escherichia* include, but are not limited to, *Escherichia coli* (*E. coli*).

The bacterium belonging to the genus *Escherichia* is not particularly limited; however, e.g., bacteria described by Neidhardt, F.C. et al. (*Escherichia coli* and *Salmonella typhimurium*, American Society for Microbiology, Washington D.C., 1208, Table 1) can be used.

The phrase “a bacterium belonging to the genus *Pantoea*” can mean that the bacterium is classified into the genus *Pantoea* according to the classification known to a person skilled in the art of microbiology. Some species of *Enterobacter agglomerans* have been recently re-classified into *Pantoea agglomerans*, *Pantoea ananatis*, *Pantoea stewartii* or the like, based on the nucleotide sequence analysis of 16S rRNA, etc. (Int. J. Syst. Bacteriol., 43, 162-173 (1993)).

The phrase “bacterium has been modified to attenuate expression of one or more genes of the *astCADBE* operon” can mean that the bacterium has been modified in such a way that the modified bacterium contains a reduced amount of one or more of the proteins, AstC, AstA, AstD, AstB and AstE, as compared with an unmodified bacterium, or it can also mean that the modified bacterium is unable to synthesize one or more of the proteins, AstC, AstA, AstD, AstB and AstE. The phrase “bacterium has been modified to attenuate expression of one or more genes of the *astCADBE* operon” also can mean that the bacterium has been modified in such a way that the modified

gene or genes encode(s) a mutant AstC, AstA, AstD, AstB or/and AstE proteins with a decreased activity. The term “gene” of the operon can mean a structural gene or cistron of the operon.

In a particular embodiment, expression of all genes of the *astCADBE* operon can be attenuated. Expression of the gene(s) of the *astCADBE* operon can be attenuated by inactivation of the *astCADBE* operon.

The phrase “inactivation of the *astCADBE* operon” can mean that the gene(s) of the modified operon encode(s) completely inactive protein(s). It is also possible that the modified DNA region of the operon is unable to naturally express the gene due to the deletion of a part of or the entire gene, or the deletion of the entire operon, the shifting of the reading frame of the gene, the introduction of missense/nonsense mutation(s), or the modification of an expression control sequence of the operon, such as promoter, enhancer, attenuator, ribosome-binding site, terminator, and etc..

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

The *astC* gene encodes the AstC protein, acetylornithine transaminase/succinylornithine transaminase (synonym – B1748). The *astC* gene of *E. coli* (nucleotides complemented to nucleotides in positions 1,828,786 to 1,830,006 in the GenBank accession number NC_000913.2; gi:49175990) is located between the *astA* gene and the *xthA* gene, oriented in opposite direction, on the chromosome of *E. coli* strain K-12. The nucleotide sequence of the *astC* gene and the amino acid sequence of AstC encoded by the *astC* gene are shown in SEQ ID NO: 1 and SEQ ID NO: 2, respectively.

The *astA* gene encodes the AstA protein, arginine succinyltransferase (synonym – B1747). The *astA* gene of *E. coli* (nucleotides complemented to nucleotides in positions 1,827,755 to 1,828,789 in the GenBank accession number NC_000913.2; gi:49175990) is located between the *astD* gene and the *astC* gene on the chromosome of *E. coli* strain K-12. The nucleotide sequence of the *astA* gene and the amino acid sequence of AstA encoded by the *astA* gene are shown in SEQ ID NO: 3 and SEQ ID NO: 4, respectively.

The *astD* gene encodes the AstD protein, aldehyde dehydrogenase (synonym – B1746). The *astD* gene of *E. coli* (nucleotides complemented to nucleotides in positions 1,826,280 to 1,827,758 in the GenBank accession number NC_000913.2; gi:49175990) is located between the *astB* gene and the *astA* gene on the chromosome of *E. coli* strain K-12. The nucleotide sequence of the *astD* gene and the amino acid sequence of AstD encoded by the *astD* gene are shown in SEQ ID NO: 5 and SEQ ID NO: 6, respectively.

The *astB* gene encodes the AstB protein, succinylarginine dihydrolase (synonym – B1745). The *astB* gene of *E. coli* (nucleotides complemented to nucleotides in positions 1,824,940 to 1,826,283 in the GenBank accession number NC_000913.2; gi:49175990) is located between the *astE* gene and the *astD* gene on the chromosome of *E. coli* strain K-12. The nucleotide sequence of the *astB* gene and the amino acid sequence of AstB encoded by the *astB* gene are shown in SEQ ID NO: 7 and SEQ ID NO: 8, respectively.

The *astE* gene encodes the AstE protein, succinylglutamate desuccinylase (synonym – B1744). The *astE* gene of *E. coli* (nucleotides complemented to nucleotides in positions 1,823,979 to 1,824,947 in the GenBank accession number NC_000913.2; gi:49175990) is located between the *spy* gene and the *astB* gene on the chromosome of *E. coli* strain K-12. The nucleotide sequence of the *astE* gene and the amino acid sequence of AstE encoded by the *astE* gene are shown in SEQ ID NO: 9 and SEQ ID NO: 10, respectively.

Since there may be some differences in DNA sequences between the genera or strains of the *Enterobacteriaceae* family, the genes of the *astCADBE* operon to be inactivated on the chromosome is not limited to the genes shown in SEQ ID No: 1, SEQ ID No: 3, SEQ ID No: 5, SEQ ID No: 7 and SEQ ID No: 9, but can include genes homologous to SEQ ID No: 1, SEQ ID No: 3, SEQ ID No: 5, SEQ ID No: 7 and SEQ ID No: 9 which encode a variant of the AstC, AstA, AstD, AstB and AstE proteins. The phrase “variant protein” can mean a protein which has changes in the sequence, whether they are deletions, insertions, additions, or substitutions of one or several amino acids, but still maintains the activity of the product as the AstC, AstA, AstD, AstB and AstE proteins. The number of changes in the variant protein depends on the position in the three dimensional structure of the protein or the type of amino acid residues. It can be 1 to 30, in another example 1 to 15, and in another example 1 to 5 in SEQ ID No: 2, SEQ ID No: 4, SEQ ID No: 6, SEQ ID No: 8 and SEQ ID No: 10. These changes in the variants can occur in regions of the protein which are not critical for the function of the protein. This is because some amino acids have high homology to one another so the three dimensional structure or activity is not affected by such a change.

Therefore, the protein variant encoded by the genes of the *astCADBE* operon can be one which has a homology of not less than 80%, in another example not less than 90%, in another example not less than 95%, in another example not less than 98%, and in another example not less than 99%, with respect to the entire amino acid sequence shown in SEQ ID No: 2, SEQ ID No: 4, SEQ ID No:6, SEQ ID No:8 and SEQ ID No: 10, as long as the activity of the AstC, AstA, AstD, AstB and AstE proteins prior to inactivation of the gene of the *astCADBE* operon is maintained. The term "homology" can mean "identity".

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

The substitution, deletion, insertion or addition of one or several amino acid residues can be conservative mutation(s) so that the activity is maintained. The representative conservative mutation is a conservative substitution. Examples of conservative substitutions include substitution of Ser or Thr for Ala, substitution of Gln, His or Lys for Arg, substitution of Glu, Gln, Lys, His or Asp for Asn, substitution of Asn, Glu or Gln for Asp, substitution of Ser or Ala for Cys, substitution of Asn, Glu, Lys, His, Asp or Arg for Gln, substitution of Asn, Gln, Lys or Asp for Glu, substitution of Pro for Gly, substitution of Asn, Lys, Gln, Arg or Tyr for His, substitution of Leu, Met, Val or Phe for Ile, substitution of Ile, Met, Val or Phe for Leu, substitution of Asn, Glu, Gln, His or Arg for Lys, substitution of Ile, Leu, Val or Phe for Met, substitution of Trp, Tyr, Met, Ile or Leu for Phe, substitution of Thr or Ala for Ser, substitution of Ser or Ala for Thr, substitution of Phe or Tyr for Trp, substitution of His, Phe or Trp for Tyr, and substitution of Met, Ile or Leu for Val.

Moreover, the genes of the *astCADBE* operon can be a variant which hybridizes under stringent conditions with the nucleotide sequence shown in SEQ ID No: 1, SEQ ID No: 3, SEQ ID No: 5, SEQ ID No:7 and SEQ ID No: 9, or a probe which can be prepared from the nucleotide sequence under stringent conditions, provided that it encodes a functional AstC, AstA, AstD, AstB and AstE proteins prior to inactivation. "Stringent conditions" include those under which a specific hybrid, for example, a hybrid having homology of not less than 60%, in another example not less than 70%, in another example not less than 80%, in another example not less than 90%, in another example not less than 95%, in another example not less than 98%, and in another example not less than 99%, is formed and a non-specific hybrid, for example, a hybrid having homology lower than the above, is not formed. For example, stringent conditions can be exemplified by washing one time or more, or in another example, two or three times at a salt concentration of $1 \times \text{SSC}$, 0.1% SDS, or in another example,

0.1 × SSC, 0.1% SDS at 60 °C. Duration of washing depends on the type of membrane used for blotting and, as a rule, should be what is recommended by the manufacturer. For example, the recommended duration of washing for the Hybond™ N+ nylon membrane (Amersham) under stringent conditions is 15 minutes. The washing step can be performed 2 to 3 times. The length of the probe can be suitably selected depending on the hybridization conditions, and is usually 100 bp to 1 kbp.

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

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

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

B.L., Proc. Natl. Acad. Sci. USA, 2000, 97:12: 6640-45), also called "Red-driven integration".

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

L-amino acid-producing bacteria

As a bacterium in accordance with the presently disclosed subject matter which is modified to attenuate expression of the gene(s) of the *astCADBE* operon, bacteria which are able to produce either an aromatic or a non-aromatic L-amino acids can be used.

The bacterium in accordance with the presently disclosed subject matter can be obtained by attenuating expression of the gene(s) of the *astCADBE* operon in a bacterium which inherently has the ability to produce L-amino acid. Alternatively, the bacterium can be obtained by imparting the ability to produce L-amino acid to a bacterium already having the attenuated expression of the gene(s) of the *astCADBE* operon.

L-threonine-producing bacteria

Examples of L-threonine-producing bacteria or parent strains which can be used to derive L-threonine-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* TDH-6/pVIC40 (VKPM B-3996) (U.S. Patent No. 5, 175, 107, U.S. Patent No. 5,705,371), *E. coli* 472T23/pYN7 (ATCC 98081) (U.S. Patent No.5,631,157), *E. coli* NRRL-21593 (U.S. Patent No. 5,939,307), *E. coli* FERM BP-3756 (U.S. Patent No. 5,474,918), *E. coli* FERM BP-3519 and FERM BP-3520 (U.S. Patent No. 5,376,538), *E. coli* MG442 (Gusyatiner et al., Genetika (in Russian), 14, 947-956 (1978)), *E. coli* VL643 and VL2055 (EP 1149911 A), and the like.

The strain TDH-6 is deficient in the *thrC* gene, as well as being sucrose-assimilative, and the *ilvA* gene has a leaky mutation. This strain also has a mutation in the *rhtA* gene, which imparts resistance to high concentrations of threonine or homoserine. The strain B-3996 contains the plasmid pVIC40 which was obtained by inserting a *thrA*BC* operon which includes a mutant *thrA* gene into a RSF1010-derived vector. This mutant *thrA* gene encodes aspartokinase homoserine dehydrogenase I which has substantially desensitized feedback inhibition by threonine.

The strain B-3996 was deposited on November 19, 1987 in the All-Union Scientific Center of Antibiotics (Russia, 117105 Moscow, Nagatinskaya Street 3-A) under the accession number RIA 1867. The strain was also deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (GNII genetika, 1 Dorozhny proezd, 1, Moscow 117545, Russian Federation) on April 7, 1987 under the accession number VKPM B-3996.

E. coli VKPM B-5318 (EP 0593792B) may also be used as a parent strain for deriving L-threonine-producing bacteria. The strain B-5318 is prototrophic with regard to isoleucine, and a temperature-sensitive lambda-phage C1 repressor and PR promoter replaces the regulatory region of the threonine operon in plasmid pVIC40. The strain VKPM B-5318 was deposited in the Russian National Collection of Industrial Microorganisms (VKPM) on May 3, 1990 under accession number of VKPM B-5318.

The bacterium can be additionally modified to enhance expression of one or more of the following genes:

- the mutant *thrA* gene which codes for aspartokinase homoserine dehydrogenase I resistant to feed back inhibition by threonine;
- the *thrB* gene which codes for homoserine kinase;
- the *thrC* gene which codes for threonine synthase;
- the *rhtA* gene which codes for a putative transmembrane protein;
- the *asd* gene which codes for aspartate- β -semialdehyde dehydrogenase; and
- the *aspC* gene which codes for aspartate aminotransferase (aspartate transaminase);

The *thrA* gene which encodes aspartokinase homoserine dehydrogenase I of *Escherichia coli* has been elucidated (nucleotide positions 337 to 2799, GenBank accession NC_000913.2, gi: 49175990). The *thrA* gene is located between the *thrL* and *thrB* genes on the chromosome of *E. coli* K-12. The *thrB* gene which encodes homoserine kinase of *Escherichia coli* has been elucidated (nucleotide positions 2801 to 3733, GenBank accession NC_000913.2, gi: 49175990). The *thrB* gene is located between the *thrA* and *thrC* genes on the chromosome of *E. coli* K-12. The *thrC* gene which encodes threonine synthase of *Escherichia coli* has been elucidated (nucleotide positions 3734 to 5020, GenBank accession NC_000913.2, gi: 49175990). The *thrC* gene is located between the *thrB* gene and the *yaaX* open reading frame on the chromosome of *E. coli* K-12. All three genes functions as a single threonine operon. To enhance expression of the threonine operon, the attenuator region which affects the transcription is desirably removed from the operon (WO2005/049808, WO2003/097839).

A mutant *thrA* gene which codes for aspartokinase homoserine dehydrogenase I resistant to feed back inhibition by threonine, as well as, the *thrB* and *thrC* genes can be obtained as one operon from well-known plasmid pVIC40 which is presented in the threonine producing *E. coli* strain VKPM B-3996. Plasmid pVIC40 is described in detail in U.S. Patent No. 5,705,371.

The *rhtA* gene exists at 18 min on the *E. coli* chromosome close to the *glnHPQ* operon, which encodes components of the glutamine transport system. The *rhtA* gene is identical to ORF1 (*ybiF* gene, nucleotide positions 764 to 1651, GenBank accession number AAA218541, gi:440181) and is located between the *pexB* and *ompX* genes. The unit expressing a protein encoded by the ORF1 has been designated the *rhtA* gene (*rht*: resistance to homoserine and threonine). Also, it was revealed that the *rhtA23* mutation is an A-for-G substitution at position -1 with respect to the ATG start codon (ABSTRACTS of the 17th International Congress of Biochemistry and Molecular Biology in conjugation with Annual Meeting of the American Society for Biochemistry and Molecular Biology, San Francisco, California August 24-29, 1997, abstract No. 457, EP 1013765 A).

The *asd* gene of *E. coli* has already been elucidated (nucleotide positions 3572511 to 3571408, GenBank accession NC_000913.1, gi:16131307), and can be obtained by PCR (polymerase chain reaction; refer to White, T.J. et al., Trends Genet., 5, 185 (1989)) utilizing primers prepared based on the nucleotide sequence of the gene. The *asd* genes of other microorganisms can be obtained in a similar manner.

Also, the *aspC* gene of *E. coli* has already been elucidated (nucleotide positions 983742 to 984932, GenBank accession NC_000913.1, gi:16128895), and can be obtained by PCR. The *aspC* genes of other microorganisms can be obtained in a similar manner.

L-lysine-producing bacteria

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

4,346,170) and *Escherichia coli* VL611. In these microorganisms, feedback inhibition of aspartokinase by L-lysine is desensitized.

The strain WC196 may be used as an L-lysine producing bacterium of *Escherichia coli*. This bacterial strain was bred by conferring AEC resistance to the strain W3110, which was derived from *Escherichia coli* K-12. The resulting strain was designated *Escherichia coli* AJ13069 strain and was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (currently National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository, Tsukuba Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) on December 6, 1994 and received an accession number of FERM P-14690. Then, it was converted to an international deposit under the provisions of the Budapest Treaty on September 29, 1995, and received an accession number of FERM BP-5252 (U.S. Patent No. 5,827,698).

Examples of L-lysine-producing bacteria or parent strains which can be used to derive L-lysine-producing bacteria also include strains in which expression of one or more genes encoding an L-lysine biosynthetic enzyme are enhanced. Examples of such genes include, but are not limited to, genes encoding dihydrodipicolinate synthase (*dapA*), aspartokinase (*lysC*), dihydrodipicolinate reductase (*dapB*), diaminopimelate decarboxylase (*lysA*), diaminopimelate dehydrogenase (*ddh*) (U.S. Patent No. 6,040,160), phosphoenolpyruvate carboxylase (*ppc*), aspartate semialdehyde dehydrogenase (*asd*), and aspartase (*aspA*) (EP 1253195 A). In addition, the parent strains may have an increased level of expression of the gene involved in energy efficiency (*cyo*) (EP 1170376 A), the gene encoding nicotinamide nucleotide transhydrogenase (*pntAB*) (U.S. Patent No. 5,830,716), the *ybjE* gene (WO2005/073390), or combinations thereof.

Examples of parent strains which can be used to derive L-lysine-producing bacteria also include strains having decreased or eliminated activity of an enzyme that catalyzes a reaction for generating a compound other than L-lysine by branching off from the biosynthetic pathway of L-lysine. Examples of the enzymes that catalyze a reaction for generating a compound other than L-lysine by branching off from the biosynthetic pathway of L-lysine include homoserine dehydrogenase, lysine decarboxylase (U.S. Patent No. 5,827,698), and the malic enzyme (WO2005/010175).

L-cysteine-producing bacteria

Examples of L-cysteine-producing bacteria or parent strains which can be used to derive L-cysteine-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* JM15 which is transformed with

different *cysE* alleles coding for feedback-resistant serine acetyltransferases (U.S. Patent No. 6,218,168, Russian patent application 2003121601); *E. coli* W3110 having over-expressed genes which encode proteins suitable for secreting substances toxic for cells (U.S. Patent No. 5,972,663); *E. coli* strains having lowered cysteine desulfohydase activity (JP11155571A2); *E. coli* W3110 with increased activity of a positive transcriptional regulator for cysteine regulon encoded by the *cysB* gene (WO0127307A1), and the like.

L-leucine-producing bacteria

Examples of L-leucine-producing bacteria or parent strains which can be used to derive L-leucine-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* strains resistant to leucine (for example, the strain 57 (VKPM B-7386, U.S. Patent No. 6,124,121)) or leucine analogs including β -2-thienylalanine, 3-hydroxyleucine, 4-azaleucine, 5,5,5-trifluoroleucine (JP 62-34397 B and JP 8-70879 A); *E. coli* strains obtained by the gene engineering method described in WO96/06926; *E. coli* H-9068 (JP 8-70879 A), and the like.

The bacterium can be improved by enhancing the expression of one or more genes involved in L-leucine biosynthesis. Examples include genes of the *leuABCD* operon, which are preferably represented by a mutant *leuA* gene coding for isopropylmalate synthase freed from feedback inhibition by L-leucine (US Patent 6,403,342). In addition, the bacterium can be improved by enhancing the expression of one or more genes coding for proteins which excrete L-amino acid from the bacterial cell. Examples of such genes include the b2682 and b2683 genes (*ygaZH* genes) (EP 1239041 A2).

L-histidine-producing bacteria

Examples of L-histidine-producing bacteria or parent strains which can be used to derive L-histidine-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* strain 24 (VKPM B-5945, RU2003677); *E. coli* strain 80 (VKPM B-7270, RU2119536); *E. coli* NRRL B-12116 – B12121 (U.S. Patent No. 4,388,405); *E. coli* H-9342 (FERM BP-6675) and H-9343 (FERM BP-6676) (U.S. Patent No. 6,344,347); *E. coli* H-9341 (FERM BP-6674) (EP1085087); *E. coli* AI80/pFM201 (U.S. Patent No. 6,258,554) and the like.

Examples of parent strains which can be used to derive L-histidine-producing bacteria also include strains in which expression of one or more genes encoding an L-histidine biosynthetic enzyme are enhanced. Examples of such genes include genes encoding ATP phosphoribosyltransferase (*hisG*), phosphoribosyl AMP cyclohydrolase

(*hisI*), phosphoribosyl-ATP pyrophosphohydrolase (*hisIE*), phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase (*hisA*), amidotransferase (*hisH*), histidinol phosphate aminotransferase (*hisC*), histidinol phosphatase (*hisB*), histidinol dehydrogenase (*hisD*), and so forth.

It is known that the L-histidine biosynthetic enzymes encoded by *hisG* and *hisBHAFI* are inhibited by L-histidine, and therefore an L-histidine-producing ability can also be efficiently enhanced by introducing a mutation conferring resistance to the feedback inhibition into ATP phosphoribosyltransferase (Russian Patent Nos. 2003677 and 2119536).

Specific examples of strains having an L-histidine-producing ability include *E. coli* FERM-P 5038 and 5048 which have been introduced with a vector carrying a DNA encoding an L-histidine-biosynthetic enzyme (JP 56-005099 A), *E. coli* strains introduced with *rht*, a gene for an amino acid-export (EP1016710A), *E. coli* 80 strain imparted with sulfaguanidine, DL-1,2,4-triazole-3-alanine, and streptomycin-resistance (VKPM B-7270, Russian Patent No. 2119536), and so forth.

L-glutamic acid-producing bacteria

Examples of L-glutamic acid-producing bacteria or parent strains which can be used to derive L-glutamic acid-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* VL334thrC⁺ (EP 1172433). *E. coli* VL334 (VKPM B-1641) is an L-isoleucine and L-threonine auxotrophic strain having mutations in *thrC* and *ilvA* genes (U.S. Patent No. 4,278,765). A wild-type allele of the *thrC* gene was transferred by the method of general transduction using a bacteriophage P1 grown on the wild-type *E. coli* strain K12 (VKPM B-7) cells. As a result, an L-isoleucine auxotrophic strain VL334thrC⁺ (VKPM B-8961), which is able to produce L-glutamic acid, was obtained.

Examples of parent strains which can be used to derive the L-glutamic acid-producing bacteria include, but are not limited to, strains in which expression of one or more genes encoding an L-glutamic acid biosynthetic enzyme are enhanced. Examples of such genes include genes encoding glutamate dehydrogenase (*gdhA*), glutamine synthetase (*glnA*), glutamate synthetase (*gltAB*), isocitrate dehydrogenase (*icdA*), aconitate hydratase (*acnA*, *acnB*), citrate synthase (*gltA*), phosphoenolpyruvate carboxylase (*ppc*), pyruvate carboxylase (*pyc*), pyruvate dehydrogenase (*aceEF*, *lpdA*), pyruvate kinase (*pykA*, *pykF*), phosphoenolpyruvate synthase (*ppsA*), enolase (*eno*), phosphoglyceromutase (*pgmA*, *pgmI*), phosphoglycerate kinase (*pgk*), glyceraldehyde-3-phosphate dehydrogenase (*gapA*), triose phosphate isomerase (*tpiA*), fructose

bisphosphate aldolase (*fbp*), phosphofructokinase (*pfkA*, *pfkB*), and glucose phosphate isomerase (*pgi*).

Examples of strains modified so that expression of the citrate synthetase gene, the phosphoenolpyruvate carboxylase gene, and/or the glutamate dehydrogenase gene is/are enhanced include those disclosed in EP1078989A, EP955368A, and EP952221A.

Examples of parent strains which can be used to derive the L-glutamic acid-producing bacteria also include strains having decreased or eliminated activity of an enzyme that catalyzes synthesis of a compound other than L-glutamic acid by branching off from an L-glutamic acid biosynthesis pathway. Examples of such enzymes include isocitrate lyase (*aceA*), α -ketoglutarate dehydrogenase (*sucA*), phosphotransacetylase (*pta*), acetate kinase (*ack*), acetohydroxy acid synthase (*ilvG*), acetolactate synthase (*ilvI*), formate acetyltransferase (*pfl*), lactate dehydrogenase (*ldh*), and glutamate decarboxylase (*gadAB*). Bacteria belonging to the genus *Escherichia* deficient in the α -ketoglutarate dehydrogenase activity or having a reduced α -ketoglutarate dehydrogenase activity and methods for obtaining them are described in U.S. Patent Nos. 5,378,616 and 5,573,945. Specifically, these strains include the following:

E. coli W3110*sucA::Km^R*

E. coli AJ12624 (FERM BP-3853)

E. coli AJ12628 (FERM BP-3854)

E. coli AJ12949 (FERM BP-4881)

E. coli W3110*sucA::Km^R* is a strain obtained by disrupting the α -ketoglutarate dehydrogenase gene (hereinafter referred to as "*sucA* gene") of *E. coli* W3110. This strain is completely deficient in the α -ketoglutarate dehydrogenase.

Other examples of L-glutamic acid-producing bacterium include those which belong to the genus *Escherichia* and have resistance to an aspartic acid antimetabolite. These strains can also be deficient in the α -ketoglutarate dehydrogenase activity and include, for example, *E. coli* AJ13199 (FERM BP-5807) (U.S. Patent No. 5,908,768), FFRM P-12379, which additionally has a low L-glutamic acid decomposing ability (U.S. Patent No. 5,393,671); AJ13138 (FERM BP-5565) (U.S. Patent No. 6,110,714), and the like.

Examples of L-glutamic acid-producing bacteria include mutant strains belonging to the genus *Pantoea* which are deficient in the α -ketoglutarate dehydrogenase activity or have a decreased α -ketoglutarate dehydrogenase activity, and can be obtained as described above. Such strains include *Pantoea ananatis* AJ13356. (U.S. Patent No. 6,331,419). *Pantoea ananatis* AJ13356 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial

Science and Technology, Ministry of International Trade and Industry (currently, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository, Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) on February 19, 1998 under an accession number of FERM P-16645. It was then converted to an international deposit under the provisions of Budapest Treaty on January 11, 1999 and received an accession number of FERM BP-6615. *Pantoea ananatis* AJ13356 is deficient in the α -ketoglutarate dehydrogenase activity as a result of disruption of the α KGDH-E1 subunit gene (*sucA*). The above strain was identified as *Enterobacter agglomerans* when it was isolated and deposited as the *Enterobacter agglomerans* AJ13356. However, it was recently re-classified as *Pantoea ananatis* on the basis of nucleotide sequencing of 16S rRNA and so forth. Although AJ13356 was deposited at the aforementioned depository as *Enterobacter agglomerans*, for the purposes of this specification, they are described as *Pantoea ananatis*.

L-phenylalanine-producing bacteria

Examples of L-phenylalanine-producing bacteria or parent strains which can be used to derive L-phenylalanine-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* AJ12739 (*tyrA*::Tn10, *tyrR*) (VKPM B-8197); *E. coli* HW1089 (ATCC 55371) harboring the mutant *pheA34* gene (U.S. Patent No. 5,354,672); *E. coli* MWEC101-b (KR8903681); *E. coli* NRRL B-12141, NRRL B-12145, NRRL B-12146 and NRRL B-12147 (U.S. Patent No. 4,407,952). Also, as a parent strain, *E. coli* K-12 [W3110 (*tyrA*)/pPHAB (FERM BP-3566), *E. coli* K-12 [W3110 (*tyrA*)/pPHAD] (FERM BP-12659), *E. coli* K-12 [W3110 (*tyrA*)/pPHATerm] (FERM BP-12662) and *E. coli* K-12 [W3110 (*tyrA*)/pBR-aroG4, pACMAB] named as AJ 12604 (FERM BP-3579) may be used (EP 488424 B1). Furthermore, L-phenylalanine producing bacteria belonging to the genus *Escherichia* with an enhanced activity of the protein encoded by the *yedA* gene or the *yddG* gene may also be used (U.S. patent applications 2003/0148473 A1 and 2003/0157667 A1).

L-tryptophan-producing bacteria

Examples of L-tryptophan-producing bacteria or parent strains which can be used to derive the L-tryptophan-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* JP4735/pMU3028 (DSM10122) and JP6015/pMU91 (DSM10123) deficient in the tryptophanyl-tRNA synthetase encoded by mutant *trpS* gene (U.S. Patent No. 5,756,345); *E. coli* SV164 (pGH5) having a *serA* allele encoding phosphoglycerate dehydrogenase free from

feedback inhibition by serine and a *trpE* allele encoding anthranilate synthase free from feedback inhibition by tryptophan (U.S. Patent No. 6,180,373); *E. coli* AGX17 (pGX44) (NRRL B-12263) and AGX6(pGX50)aroP (NRRL B-12264) deficient in the enzyme tryptophanase (U.S. Patent No. 4,371,614); *E. coli* AGX17/pGX50,pACKG4-pps in which a phosphoenolpyruvate-producing ability is enhanced (WO9708333, U.S. Patent No. 6,319,696), and the like may be used. L-tryptophan-producing bacteria belonging to the genus *Escherichia* with an enhanced activity of the identified protein encoded by and the *yedA* gene or the *yddG* gene may also be used (U.S. patent applications 2003/0148473 A1 and 2003/0157667 A1).

Examples of parent strains which can be used to derive the L-tryptophan-producing bacteria also include strains in which one or more activities of the enzymes selected from anthranilate synthase, phosphoglycerate dehydrogenase, and tryptophan synthase are enhanced. The anthranilate synthase and phosphoglycerate dehydrogenase are both subject to feedback inhibition by L-tryptophan and L-serine, so that a mutation desensitizing the feedback inhibition may be introduced into these enzymes. Specific examples of strains having such a mutation include a *E. coli* SV164 which harbors desensitized anthranilate synthase and a transformant strain obtained by introducing into the *E. coli* SV164 the plasmid pGH5 (WO 94/08031), which contains a mutant *serA* gene encoding feedback-desensitized phosphoglycerate dehydrogenase.

Examples of parent strains which can be used to derive the L-tryptophan-producing bacteria also include strains into which the tryptophan operon which contains a gene encoding desensitized anthranilate synthase has been introduced (JP 57-71397 A, JP 62-244382 A, U.S. Patent No. 4,371,614). Moreover, L-tryptophan-producing ability may be imparted by enhancing expression of a gene which encodes tryptophan synthase, among tryptophan operons (*trpBA*). The tryptophan synthase consists of α and β subunits which are encoded by the *trpA* and *trpB* genes, respectively. In addition, L-tryptophan-producing ability may be improved by enhancing expression of the isocitrate lyase-malate synthase operon (WO2005/103275).

L-proline-producing bacteria

Examples of L-proline-producing bacteria or parent strains which can be used to derive L-proline-producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* 702ilvA (VKPM B-8012) which is deficient in the *ilvA* gene and is able to produce L-proline (EP 1172433). The bacterium can be improved by enhancing the expression of one or more genes involved in L-proline biosynthesis. Examples of such genes for L-proline producing bacteria which are

preferred include the *proB* gene coding for glutamate kinase of which feedback inhibition by L-proline is desensitized (DE Patent 3127361). In addition, the bacterium can be improved by enhancing the expression of one or more genes coding for proteins excreting L-amino acid from bacterial cell. Such genes are exemplified by b2682 and b2683 genes (*ygaZH* genes) (EP1239041 A2).

Examples of bacteria belonging to the genus *Escherichia*, which have an activity to produce L-proline include the following *E. coli* strains: NRRL B-12403 and NRRL B-12404 (GB Patent 2075056), VKPM B-8012 (Russian patent application 2000124295), plasmid mutants described in DE Patent 3127361, plasmid mutants described by Bloom F.R. et al (The 15th Miami winter symposium, 1983, p.34), and the like.

L-arginine-producing bacteria

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

Examples of parent strains which can be used to derive L-arginine producing bacteria also include strains in which expression of one or more genes encoding an L-arginine biosynthetic enzyme are enhanced. Examples of such genes include genes encoding N-acetylglutamyl phosphate reductase (*argC*), ornithine acetyl transferase (*argJ*), N-acetylglutamate kinase (*argB*), acetylornithine transaminase (*argD*), ornithine carbamoyl transferase (*argF*), argininosuccinic acid synthetase (*argG*), argininosuccinic acid lyase (*argH*), and carbamoyl phosphate synthetase (*carAB*).

L-citrulline producing bacteria

Examples of L-citrulline-producing bacteria or parent strains which can be used to derive L-citrulline producing bacteria include, but are not limited to, strains belonging to the genus *Escherichia*, such as *E. coli* mutant N-acetylglutamate synthase strains 237/pMADS11, 237/pMADS12 and 237/pMADS13 (RU2215783, EP1170361B1, US6790647B2), *E. coli* strains 333 (VKPM B-8084) and 374 (VKPM B-8086), both harboring mutant feedback-resistant carbamoyl phosphate synthetase (Russian Patent RU2264459 C2), strains *E. coli*, in which α -ketoglutarate synthase

activity is increased, and ferredoxin NADP⁺ reductase, pyruvate synthase or α -ketoglutarate dehydrogenase activities are additionally modified (EP 2133417 A1), and strain *P. ananantis* NA1sucAsdhA, in which succinate dehydrogenase and α -ketoglutarate dehydrogenase activities are decreased (US Patent Application No 2009286290), and the like.

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

Also citrulline producing bacterium can be easily obtained from any arginine producing bacterium, for example *E. coli* stain 382 (VKPM B-7926), by inactivation of argininosuccinate synthase encoded by *argG* gene. The *argG* gene can be inactivated in the same manner as that for inactivation of the *astCADBE* operon

L-ornithine producing bacteria

L-ornithine producing bacterium can be easily obtained from any arginine producing bacterium, for example *E. coli* stain 382 (VKPM B-7926), by inactivation of ornithine carbamoyltransferase encoded by both *argF* and *argI* genes. Methods for inactivation of ornithine carbamoyltransferase are described above.

L-valine-producing bacteria

Examples of L-valine-producing bacteria or parent strains which can be used to derive L-valine-producing bacteria include, but are not limited to, strains which have been modified to overexpress the *ilvGMEDA* operon (U.S. Patent No. 5,998,178). It is desirable to remove the region of the *ilvGMEDA* operon which is required for attenuation so that expression of the operon is not attenuated by L-valine that is produced. Furthermore, the *ilvA* gene in the operon is desirably disrupted so that threonine deaminase activity is decreased.

Examples of parent strains for deriving L-valine-producing bacteria include also include mutants having a mutation of amino-acyl t-RNA synthetase (U.S. Patent No. 5,658,766). For example, *E. coli* VL1970, which has a mutation in the *ileS* gene encoding isoleucine tRNA synthetase, can be used. *E. coli* VL1970 was deposited in

the Russian National Collection of Industrial Microorganisms (VKPM) (GNII genetika, 1 Dorozhny proezd, 1, Moscow 117545, Russian Federation) on June 24, 1988 under accession number VKPM B-4411.

Furthermore, mutants requiring lipoic acid for growth and/or lacking H⁺-ATPase can also be used as parent strains (WO96/06926).

L-isoleucine-producing bacteria

Examples of L-isoleucine-producing bacteria or parent strains which can be used to derive L-isoleucine producing bacteria include, but are not limited to, mutants having resistance to 6-dimethylaminopurine (JP 5-304969 A), mutants having resistance to an isoleucine analogue such as thiaisoleucine and isoleucine hydroxamate, and mutants additionally having resistance to DL-ethionine and/or arginine hydroxamate (JP 5-130882 A). In addition, recombinant strains transformed with genes encoding proteins involved in L-isoleucine biosynthesis, such as threonine deaminase and acetohydroxate synthase, can also be used as parent strains (JP 2-458 A, FR 0356739, and U.S. Patent No. 5,998,178).

L-methionine-producing bacteria

Examples of L-methionine-producing bacteria and parent strains for deriving L-methionine producing bacteria include, but are not limited to, L-threonine-auxotrophic mutant strain and norleucine-resistant mutant strain (JP 2000-139471 A). Furthermore, a methionine repressor-deficient strain and recombinant strains transformed with genes encoding proteins involved in L-methionine biosynthesis such as homoserine transsuccinylase and cystathionine γ -synthase (JP 2000-139471 A) can also be used as parent strains.

2. Method

Exemplary methods in accordance with the presently disclosed subject matter include producing an L-amino acid by cultivating the bacterium in accordance with the presently disclosed subject matter in a culture medium to produce and excrete the L-amino acid into the medium, and collecting the L-amino acid from the medium.

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

The medium chosen for the culture can be either a synthetic or natural medium, so long as the medium includes a carbon source and a nitrogen source and minerals and, if necessary, appropriate amounts of nutrients which the chosen bacterium

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

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

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

Examples

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

Example 1. Construction of a strain with an inactivated *astCADBE* operon

1. Deletion of the *astCADB* operon

A strain having deletion of the *astCADBE* operon was constructed by the “Red-driven integration”. The DNA fragment containing the Cm^R marker encoded by the *cat* gene was obtained by PCR, using primers P1 (SEQ ID NO: 11) and P2 (SEQ ID NO: 12) and plasmid pMW118-attL-Cm-attR as a template. Conditions for PCR were as follows: denaturation step for 30 sec at 94 °C; profile for 25 cycles: 30 sec at 94 °C, 30 sec at 55°C, 90 sec at 72 °C; final step: 2 min at 72 °C.

The PCR product was purified from agarose gel and was used for electroporation of the *E. coli* strain MG1655. The pKD46 plasmid (Datsenko, K.A. and Wanner, B.L., Proc. Natl. Acad. Sci. USA, 2000, 97:12:6640-45) contains a temperature-sensitive replication origin, and includes a 2,154 nucleotide DNA fragment of phage λ (nucleotide positions 31088 to 33241, GenBank accession no.

J02459), as well as the genes of the λ Red homologous recombination system (γ , β , exo genes), which are under the control of the arabinose-inducible P_{araB} promoter. The pKD46 plasmid is necessary for integration of the PCR product into the chromosome of the MG1655 strain. The strain MG1655 can be obtained from American Type Culture Collection. (P.O. Box 1549 Manassas, VA 20108, U.S.A.).

Electrocompetent cells were prepared as follows: *E. coli* MG1655/pKD46 was grown overnight at 30 °C in LB medium containing ampicillin (100 mg/l), and the culture was diluted 100 times with 5 ml of SOB medium (Sambrook et al, "Molecular Cloning: A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press, 1989) containing ampicillin and L-arabinose (1 mM). The cells were grown with aeration at 30 °C to an OD_{600} of ≈ 0.6 and then were made electrocompetent by concentrating 100-fold and washing three times with ice-cold deionized H_2O . Electroporation was performed using 70 μl of cells and ≈ 100 ng of the PCR product. Cells after electroporation were incubated with 1 ml of SOC medium (Sambrook et al, "Molecular Cloning: A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press, 1989) at 37 °C for 2.5 hours and then were plated onto L-agar containing chloramphenicol (30 $\mu\text{g}/\text{ml}$) and grown at 37 °C to select Cm^{R} recombinants. Then, to eliminate the pKD46 plasmid, two passages on L-agar with Cm at 42 °C were performed and the obtained colonies were tested for sensitivity to ampicillin.

2. Verification of the *astCADBE* operon deletion by PCR

The mutants having the *astCADBE* operon deleted and marked with the Cm resistance gene were verified by PCR. Locus-specific primers P3 (SEQ ID NO: 13) and P4 (SEQ ID NO: 14) were used in PCR for the verification. Conditions for PCR verification were as follows: denaturation step for 30 sec at 94 °C; profile for 30 cycles: 30 sec at 94 °C, 30 sec at 55 °C, 2 min at 72 °C; final step: 2 min at 72 °C. The PCR product obtained in the reaction with the cells of parental strain MG1655 as a template, was ~ 6.1 kbp in length. The PCR product obtained in the reaction with the cells of mutant strain as the template was ~ 1.7 kbp in length. The mutant strain was named MG1655 Δ *astCADBE::cat*.

Example 2. Production of L-threonine by *E. coli* strain B-3996- Δ *astCADBE*

To test the effect of inactivation of the *astCADBE* operon on threonine production, the DNA fragments from the chromosome of the above-described *E. coli* MG1655 Δ *astCADBE::cat* can be transferred to the threonine-producing *E. coli* strain VKPM B-3996 by P1 transduction (Miller, J.H. Experiments in Molecular Genetics,

Cold Spring Harbor Lab. Press, 1972, Plainview, NY) to obtain the strain B-3996- Δ astCADBE. The strain B-3996 was deposited on November 19, 1987 in the All-Union Scientific Center of Antibiotics (Russia, 117105 Moscow, Nagatinskaya Street, 3-A) under the accession number RIA 1867. The strain was also deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (GNII genetika, 1 Dorozhny proezd, 1, Moscow 117545, Russian Federation) under the accession number B-3996.

Both *E. coli* strains, B-3996 and B-3996- Δ astCADBE, can be grown for 18-24 hours at 37°C on L-agar plates. To obtain a seed culture, the strains can be grown on a rotary shaker (250 rpm) at 32 °C for 18 hours in 20x200-mm test tubes containing 2 ml of L-broth supplemented with 4% glucose. Then, the fermentation medium can be inoculated with 0.21 ml (10%) of seed material. The fermentation can be performed in 2 ml of minimal medium for fermentation in 20x200-mm test tubes. Cells can be grown for 65 hours at 32 °C with shaking at 250 rpm.

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

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

Glucose	80.0
(NH ₄) ₂ SO ₄	22.0
NaCl	0.8
KH ₂ PO ₄	2.0
MgSO ₄ ·7H ₂ O	0.8
FeSO ₄ ·7H ₂ O	0.02
MnSO ₄ ·5H ₂ O	0.02
Thiamine HCl	0.0002
Yeast extract	1.0
CaCO ₃	30.0

Glucose and magnesium sulfate are sterilized separately. CaCO₃ is sterilized by dry-heat at 180 °C for 2 hours. The pH is adjusted to 7.0. The antibiotic is introduced into the medium after sterilization.

Example 3. Production of L-lysine by *E. coli* AJ11442- Δ astCADBE

To test the effect of inactivation of the *astCADBE* operon on lysine production, the DNA fragments from the chromosome of the above-described *E. coli* strain MG1655 Δ astCADBE::cat can be transferred to the lysine-producing *E. coli* strain AJ11442 by P1 transduction (Miller, J.H. Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, 1972, Plainview, NY) to obtain AJ11442- Δ astCADBE strain. The strain AJ11442 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (currently National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository, Tsukuba Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) on May 1, 1981 and received an accession number of FERM P-5084. Then, it was converted to an international deposit under the provisions of the Budapest Treaty on October 29, 1987, and received an accession number of FERM BP-1543.

E. coli strains, AJ11442 and AJ11442- Δ astCADBE, can be separately cultured in L-medium containing streptomycin (20 mg/l) at 37 °C, and 0.3 ml of the obtained culture can be inoculated into 20 ml of the fermentation medium containing the required drugs in a 500-ml flask. The cultivation can be carried out at 37 °C for 16 h by using a reciprocal shaker at the agitation speed of 115 rpm. After the cultivation, the amounts of L-lysine and residual glucose in the medium can be measured by a known method (Biotech-analyzer AS210 manufactured by Sakura Seiki Co.). Then, the yield of L-lysine can be calculated relative to consumed glucose for each of the strains.

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

Glucose	40.0
(NH ₄) ₂ SO ₄	24.0
K ₂ HPO ₄	1.0
MgSO ₄ ·7H ₂ O	1.0
FeSO ₄ ·7H ₂ O	0.01
MnSO ₄ ·5H ₂ O	0.01
Yeast extract	2.0

The pH is adjusted to 7.0 by KOH and the medium is autoclaved at 115 °C for 10 min. Glucose and MgSO₄·7H₂O are sterilized separately. CaCO₃ is dry-heat sterilized at 180 °C for 2 hours and added to the medium for a final concentration of 30 g/l.

Example 4. Production of L-cysteine by *E. coli* JM15(ydeD)- Δ astCADBE

To test the effect of inactivation of the *astCADBE* operon on L-cysteine production, the DNA fragments from the chromosome of the above-described *E. coli*

MG1655 Δ astCADBE::cat can be transferred to the *E. coli* L-cysteine-producing strain JM15(ydeD) by P1 transduction (Miller, J.H. Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, 1972, Plainview, NY) to obtain the strain JM15(ydeD)- Δ astCADBE.

E. coli JM15(ydeD) is a derivative of *E. coli* JM15 (US Patent 6,218,168), which can be transformed with DNA having the *ydeD* gene encoding a membrane protein, and is not involved in a biosynthetic pathway of any L-amino acid (US Patent No. 5,972,663). The strain JM15 (CGSC# 5042) can be obtained from The Coli Genetic Stock Collection at the *E. coli* Genetic Resource Center, MCD Biology Department, Yale University (<http://cgsc.biology.yale.edu/>).

Fermentation conditions for evaluation of L-cysteine production were described in detail in Example 6 of US Patent No. 6,218,168.

Example 5. Production of L-leucine by *E. coli* 57- Δ astCADBE

To test the effect of inactivation of the *astCADBE* operon on L-leucine production, the DNA fragments from the chromosome of the above-described *E. coli* strain MG1655 Δ astCADBE::cat can be transferred to the *E. coli* L-leucine-producing strain 57 (VKPM B-7386, US Patent No. 6,124,121) by P1 transduction (Miller, J.H. Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, 1972, Plainview, NY) to obtain the strain 57- Δ astCADBE. The strain 57 was deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (GNII genetika, 1 Dorozhny proezd, 1, Moscow 117545, Russian Federation) on May 19, 1997 under the accession number VKPM B-7386.

E. coli strains, 57 and 57- Δ astCADBE, can be separately cultured for 18-24 hours at 37 °C on L-agar plates. To obtain a seed culture, the strains can be grown on a rotary shaker (250 rpm) at 32 °C for 18 hours in 20x200-mm test tubes containing 2 ml of L-broth supplemented with 4% sucrose. Then, the fermentation medium can be inoculated with 0.21 ml of seed material (10%). The fermentation can be performed in 2 ml of a minimal fermentation medium in 20x200-mm test tubes. Cells can be grown for 48-72 hours at 32 °C with shaking at 250 rpm. The amount of L-leucine can be measured by paper chromatography (liquid phase composition: butanol - acetic acid - water = 4:1:1).

The composition of the fermentation medium (g/l) (pH 7.2) is as follows:

Glucose	60.0
(NH ₄) ₂ SO ₄	25.0
K ₂ HPO ₄	2.0
MgSO ₄ ·7H ₂ O	1.0

Thiamine	0.01
----------	------

CaCO ₃	25.0
-------------------	------

Glucose and CaCO₃ are sterilized separately.

Example 6. Production of L-histidine by *E. coli* 80- ΔastCADBE

To test the effect of inactivation of the *astCADBE* operon on L-histidine production, the DNA fragments from the chromosome of the above-described *E. coli* MG1655 ΔastCADBE::cat can be transferred to the histidine-producing *E. coli* strain 80 by P1 transduction (Miller, J.H. Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, 1972, Plainview, NY) to obtain strain 80-ΔastCADBE. The strain 80 was described in Russian patent 2119536 and deposited in the Russian National Collection of Industrial Microorganisms (GNII genetika, 1 Dorozhny proezd, 1, Moscow 117545, Russian Federation) on October 15, 1999 under accession no. VKPM B-7270 and then converted to a deposit under the Budapest Treaty on July 12, 2004.

E. coli strains, 80 and 80-ΔastCADBE, can be separately cultured in L-broth for 6 h at 29 °C. Then, 0.1 ml of obtained cultures can be each inoculated into 2 ml of fermentation medium in a 20x200-mm test tube and cultivated for 65 hours at 29 °C with shaking on a rotary shaker (350 rpm). After cultivation, the amount of histidine which accumulates in the medium can be determined by paper chromatography. The paper can be developed with a mobile phase consisting of n-butanol : acetic acid : water = 4 : 1 : 1 (v/v). A solution of ninhydrin (0.5%) in acetone can be used as a visualizing reagent.

The composition of the fermentation medium (g/l) (pH 6.0) is as follows:

Glucose	100.0
Mameno (soybean hydrolysate)	0.2 of as total nitrogen
L-proline	1.0
(NH ₄) ₂ SO ₄	25.0
KH ₂ PO ₄	2.0
MgSO ₄ ·7H ₂ O	1.0
FeSO ₄ ·7H ₂ O	0.01
MnSO ₄	0.01
Thiamine	0.001
Betaine	2.0
CaCO ₃	60.0

Glucose, proline, betaine and CaCO₃ are sterilized separately. The pH is adjusted to 6.0 before sterilization.

Example 7. Production of L-glutamic acid by *E. coli* VL334thrC⁺- Δ astCADBE

To test the effect of inactivation of the *astCADBE* operon on L-glutamic acid production, the DNA fragments from the chromosome of the above-described *E. coli* strain MG1655 Δ astCADBE::cat can be transferred to the *E. coli* L-glutamic acid-producing strain VL334thrC⁺ (EP 1172433) by P1 transduction (Miller, J.H. Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, 1972, Plainview, NY) to obtain the strain VL334thrC⁺- Δ astCADBE. The strain VL334thrC⁺ was deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (GNII genetika, 1 Dorozhny proezd, 1, Moscow 117545, Russian Federation) on December 6, 2004 under the accession number VKPM B-8961 and then converted to an international deposit under the Budapest Treaty on December 8, 2004.

E. coli strains, VL334thrC⁺ and VL334thrC⁺- Δ astCADBE, can be separately grown for 18-24 hours at 37 °C on L-agar plates. Then, one loop of the cells can be transferred into test tubes containing 2ml of fermentation medium. The fermentation medium contains glucose (60g/l), ammonium sulfate (25 g/l), KH₂PO₄ (2g/l), MgSO₄ (1 g/l), thiamine (0.1 mg/ml), L-isoleucine (70 µg/ml), and CaCO₃ (25 g/l). The pH is adjusted to 7.2. Glucose and CaCO₃ are sterilized separately. Cultivation can be carried out at 30 °C for 3 days with shaking. After the cultivation, the amount of L-glutamic acid which is produced can be determined by paper chromatography (liquid phase composition of butanol-acetic acid-water=4:1:1) with subsequent staining by ninhydrin (1% solution in acetone) and further elution of the compounds in 50% ethanol with 0.5% CdCl₂.

Example 8. Production of L-phenylalanine by *E. coli* AJ12739- Δ astCADBE

To test the effect of inactivation of the *astCADBE* operon on L-phenylalanine production, the DNA fragments from the chromosome of the above-described *E. coli* MG1655 Δ astCADBE::cat can be transferred to the phenylalanine-producing *E. coli* strain AJ12739 by P1 transduction (Miller, J.H. Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, 1972, Plainview, NY) to obtain strain AJ12739- Δ astCADBE. The strain AJ12739 was deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (GNII genetika, 1 Dorozhny proezd, 1, Moscow 117545, Russian Federation) on November 6, 2001 under accession no. VKPM B-8197 and then converted to an international deposit under the Budapest Treaty on August 23, 2002.

E. coli strains, AJ12739 and AJ12739- Δ astCADBE, can be separately cultivated at 37 °C for 18 hours in a nutrient broth, and 0.3 ml of the obtained culture can be each inoculated into 3 ml of a fermentation medium in a 20x200-mm test tube and

cultivated at 37 °C for 48 hours with shaking on a rotary shaker. After cultivation, the amount of phenylalanine which accumulates in the medium can be determined by TLC. The 10x15-cm TLC plates coated with 0.11-mm layers of Sorbfil silica gel containing no fluorescent indicator (Stock Company Sorbpolimer, Krasnodar, Russia) can be used. The Sorbfil plates can be developed with a mobile phase consisting of propan-2-ol : ethylacetate : 25% aqueous ammonia : water = 40 : 40 : 7 : 16 (v/v). A solution of ninhydrin (2%) in acetone can be used as a visualizing reagent.

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

Glucose	40.0
(NH ₄) ₂ SO ₄	16.0
K ₂ HPO ₄	0.1
MgSO ₄ · 7H ₂ O	1.0
FeSO ₄ · 7H ₂ O	0.01
MnSO ₄ · 5H ₂ O	0.01
Thiamine HCl	0.0002
Yeast extract	2.0
Tyrosine	0.125
CaCO ₃	20.0

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

Example 9. Production of L-tryptophan by *E. coli* SV164 (pGH5)-ΔastCADBE

To test the effect of inactivation of the *astCADBE* operon on L-tryptophan production, the DNA fragments from the chromosome of the above-described *E. coli* strain MG1655 ΔastCADBE::cat can be transferred to the tryptophan-producing *E. coli* strain SV164 (pGH5) by P1 transduction (Miller, J.H. Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, 1972, Plainview, NY) to obtain the strain SV164(pGH5)-ΔastCADBE. The strain SV164 was obtained by introducing the *trpE* allele encoding anthranilate synthase free from feedback inhibition by tryptophan into a *trpE* deficient strain, *E. coli* KB862 (DSM7196) (WO94/08031, Japanese Patent Laid-open No. 7-507693). The plasmid pGH5 harbors a mutant *serA* gene encoding phosphoglycerate dehydrogenase free from feedback inhibition by serine. The strain SV164 (pGH5) was described in detail in US patent No. 6,180,373 or European patent 0662143. The KB862 strain was designated AJ13828 and was deposited on December 21, 2000 in the National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology (currently independent administrative agency, National Institute of Advanced Industrial Science and Technology, International Patent

Organism Depository, Tsukuba Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) as an international deposit under the provisions of the Budapest Treaty with a deposit number of FERM BP-7405.

E. coli strains, SV164(pGH5) and SV164(pGH5)- Δ astCADBE, can be separately cultivated with shaking at 37 °C for 18 hours in 3 ml of nutrient broth supplemented with tetracycline (20 mg/ml, marker of pGH5 plasmid). The obtained cultures (0.3 ml each) can be inoculated into 3 ml of a fermentation medium containing tetracycline (20 mg/ml) in 20 x 200-mm test tubes, and cultivated at 37 °C for 48 hours with a rotary shaker at 250 rpm. After cultivation, the amount of tryptophan which accumulates in the medium can be determined by TLC as described in Example 8. The fermentation medium components are listed in Table 1, but should be sterilized in separate groups (A, B, C, D, E, F, and H), as shown, to avoid adverse interactions during sterilization.

Table 1

Solutions	Component	Final concentration, g/l
A	KH ₂ PO ₄	1.5
	NaCl	0.5
	(NH ₄) ₂ SO ₄	1.5
	L-Methionine	0.05
	L-Phenylalanine	0.1
	L-Tyrosine	0.1
	Mameno (total N)	0.07
	Glucose	40.0
B	MgSO ₄ ·7H ₂ O	0.3
C	CaCl ₂	0.011
D	FeSO ₄ ·7H ₂ O	0.075
	Sodium citrate	1.0
E	Na ₂ MoO ₄ ·2H ₂ O	0.00015
	H ₃ BO ₃	0.0025
	CoCl ₂ ·6H ₂ O	0.00007
	CuSO ₄ ·5H ₂ O	0.00025
	MnCl ₂ ·4H ₂ O	0.0016
	ZnSO ₄ ·7H ₂ O	0.0003
F	Thiamine HCl	0.005
G	CaCO ₃	30.0
H	Pyridoxine	0.03

The pH of solution A is adjusted to 7.1 with NH₄OH.

Example 10. Production of L-proline by *E. coli* 702ilvA- Δ astCADBE

To test the effect of inactivation of the *astCADBE* operon on L-proline production, the DNA fragments from the chromosome of the above-described *E. coli*

strain MG1655 $\Delta astCADBE::cat$ can be transferred to the proline-producing *E. coli* strain 702ilvA by P1 transduction (Miller, J.H. Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, 1972, Plainview, NY) to obtain the strain 702ilvA- $\Delta astCADBE$. The strain 702ilvA was deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (GNII genetika, 1 Dorozhny proezd, 1, Moscow 117545, Russian Federation) on July 18, 2000 under accession number VKPM B-8012 and then converted to an international deposit under the Budapest Treaty on May 18, 2001.

E. coli strains, 702ilvA and 702ilvA- $\Delta astCADBE$, can be separately grown for 18-24 hours at 37 °C on L-agar plates. Then, these strains can be cultivated under the same conditions as in Example 7.

Example 11. Production of L-arginine by *E. coli* 382- $\Delta astCADBE$

To test the effect of inactivation of the *astCADBE* operon on L-arginine production, the DNA fragments from the chromosome of the above-described *E. coli* strain MG1655 $\Delta astCADBE::cat$ were transferred to the arginine-producing *E. coli* strain 382 by P1 transduction (Miller, J.H. Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, 1972, Plainview, NY) to obtain the strain 382- $\Delta astCADBE$. The strain 382 was deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (GNII genetika, 1 Dorozhny proezd, 1, Moscow 117545, Russian Federation) on April 10, 2000 under accession number VKPM B-7926 and then converted to an international deposit under the Budapest Treaty on May 18, 2001. Thus the strain 382- $\Delta astCADBE$ was obtained.

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

After the cultivation, the amount of L-arginine which accumulates in the medium was determined by paper chromatography using the following mobile phase: butanol : acetic acid : water = 4 : 1 : 1 (v/v). A solution of ninhydrin (2%) in acetone was used as a visualizing reagent. A spot containing L-arginine was cut out, L-arginine was eluted with 0.5% water solution of $CdCl_2$, and the amount of L-arginine was estimated spectrophotometrically at 540 nm. The results of eight independent test tube fermentations are shown in Table 2. As follows from Table 2, strain 382- $\Delta astCADBE$ was able to produce a higher amount of L-arginine, as compared with the parent strain 382.

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

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

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

Table 2

Strain	OD ₅₄₀	Amount of L-arginine, g/l
382	15.7 ± 0.5	8.0 ± 0.2
382-ΔastCADBE	15.0 ± 0.5	10.5 ± 0.9

Example 12. Production of L-citrulline by *E. coli* strain 382ΔargG ΔastCADBE.

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

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

After the cultivation, the amount of citrulline which accumulates in the medium can be determined by paper chromatography using the following mobile phase:

butanol : acetic acid : water = 4 : 1 : 1 (v/v). A solution of ninhydrin (2%) in acetone can be used as a visualizing reagent. A spot containing citrulline can be cut out, citrulline can be eluted with 0.5% water solution of CdCl₂, and the amount of citrulline can be estimated spectrophotometrically at 540 nm.

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

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

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

Example 13. Production of L-ornithine by *E. coli* strain 382ΔargFΔargI ΔastCADBE.

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

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

After the cultivation, the amount of ornithine which accumulates in the medium can be determined by paper chromatography using the following mobile phase:

butanol : acetic acid : water = 4 : 1 : 1 (v/v). A solution of ninhydrin (2%) in acetone can be used as a visualizing reagent. A spot containing ornithine can be cut out, ornithine can be eluted with 0.5% water solution of CdCl₂, and the amount of ornithine can be estimated spectrophotometrically at 540 nm.

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

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

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

Example 14. Production of L-valine by *E. coli* strain H-81 ΔastCADBE

To test the effect of inactivation of the *astCADBE* operon on L-valine production, the DNA fragments from the chromosome of the above-described *E. coli* strain MG1655 ΔastCADBE::cat can be transferred to the valine-producing *E. coli* strain H-81 by P1 transduction (Miller, J.H. Experiments in Molecular Genetics, Cold Spring Harbor Lab. Press, 1972, Plainview, NY) to obtain strain H-81 ΔastCADBE. The H-81 strain was deposited at the Russian National Collection of Industrial Microorganisms (VKPM) (GNII genetika, 1 Dorozhny proezd, 1, Moscow 117545, Russian Federation) on January 30, 2001 under the accession number VKPM B-8066, and it was then converted to an international deposit under the provisions of the Budapest Treaty on February 1, 2002.

Both *E. coli* strains, H-81 and H-81 ΔastCADBE, can be cultivated at 37 °C for 18 hours in a nutrient broth and 0.1 ml of each of the obtained cultures can be inoculated into 2 ml of fermentation medium in a 20x200 mm test tube and cultivated at 32 °C for 72 hours with a rotary shaker. After cultivation for 48 hours and for 72 hours accumulated amounts of L-valine can be measured by TLC. The 10x15-cm TLC plates coated with 0.11-mm layers of Sorbfil silica gel containing no fluorescent indicator (Stock Company Sorbpolymer, Krasnodar, Russia) can be used. The Sorbfil plates can be developed with a mobile phase consisting of propan-2-ol: ethylacetate :

25% aqueous ammonia : water = 40 : 40 : 7 : 16 (v/v). A solution of ninhydrin (2%) in acetone can be used as a visualizing reagent.

Fermentation medium composition (g/l):

Glucose	60.0
(NH ₄) ₂ SO ₄	15.0
KH ₂ PO ₄	1.5
MgSO ₄ ·7H ₂ O	1.0
Mameno (TN)	0.4
CaCO ₃	25.0

CaCO₃ is dry-heat sterilized at 180 °C for 2 hours. The pH is adjusted to 7.0.

While the invention has been described in detail with reference to preferred embodiments thereof, it will be apparent to one skilled in the art that various changes can be made, and equivalents employed, without departing from the scope of the invention. All the cited references herein are incorporated as a part of this application by reference.

Industrial Applicability

According to the present invention, production of L-amino acid by a bacterium of the *Enterobacteriaceae* family can be improved.

CLAIMS

1. An L-amino acid-producing bacterium of the *Enterobacteriaceae* family, wherein said bacterium has been modified to attenuate expression of one or more genes of the *astCADBE* operon.
2. The bacterium according to claim 1, wherein expression of the genes is attenuated by inactivation of the *astCADBE* operon.
3. The bacterium according to claim 1, wherein said bacterium belongs to genus *Escherichia*.
4. The bacterium according to claim 3, wherein said bacterium is *Escherichia coli*.
5. The bacterium according to claim 1 or 2, wherein said bacterium belongs to genus *Pantoea*.
6. The L-amino acid-producing bacterium according to any of claims 1 to 5, wherein said L-amino acid is selected from the group consisting of an aromatic L-amino acid and a non-aromatic L-amino acid.
7. The L-amino acid-producing bacterium according to claim 6, wherein said aromatic L-amino acid is selected from the group consisting of L-phenylalanine, L-tyrosine, and L-tryptophan.
8. The L-amino acid-producing bacterium according to claim 6, wherein said non-aromatic L-amino acid is selected from the group consisting of L-threonine, L-lysine, L-cysteine, L-methionine, L-leucine, L-isoleucine, L-valine, L-histidine, glycine, L-serine, L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-proline, L-arginine, L-citrulline and L-ornithine.
9. The L-amino acid-producing bacterium according to any of claims 1 to 5, wherein said L-amino acid is L-arginine.
10. A method for producing an L-amino acid comprising:
 - cultivating the bacterium according to any of claims 1 to 9 in a medium, and
 - collecting said L-amino acid from the medium.
11. The method according to claim 10, wherein said L-amino acid is selected from the group consisting of an aromatic L-amino acid and a non-aromatic L-amino acid.
12. The method according to claim 11, wherein said aromatic L-amino acid is selected from the group consisting of L-phenylalanine, L-tyrosine, and L-tryptophan.
13. The method according to claim 11, wherein said non-aromatic L-amino acid is selected from the group consisting of L-threonine, L-lysine, L-cysteine, L-methionine, L-leucine, L-isoleucine, L-valine, L-histidine, glycine, L-serine, L-alanine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-proline, L-arginine, L-citrulline and L-ornithine.

14. The method according to claim 10, wherein said L-amino acid is L-arginine.

INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2011/066786

A. CLASSIFICATION OF SUBJECT MATTER		
INV.	C12P13/10 C12N9/80	C12R1/01 C12N1/20
	C12N9/10	C12N9/02 C12N9/78
ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) C12P C12R C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2011/024555 A1 (AJINOMOTO KK [JP]; DOI HIDETAKA [JP]; USUDA YOSHIHIRO [JP]) 3 March 2011 (2011-03-03) the whole document	1-14
X	----- SCHNEIDER ET AL: "Arginine catabolism and the arginine succinyltransferase pathway in Escherichia coli.", JOURNAL OF BACTERIOLOGY, vol. 180, no. 16, 1 August 1998 (1998-08-01), pages 4278-4286, XP55009025, ISSN: 0021-9193 cited in the application	1-4,6-9
Y	page 4278, column 2, paragraph 2 page 4283, column 1, paragraph 3 - column 2, paragraph 1; figure 4 ----- -/-	5,10-14
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
Date of the actual completion of the international search 24 October 2011		Date of mailing of the international search report 31/10/2011
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer van Heusden, Miranda

INTERNATIONAL SEARCH REPORT

International application No

PCT/JP2011/066786

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 2 196 535 A1 (AJINOMOTO KK [JP]) 16 June 2010 (2010-06-16) paragraph [0004] -----	5,10-14
X	WEERASINGHE JEEVAKA P ET AL: "Stationary phase expression of the arginine biosynthetic operon argCBH in Escherichia coli", BMC MICROBIOLOGY, BIOMED CENTRAL, LONDON, GB, vol. 6, no. 1, 22 February 2006 (2006-02-22), page 14, XP021014843, ISSN: 1471-2180, DOI: 10.1186/1471-2180-6-14 page 9, column 1, paragraph 4 - page 10, column 1; table 5 page 4, column 2, paragraph 2 -----	1-9
X	Y. ELBAHLOUL ET AL: "Engineering the Genotype of Acinetobacter sp. Strain ADP1 To Enhance Biosynthesis of Cyanophycin", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 72, no. 2, 1 February 2006 (2006-02-01), pages 1410-1419, XP55009317, ISSN: 0099-2240, DOI: 10.1128/AEM.72.2.1410-1419.2006 abstract page 1412, column 2, paragraph 4 - paragraph 6; table 1 -----	1,6-9
X	Z. YANG ET AL: "Functional Genomics Enables Identification of Genes of the Arginine Transaminase Pathway in Pseudomonas aeruginosa", JOURNAL OF BACTERIOLOGY, vol. 189, no. 11, 6 April 2007 (2007-04-06), pages 3945-3953, XP55009039, ISSN: 0021-9193, DOI: 10.1128/JB.00261-07 abstract; table 1 -----	1,6-9
X	SOUPENE E., LEE H., KUSTU S.: "Ammonium/methylammonium transport (Amt) proteins facilitate diffusion of NH ₃ bidirectionally", PNAS, vol. 99, no. 6, 19 March 2002 (2002-03-19) , pages 3926-3931, XP055009289, page 3927, column 2, paragraph 2; table 1 ----- -/--	1,2,6-9

INTERNATIONAL SEARCH REPORT

International application No

PCT/JP2011/066786

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TRICOT C. ET AL.: "Isolation and characterization of <i>Pseudomonas putida</i> mutants affected in arginine, ornithine and citrulline catabolism: function of the arginine oxidase and arginine succinyltransferase pathways", J. GENERAL MICROBIOLOGY, vol. 137, 1991, pages 2911-2918, XP55009331, table 2 -----	1,6-9

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/JP2011/066786

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011024555	A1	03-03-2011	NONE

EP 2196535	A1	16-06-2010	CN 101760441 A 30-06-2010
			RU 2008148283 A 20-06-2010
			US 2010143983 A1 10-06-2010
