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(54) Title: METHOD FOR PRODUCING L-ARGININE USING BACTERIUM OF THE FAMILY ENTEROBACTERIACEAE HAVING N-ACETYLRNITHINE DEACETYLASE WITH DOWNREGULATED ACTIVITY

(57) Abstract: The present invention describes a method for producing L-amino acid such as L-arginine by fermentation using a bacterium of the family *Enterobacteriaceae*, particularly a bacterium belonging to the genus *Escherichia*, which has been modified to contain the *argJ* gene, encoding an enzyme having at least an ornithine acetyltransferase activity, and the attenuated or inactivated the *argE* gene encoding N-acetylornithine deacetylase.



DESCRIPTION

METHOD FOR PRODUCING L-ARGININE USING BACTERIUM OF THE FAMILY *ENTEROBACTERIACEAE* HAVING N-ACETYLORNITHINE DEACETYLASE WITH DOWNREGULATED ACTIVITY

Technical Field

The present invention relates to the microbiological industry, and specifically to a method for producing L-amino acids such as L-arginine by fermentation of a bacterium of the family *Enterobacteriaceae* which has been modified to contain the *argJ* gene, encoding an enzyme having at least an ornithine acetyltransferase activity, and having downregulated the *argE* gene encoding N-acetylornithine deacetylase.

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 transformation of microorganisms with recombinant DNA (see, for example, U.S. Patent No. 4,278,765 A) and alteration of regulatory regions such as promoter, leader sequence, and/or attenuator, or others known to the person skilled in the art (see, for example, US20060216796 A1 and WO9615246 A1). Other techniques for enhancing production yields include increasing the activities of enzymes involved in amino acid biosynthesis and/or desensitizing the target enzymes to the feedback inhibition by the resulting L-amino acid (see, for example, WO9516042 A1, EP0685555 A1 or U.S. Patent Nos. 4,346,170 A, 5,661,012 A, and 6,040,160 A).

Another method for enhancing L-amino acids production yields is to attenuate expression of a gene or several genes which are involved in degradation of the target L-amino acid, genes which divert the precursors of

the target L-amino acid from the L-amino acid biosynthetic pathway, genes involved in the redistribution of the carbon, nitrogen, and phosphate fluxes, and genes encoding toxins, etc.

It is generally accepted that, for example, in bacteria biosynthesis of L-arginine from L-glutamate can proceed through two pathways, the linear or the cyclic one, depending on the microorganism concerned (Cunin R. et al., *Microbiol. Rev.*, 1986, 50:314-352). In bacteria such as bacteria belonging to the family *Enterobacteriaceae*, in particular, *Escherichia coli* (*E. coli*) the linear pathway takes place, and it includes eight steps until L-arginine is formed from L-glutamate (Vogel H.J. and MacLellan W.L., *Methods Enzymol.*, 1970, 17A, 265-269). The biosynthesis is initiated by acetylating L-glutamate with amino acid N-acetyltransferase (EC 2.3.1.1, also referred to as N-acetyl-L-glutamate synthetase) encoded by the *argA* gene. The follow-up biosynthetic reactions are catalyzed by the enzymes commonly referred to as N-acetylglutamate kinase, N-acetyl- γ -glutamylphosphate reductase, N-acetylornithine aminotransferase, N-acetylornithine deacetylase, ornithine carbamoyltransferase, argininosuccinate synthase, and argininosuccinate lyase encoded by the *argB*, *argC*, *argD*, *argE*, *argF*, *argG* and *argH* genes, respectively. The acetyl group, cleaved off by N-acetylornithine deacetylase (ArgE) from N-acetylornithine to produce ornithine, is recruited further by the coenzyme A (HS-CoA, CoA) to form acetylated coenzyme A (AcS-CoA, acetyl-CoA) which acts as the acetyl group donor in the amino acid N-acetyltransferase (*argA*) catalyzed acetylation of L-glutamate.

The cyclic pathway of L-arginine biosynthesis was found in prokaryotes such as *Corynebacterium glutamicum* (Udaka S. and Kinoshita S., *J. Gen. Appl. Microbiol.*, 1958, 4:272-282; Sakanyan V. et al., *Microbiology*, 1996, 142:99-108), *Bacillus* species (Sakanyan V. et al., *J. Gen. Microbiol.*, 1992, 138:125-130), *Thermotoga neapolitana* (Marc F. et al., *Eur. J. Biochem.*, 2000, 267(16):5217-5226), and eukaryotic organisms (De Deken R.H., *Biochim. Biophys. Acta.*, 1962, 78:606-616). Contrary to the linear pathway, in the cyclic pathway the transfer of the acetyl group from N-acetylornithine to L-glutamate is catalyzed by bifunctional ornithine acetyltransferase/N-acetylglutamate synthase (EC 2.3.1.35/2.3.1.1) encoded by the *argJ* gene.

Apart from the bifunctional enzyme encoded by the *argJ* gene, the ArgJ protein encoded by *argJ* and exhibiting solely the ornithine acetyltransferase activity has been also described (Haas D. et al., *Eur. J. Biochem.*, 1972, 31:290-295; Marc F. et al., *Eur. J. Biochem.*, 2000, 267(16):5217-5226). The monofunctional and bifunctional ArgJ enzymes can be distinguished, for example, by two means: (i) by enzymatic assay using two acetyl group donors such as N-acetylornithine and AcS-CoA; and (ii) by complementation test using *argE* and *argA* mutants of *E. coli* for the cloned *argJ* gene. The monofunctional ArgJ enzyme transfers the acetyl group from N-acetylornithine to L-glutamate and complements the *argE* mutant, whereas the bifunctional ArgJ enzyme transfers the acetyl group from N-acetylornithine and AcS-CoA and complements *argE* and *argA* mutant strains.

A new L-arginine biosynthetic pathway has been found recently in *Xanthomonas campestris* where N-acetylornithine is converted into N-acetylcitrulline by acetylornithine carbamoyltransferase encoded by *argF'*, and citrulline is produced from N-acetylcitrulline by ArgE (Shi D. et al., *J. Biol. Chem.*, 2005, 280:14366-14369).

An *E. coli* bacterium natively having the linear pathway has been modified to contain the *argJ* gene from *Bacillus stearothermophilus* or *Thermotoga neapolitana* (*T. neapolitana*), which encodes the bifunctional ArgJ enzyme, to initiate the less energy consuming cyclic pathway and thus to increase production of L-arginine by the modified bacterium (U.S. Patent No. 6,897,048 B2).

In microorganisms natively utilizing the linear biosynthetic pathway or having the modified linear pathway which functions as the cyclic pathway, the amino acid N-acetyltransferase (N-acetyl-L-glutamate synthetase) (ArgA) may be required to initiate and support L-arginine biosynthesis through the N-acetylglutamate supply. Therefore, to increase production of L-arginine by a recombinant *E. coli* strain, the number of copies of the *argA* gene can be increased by cloning the wild-type *argA* gene on plasmid vectors and incorporating them into the strain having the *argJ* gene cloned (U.S. Patent No. 6,897,048 B2). Alternatively, the *argA* gene encoding the mutant amino

acid N-acetyltransferase resistant to the feedback inhibition by L-arginine (Eckhardt T. et al., *Mol. Gen. Genet.*, 1975, 138:225-232) can be introduced into *E. coli* strain to improve L-arginine production (EP1170361 A2).

Despite positive effects on L-arginine production from the use of bifunctional ArgJ protein, additional copies of the *argA* gene, and/or the mutant N-acetyl-L-glutamate synthetase (mutant ArgA), and the like are clearly understood, the approaches for further increasing of L-arginine production by a microorganism are not obvious.

Until now, no data has been reported demonstrating the effect from the downregulated *argE* gene, so that the activity of N-acetylornithine deacetylase (ArgE) is decreased or abolished, on L-arginine production by the modified bacterial strains of the family *Enterobacteriaceae* having the heterologous *argJ* gene.

Summary of Invention

The inventors of the present invention have assumed that in a microorganism, for example, in a bacterium having the heterologous *argJ* gene, which encodes monofunctional ornithine acetyltransferase or bifunctional ornithine acetyltransferase/N-acetylglutamate synthase, attenuation of expression of the *argE* gene or inactivation of the *argE* gene may retrieve additional energy from the processes related to the acetyl group transfer from N-acetylornithine to L-glutamate thus promoting increased L-arginine production by the modified strain as compared with a parent strain. It is presumed by the inventors that the ArgJ enzyme having mono or dual activity may rescue the L-arginine biosynthesis in *argE* or *argA* and *argE* mutants upon cloning of the *argJ* gene from a donor microorganism. On this account, once initiated by realization of the first biosynthetic reaction to produce N-acetylglutamate, the artificial cyclic pathway may function through direct acetylation of L-glutamate by the acetyl group originated from N-acetylornithine rather than from energetically wealthy AcS-CoA.

An aspect of the present invention is to provide a bacterium belonging to the family *Enterobacteriaceae*, which may belong to the genus *Escherichia*, and more specifically, to the species *Escherichia coli*, which has the activity of

ornithine acetyltransferase or ornithine acetyltransferase/N-acetylglutamate synthase (ArgJ), and in which the N-acetylornithine deacetylase (ArgE) is inactive or the activity of ArgE is decreased.

Another aspect of the present invention is to provide a method for producing L-amino acids such as L-arginine using a bacterium of the family *Enterobacteriaceae* as described herein.

These aims were achieved by the finding that inactivation or attenuation of expression of the *argE* gene in a bacterium of the family *Enterobacteriaceae* having the heterologous *argJ* gene results in increased production of L-arginine.

An aspect of the present invention is to provide L-arginine-producing bacterium of the family *Enterobacteriaceae* having a recombinant DNA comprising the *argJ* gene encoding an enzyme having at least an ornithine acetyltransferase activity, wherein the bacterium has been modified to contain the downregulated N-acetylornithine deacetylase activity.

It is a further aspect of the present invention to provide the bacterium as described above, wherein the *argJ* gene is derived from a microorganism having an enzyme having the ornithine acetyltransferase or ornithine acetyltransferase/N-acetylglutamate synthase activity.

It is a further aspect of the present invention to provide the bacterium as described above, wherein the *argJ* gene is derived from the microorganism belonging to the family selected from the group consisting of the families *Thermotogaceae*, *Bacillaceae* and *Methanocaldococcaceae*.

It is a further aspect of the present invention to provide the bacterium as described above, wherein the *argJ* gene is derived from the species *Thermotoga neapolitana*.

It is a further aspect of the present invention to provide the bacterium as described above, wherein the *argJ* gene encodes a protein selected from the group consisting of:

- (A) a protein having the amino acid sequence of SEQ ID NO: 2 which has ornithine acetyltransferase/N-acetylglutamate synthase activity; and

(B) a variant protein having the amino acid sequence of SEQ ID NO: 2, but which includes substitution, deletion, insertion, and/or addition of one or several amino acid residues and has ornithine acetyltransferase/N-acetylglutamate synthase activity according to the amino acid sequence of SEQ ID NO: 2.

It is a further aspect of the present invention to provide the bacterium as described above, wherein the N-acetylornithine deacetylase is a protein selected from the group consisting of:

(D) a protein having the amino acid sequence of SEQ ID NO: 4; and

(E) a variant protein having the amino acid sequence of SEQ ID NO: 4, but which includes substitution, deletion, insertion, and/or addition of one or several amino acid residues and has N-acetylornithine deacetylase activity according to the amino acid sequence of SEQ ID NO: 4.

It is a further aspect of the present invention to provide the bacterium as described above, wherein the activity of N-acetylornithine deacetylase is decreased.

It is a further aspect of the present invention to provide the bacterium as described above, wherein the activity of N-acetylornithine deacetylase is abolished.

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

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

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

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

An aspect of the present invention is to provide a method for producing L-arginine or a salt thereof comprising:

(i) cultivating the bacterium as described above in a culture medium to accumulate L-arginine or the salt thereof in the bacterium or culture medium, or both; and, if necessary,

(ii) collecting L-arginine or the salt thereof from the bacterium or culture medium.

The present invention is described in details below.

Brief Description of Drawings

FIG. 1 shows the scheme for integration of the *argJ* gene into the chromosome of the *E. coli* MG1655 Δ *argA* Δ *argR* strain.

FIG. 2 shows the scheme for construction of the *E. coli* MG1655 Δ *argA* Δ *argR* Δ *artP-J::P_{nlp8p10}argJargEm24::Cm* strain.

Description of Embodiments

1. Bacterium

The phrase “an L-amino acid-producing bacterium” can mean a bacterium of the family *Enterobacteriaceae* which has an ability to produce, excrete or secrete, and cause accumulation of an L-amino acid in a culture medium or the bacterial cells when the bacterium is cultured in the medium.

The phrase “L-amino acid-producing ability” can mean the ability of the bacterium to produce, excrete or secrete, and cause accumulation of the L-amino acid in a medium or the bacterial cells to such a level that the L-amino acid can be collected from the medium or the bacterial cells, when the bacterium is cultured in the medium.

The phrase “L-amino acid” can mean L-alanine, L-arginine, 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 bacterium may inherently have the L-amino acid-producing ability or may be modified to have an L-amino acid-producing ability by using a mutation method or DNA recombination techniques.

The bacteria belonging to the family *Enterobacteriaceae* can be from the genera *Enterobacter*, *Erwinia*, *Escherichia*, *Klebsiella*, *Morganella*, *Pantoea*, *Photobacterium*, *Providencia*, *Salmonella*, *Yersinia*, and so forth, and can have the ability to produce an L-amino acid. Specifically, those classified into the family *Enterobacteriaceae* according to the taxonomy used in the NCBI (National Center for Biotechnology Information) database (www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=543) can be used. Examples of strains from the family *Enterobacteriaceae* which can be modified include a bacterium of the genus *Escherichia*, *Enterobacter* or *Pantoea*.

Strains of *Escherichia* bacterium which can be modified to obtain *Escherichia* bacteria in accordance with the presently disclosed subject matter are not particularly limited, and specifically, those described in the work of Neidhardt et al. can be used (Bachmann, B.J., Derivations and genotypes of some mutant derivatives of *E. coli* K-12, p. 2460-2488. In F.C. Neidhardt et al. (ed.), *E. coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C., 1996). The species *E. coli* is a particular example. Specific examples of *E. coli* include *E. coli* W3110 (ATCC 27325), *E. coli* MG1655 (ATCC 47076), and so forth, which are derived from the prototype wild-type strain, *E. coli* K-12 strain. These strains are available from, for example, the American Type Culture Collection (P.O. Box 1549, Manassas, VA 20108, United States of America). That is, registration numbers are given to each of the strains, and the strains can be ordered by using these registration numbers (refer to www.atcc.org). The registration numbers of the strains are listed in the catalogue of the American Type Culture Collection.

Examples of the *Enterobacter* bacteria include *Enterobacter agglomerans*, *Enterobacter aerogenes*, and so forth. Examples of the *Pantoea* bacteria include *Pantoea ananatis*, and so forth. Some strains of *Enterobacter agglomerans* were recently reclassified into *Pantoea agglomerans*, *Pantoea ananatis* or *Pantoea stewartii* on the basis of nucleotide sequence analysis of 16S rRNA, etc. A bacterium belonging to any of the genus *Enterobacter* or *Pantoea* may be used so long as it is a bacterium classified into the family

Enterobacteriaceae. When a *Pantoea ananatis* strain is bred by genetic engineering techniques, *Pantoea ananatis* AJ13355 strain (FERM BP-6614), AJ13356 strain (FERM BP-6615), AJ13601 strain (FERM BP-7207) and derivatives thereof can be used. These strains were identified as *Enterobacter agglomerans* when they were isolated, and deposited as *Enterobacter agglomerans*. However, they were recently re-classified as *Pantoea ananatis* on the basis of nucleotide sequencing of 16S rRNA and so forth as described above.

The phrase "an L-arginine-producing bacterium" can mean a bacterium which has an ability to produce, excrete or secrete, and cause accumulation of L-arginine in a culture medium or the bacterial cells in an amount larger than a wild-type or a parent strain of *E. coli* such as *E. coli* K-12 when the bacterium is cultured in the medium. The L-arginine-producing ability can mean the ability of the bacterium to produce, excrete or secrete, and cause accumulation of L-arginine in a medium in an amount of not less than 0.5 g/L, or not less than 1.0 g/L, so that L-arginine can be collected from the medium and/or the bacterial cells when the bacterium is cultured in the medium.

Examples of 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 (EP1170361 A2), *E. coli* strain 382 (VKPM B-7926) (EP1170358 A1), an L-arginine-producing strain having the *argA* gene introduced (EP1170361 A1), and the like.

The L-arginine-producing bacterium of the family *Enterobacteriaceae* can be modified further to contain the heterologous *argJ* gene which encodes monofunctional ornithine acetyltransferase or bifunctional ornithine acetyltransferase/N-acetylglutamate synthase (ArgJ). The exemplary bacterium of the family *Enterobacteriaceae* may have the *argJ* gene encoding the mono- or bifunctional ArgJ enzyme resistant to feedback inhibition by L-arginine. The *argJ* gene can be derived from a thermophilic microorganism such as a microorganism belonging to the family *Thermotogaceae*, and more

specifically to the genus *Thermotoga*, for example, *T. neapolitana* (*T. neapolitana*). The *argJ* gene may also derive from other bacterial sources such as *Bacillus stearothermophilus* (or *Geobacillus stearothermophilus*) of the family *Bacillaceae* or Archaea sources such as *Methanocaldococcus jannaschii* (*M. jannaschii*) (formerly *Methanococcus jannaschii*) of the family *Methanocaldococcaceae*, and the like. However, various bacterial sources of the *argJ* gene may be used as soon as the *argJ* gene encodes an enzyme having at least an ornithine acetyltransferase activity.

The L-arginine-producing bacterium of the family *Enterobacteriaceae* can be modified further to contain inactive *argE* gene or the *argE* gene with attenuated expression so that the N-acetylornithine deacetylase (ArgE) in the modified bacterium is not active or the activity of the N-acetylornithine deacetylase is decreased as compared with a non-modified bacterium.

The phrase “an L-arginine-producing bacterium” can also mean the bacterium of the family *Enterobacteriaceae* having the *argA* gene encoding the wild-type or a mutant amino acid N-acetyltransferase (ArgA). The ArgA enzyme resistant to feedback inhibition by L-arginine may be referred to as the mutant amino acid N-acetyltransferase. The exemplary mutant ArgA protein resistant to feedback inhibition by L-arginine may derive from the wild-type ArgA protein by substitution of the amino acid sequence at the position from 15 to 19 to another amino acid sequence as disclosed in EP1170361 A2. The mutant ArgA protein having substitution, deletion, insertion, and/or addition of one or several amino acid residues at one or more positions other than from 15 to 19 in the amino acid sequence may also be referred to as the mutant ArgA protein provided that the three-dimensional structure of the mutant amino acid N-acetyltransferase is not significantly changed relative to the wild-type protein or an activity of the mutant enzyme is not deteriorated and it is resistant to feedback inhibition by L-arginine.

The phrase “an L-arginine-producing bacterium” can also mean the bacterium of the family *Enterobacteriaceae* as described above having enhanced, attenuated, and/or inactivated the *argA* gene encoding the wild-type or a mutant amino acid N-acetyltransferase.

The phrase “an L-arginine-producing bacterium” can also mean the bacterium of the family *Enterobacteriaceae* modified further to be devoid of the ArgR-mediated transcriptional repression. The DNA-binding transcriptional dual regulator ArgR involved in the negative control of L-arginine biosynthesis pathway in microorganisms can be inactivated by, for example, inactivation of the ArgR encoding gene (*argR*).

The phrase “a wild-type protein” can mean a native protein naturally produced by a wild-type or a parent bacterial strain of the family *Enterobacteriaceae*, for example, by the wild-type *E. coli* MG1655 strain. A wild-type protein can be encoded by the wild-type, or non-modified, gene naturally occurring in a genome of wild-type bacterium.

The phrase “a bacterium modified to contain the *argJ* gene” can mean that the bacterium belonging to the first bacterial species, naturally not containing the *argJ* gene and thus referred to as a recipient bacterium, has been modified to contain one or more recombinant DNA molecules, having the *argJ* gene, synthesized and/or originated and introduced from a donor bacterium belonging to the second bacterial species, which is different from the first species. The exemplary modification to introduce a recombinant DNA can include the heterologous gene expression. Exemplary, the recipient bacterium may belong to the family *Enterobacteriaceae*, for example, to the species *E. coli*; the donor bacterium may belong to a thermophilic bacterium, for example, to the species *T. neapolitana*.

The phrase “a bacterium modified to contain the recombinant DNA” can mean the bacterium modified to contain an exogenous DNA by, for example, conventional methods such as, for example, transformation, transfection, infection, conjugation and mobilization. Transformation, transfection, infection, conjugation or mobilization of a bacterium with the DNA encoding a protein can impart the bacterium an ability to synthesize the protein encoded by the DNA. Methods of transformation, transfection, infection, conjugation and mobilization include any known methods that have been reported. For example, a method of treating recipient cells with calcium chloride so as to increase permeability of the cells of *Escherichia coli* K-12 to DNA has been reported for efficient DNA transformation and

transfection (Mandel M. and Higa A., Calcium-dependent bacteriophage DNA infection, *J. Mol. Biol.*, 1970, 53:159-162). Methods of specialized and/or generalized transduction are described (Morse M.L. et al., Transduction in *Escherichia coli* K-12, *Genetics*, 1956, 41(1):142-156; Miller J.H., *Experiments in Molecular Genetics*. Cold Spring Harbor, N.Y.: Cold Spring Harbor La. Press, 1972). Other methods for random and/or targeted integration of DNA into the host genome can be applied, for example, "Mu-driven integration/amplification" (Akhverdyan et al., *Appl. Microbiol. Biotechnol.*, 2011, 91:857-871), "Red/ET-driven integration" or " λ Red/ET-mediated integration" (Datsenko K.A. and Wanner B.L., *Proc. Natl. Acad. Sci. USA* 2000, 97(12):6640-45; Zhang Y., et al., *Nature Genet.*, 1998, 20:123-128). Moreover, for multiple insertions of desired genes in addition to Mu-driven replicative transposition (Akhverdyan et al., *Appl. Microbiol. Biotechnol.*, 2011, 91:857-871) and chemically inducible chromosomal evolution based on *recA*-dependent homologous recombination resulted in an amplification of desired genes (Tyo K.E.J. et al., *Nature Biotechnol.*, 2009, 27:760-765), another methods can be used, which utilize different combinations of transposition, site-specific and/or homologous Red/ET-mediated recombinations, and/or P1-mediated generalized transduction (see, for example, Minaeva N. et al., *BMC Biotechnology*, 2008, 8:63; Koma D. et al., *Appl. Microbiol. Biotechnol.*, 2012, 93(2):815-829).

The heterologous expression of the *argJ* gene in host microorganisms can be achieved by substituting rare (low-usage in the host organism) codons for synonymous middle- or high-usage codons, where codon usage can be defined as the number of times (frequency) a codon is translated per unit time in the cell of an organism or an average codon frequency of the sequenced protein-coding reading frames of an organism (Zhang S.P. et al., *Gene*, 1991, 105(1):61-72). The codon usage per organism can be found in the Codon Usage Database, which is an extended web-version of the CUTG (Codon Usage Tabulated from GenBank) (<http://www.kazusa.or.jp/codon/>; Nakamura Y. et al., Codon usage tabulated from the international DNA sequence databases: status for the year 2000, *Nucleic Acids Res.*, 2000, 28(1):292). In *E. coli* such mutations can include, without limiting, the

substitution of rare Arg codons AGA, AGG, CGG, CGA for CGT or CGC; rare Ile codon ATA for ATC or ATT; rare Leu codon CTA for CTG, CTC, CTT, TTA or TTG; rare Pro codon CCC for CCG or CCA; rare Ser codon TCG for TCT, TCA, TCC, AGC or AGT; rare Gly codons GGA, GGG for GGT or GGC; and so forth. The substitution of low-usage codons for synonymous high-usage codons can be preferable. The substituting rare and/or low-usage codons for synonymous middle- or high-usage codons may be combined with co-expression of the genes which encode tRNAs recognizing rare codons.

The phrase “a bacterium modified to contain the downregulated *argE* gene” can mean that the bacterium has been modified in such a way that in the modified bacterium the expression of the *argE* gene is attenuated or the *argE* gene is inactivated.

The phrase “expression of the *argE* gene is attenuated” can mean that the amount of the ArgE protein in the modified bacterium in which expression of the *argE* gene is attenuated, is reduced as compared with a non-modified bacterium, for example, a wild-type or parent strain.

The phrase “expression of the *argE* gene is attenuated” can also mean that the modified bacterium includes a modified *argE* gene, which encodes a mutant ArgE protein having decreased activity as compared with the wild-type ArgE protein, or a region operably linked to the gene, including sequences controlling gene expression, such as promoters, enhancers, attenuators and transcription termination signals, ribosome-binding sites (RBS), and other expression control elements is modified resulting in a decrease in the expression level of the *argE* gene, and other examples (see, for example, WO95/34672; Carrier T.A. and Keasling J.D., *Biotechnol. Prog.* 1999, 15:58-64).

The similar definitions may be given to the phrases “expression of the *argR* gene is attenuated” and “expression of the *argA* gene is attenuated”.

Expression of the *argE*, *argA*, and/or *argR* gene(s) can be attenuated by replacing an expression control sequence of the gene(s), such as a promoter on the chromosomal DNA, with a weaker one. The strength of a promoter is defined by the frequency of initiation acts of RNA synthesis. Examples of methods for evaluating the strength of promoters and strong promoters are

described in Goldstein et al., Prokaryotic promoters in biotechnology, *Biotechnol. Annu. Rev.*, 1995, 1:105-128), and so forth. Furthermore, it is also possible to introduce nucleotide substitution for several nucleotides in a promoter region of a target gene and thereby modify the promoter to be weakened as disclosed in International Patent Publication WO00/18935. Furthermore, it is known that substitution of several nucleotides in the SD sequence, and/or in the spacer between the SD sequence and the start codon, and/or a sequence immediately upstream and/or downstream from the start codon in the ribosome-binding site (RBS) greatly affects the translation efficiency of mRNA. This modification of the RBS may be combined with decreasing transcription of the *argE*, *argA*, and/or *argR* gene(s).

Expression of the *argE*, *argA*, and/or *argR* gene(s) can also be attenuated by insertion of a transposon or an IS (insertion sequence) into the coding region of the gene (U.S. Patent No. 5,175,107) or in the region controlling gene expression, or in the proximal part of the *argE*, *argA*, and/or *argR* gene(s) structure, where the *argE*, *argA*, and/or *argR* gene(s) is/are the distal part, or by conventional methods such as mutagenesis with ultraviolet irradiation (UV) irradiation or nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine). Furthermore, the incorporation of a site-specific mutation can be conducted by known chromosomal editing methods based, for example, on λ Red/ET-mediated recombination.

The phrases "enzymatic activity of ArgE is decreased", "enzymatic activity of ArgA is decreased" and "enzymatic activity of ArgR is decreased" can mean that the enzymatic activity of N-acetylornithine deacetylase (ArgE), amino acid N-acetyltransferase (ArgA), and/or ArgR regulator, respectively, is lower than that in a non-modified strain, for example, a wild-type strain of the bacterium belonging to the family *Enterobacteriaceae*, or more specifically, the species *E. coli*. Exemplary, the enzymatic activity of N-acetylornithine deacetylase (ArgE), amino acid N-acetyltransferase (ArgA), and/or ArgR regulator can be decreased by the gene inactivation.

The enzymatic activity of enzymes encoded by the *argE*, *argA*, and/or *argR* gene(s) can also be decreased by introducing a mutation into the

chromosome so that intracellular activity of the proteins encoded the *argE*, *argA*, and/or *argR* gene(s) is decreased as compared with a non-modified strain. Such a mutation on the gene(s) or upstream the genes in the operon structure can be the replacement of one base or more to cause an amino acid substitution in the protein encoded by the gene(s) (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 and/or transcription termination signal, deletion of a part of the gene(s) or deletion of the entire gene(s) (Qiu Z. and Goodman M.F., *J. Biol. Chem.*, 1997, 272:8611-8617; Kwon D.H. et al., *J. Antimicrob. Chemother.*, 2000, 46:793-796).

In the modified bacterium, the amount of the ArgE protein encoded by the *argE* gene can be decreased to such a level that the residual ArgE activity is less than about 3%, or less than about 2%, or less than about 1% of the initial activity but higher than zero, as compared with a non-modified bacterium.

In the modified bacterium, the specific activity of ArgE encoded by the *argE* gene can be decreased to such a level that the residual specific activity of ArgE is not more than 1000 nmol/mg min, 750 nmol/mg min, 500 nmol/mg min, 250 nmol/mg min, 100 nmol/mg min, 50 nmol/mg min, 25 nmol/mg min, 10 nmol/mg min, 5 nmol/mg min, or about 3 nmol/mg min as resulted from the spread of experimental data. The residual specific activity can be more than 0.5 nmol/mg min, 1 nmol/mg min, 2 nmol/mg min, or about 3 nmol/mg min as resulted from the spread of experimental data. The specific activity of ArgE of the bacterium per mg crude protein can be determined in crude extracts of the sonicated bacterial cells by the described method (Takahara K. et al. *FEBS J.*, 2005, 272:5353-5364). The crude protein concentration can be determined by the Bradford protein assay (Bradford M.M., *Anal. Biochem.*, 1976, 72:248-254) using bovine serum albumin as a standard.

The enzymatic activity of N-acetylornithine deacetylase (ArgE) may be decreased by varying the cultivation conditions such as the acidity of the culture medium (pH), the concentration of co-factor(s) such as metal-ions, temperature, ionic strength, and so forth.

The phrase “the *argE* gene is inactivated” can mean that the modified gene encodes a completely inactive or non-functional enzyme. It is also possible that the modified DNA region is unable to naturally express the gene due to deletion of a part of or the entire gene, shifting of the reading frame of the gene, introduction of missense/nonsense mutation(s), or modification of an adjacent region of the gene, including sequences controlling gene expression such as promoter(s), enhancer(s), attenuator(s), ribosome-binding site(s), etc. Inactivation of the gene can also be performed, for example, by conventional methods such as a mutagenesis treatment using 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; Datsenko K.A. and Wanner B.L., *Proc. Natl. Acad. Sci. USA* 2000, 97(12):6640-45; Zhang Y., et al., *Nature Genet.*, 1998, 20:123-128) based on “Red/ET-driven integration” or “λRed/ET-mediated integration”.

The similar definitions may be given to the phrases “the *argR* gene is inactivated” and “the *argA* gene is inactivated”.

The phrase “activity of ArgE is abolished” is equivalent to the phrase “the ArgE protein is inactive” and can mean that the activity of ArgE is completely absent, that is equal to zero, or the activity of ArgE is below the detection level when the activity is measured using the method described in Takahara K. et al. *FEBS J.*, 2005, 272:5353-5364. Examples of a wild-type *Enterobacteriaceae* bacterium, which may serve as controls for the above comparison, include the *E. coli* MG1655 strain, and so forth. In the modified bacterium, the specific activity of ArgE encoded by the *argE* gene can be abolished to such a level that the residual specific activity of ArgE is zero or not more than 0.01 nmol/mg min, 0.05 nmol/mg min, 0.1 nmol/mg min, or about 0.5 nmol/mg min as resulted from the spread of experimental data and the detection level, when measuring activity as described above.

As described above, the activity of the ArgE protein (N-acetylornithine deacetylase) can be decreased and/or abolished. Therefore, the phrase “activity of the ArgE is downregulated” can be understood as the phrase(s)

“activity of the ArgE is decreased” and/or “activity of ArgE is abolished” as specified above.

The phrase “enhanced expression of the *argA* gene” can mean that the total enzymatic activity of the corresponding gene protein product, ArgA, is increased by, for example, introducing and/or increasing the copy number of the *argA* gene in bacterial genome, or enhancing the activity per molecule (may be referred to as a specific activity) of the proteins encoded by said gene, as compared with a non-modified strain such as a wild-type or a parent strain. The bacterium can be modified so that the activity of the ArgA protein per cell is increased to 150% or more, 200% or more, 300% or more, of the activity of a non-modified strain. Examples of a non-modified strain serving as a reference for the above comparison include a wild-type strain of a microorganism belonging to the family *Enterobacteriaceae* such as the *E. coli* MG1655 strain (ATCC 47076), W3110 strain (ATCC 27325), *Pantoea ananatis* AJ13335 strain (FERM BP-6614), and so forth.

The phrase “enhanced expression of the *argA* gene can also mean that the expression level of the *argA* gene is higher than that level in a non-modified strain, for example, a wild-type or parent strain.

Methods which can be used to enhance expression of the *argA* gene include, but are not limited to, increasing the *argA* gene copy number in bacterial genome (in the chromosome and/or in the autonomously replicated plasmid) and/or introducing the *argA* gene into a vector that is able to increase the copy number and/or the expression level of the *argA* gene in a bacterium of the family *Enterobacteriaceae* according to genetic engineering methods known to the one skilled in the art.

Examples of the vectors include, but are not limited to, broad-host-range plasmids such as pCM110, pRK310, pVK101, pBBR122, pBHR1, and the like. Multiple copies of the *argA* gene can also be introduced into the chromosomal DNA of a bacterium by, for example, homologous recombination, Mu-driven integration, or the like. Homologous recombination can be carried out using a sequence multiple copies in the chromosomal DNA. Sequences with multiple copies in the chromosomal DNA include, but are not limited to, repetitive DNA or inverted repeats present at the end of a

transposable element. In addition, it is possible to incorporate the *argA* gene into a transposon and allow it to be transferred to introduce multiple copies of the *argA* gene into the chromosomal DNA. By using Mu-driven integration, more than 3 copies of the gene can be introduced into the chromosomal DNA during a single act (Akhverdyan V.Z. et al., *Biotechnol. (Russian)*, 2007, 3:3-20).

Enhancing of the *argA* gene expression can also be achieved by increasing the expression level of the *argA* gene by modification of adjacent regulatory regions of the *argA* gene or introducing native and/or modified foreign regulatory regions. Regulatory regions or sequences can be exemplified by promoters, enhancers, attenuators and termination signals, anti-termination signals, ribosome-binding sites (RBS) and other expression control elements (e.g., regions to which repressors or inducers bind and/or binding sites for transcriptional and translational regulatory proteins, for example, in the transcribed mRNA). Such regulatory regions are described, for example, in Sambrook J., Fritsch E.F. and Maniatis T., "Molecular Cloning: A Laboratory Manual", 2nd ed., Cold Spring Harbor Laboratory Press (1989). Modifications of regions controlling gene(s) expression can be combined with increasing the copy number of the modified gene(s) in bacterial genome using the known methods (see, for example, Akhverdyan V.Z. et al., *Appl. Microbiol. Biotechnol.*, 2011, 91:857-871; Tyo K.E.J. et al., *Nature Biotechnol.*, 2009, 27:760-765).

The exemplary promoters enhancing the *argA* gene expression can be the potent promoters. For example, the *lac* promoter, the *trp* promoter, the *trc* promoter, the *tac* promoter, the P_R or the P_L promoters of lambda phage are all known to be potent promoters. Potent promoters providing a high level of gene expression in a bacterium belonging to the family *Enterobacteriaceae* can be used. Alternatively, the effect of a promoter can be enhanced by, for example, introducing a mutation into the promoter region of the *argA* gene to obtain a stronger promoter function, thus resulting in the increased transcription level of the *argA* gene located downstream of the promoter. Furthermore, it is known that substitution of several nucleotides in the SD sequence, and/or in the spacer between the SD sequence and the start

codon, and/or a sequence immediately upstream and/or downstream from the start codon in the ribosome-binding site (RBS) greatly affects the translation efficiency of mRNA. For example, a 20-fold range in the expression levels was found, depending on the nature of the three nucleotides preceding the start codon (Gold L. et al., *Annu. Rev. Microbiol.*, 1981: 35, 365-403; Hui A. et al., *EMBO J.*, 1984: 3, 623-629).

The expression level of the heterologous *argJ* gene can be enhanced using the same approaches as described above for the *argA* gene.

The copy number, presence or absence of the gene and/or operon genes can be measured, for example, by restricting the chromosomal DNA followed by Southern blotting using a probe based on the gene sequence, fluorescence *in situ* hybridization (FISH), and the like. The level of gene expression can be determined by measuring the amount of mRNA transcribed from the gene using various well-known methods, including Northern blotting, quantitative RT-PCR, and the like. The amount of the protein coded by the gene can be measured by known methods including SDS-PAGE followed by immunoblotting assay (Western blotting analysis), or mass spectrometry analysis of the protein samples, and the like.

Methods for manipulation with recombinant molecules of DNA, molecular cloning and heterologous gene expression such as preparation of plasmid DNA, digestion, ligation and transformation of DNA, selection of an oligonucleotide as a primer, and the like may be ordinary methods well-known to the person skilled in the art. These methods are described, for example, in Sambrook J., Fritsch E.F. and Maniatis T., "Molecular Cloning: A Laboratory Manual", 2nd ed., Cold Spring Harbor Laboratory Press (1989) or Green M.R. and Sambrook J.R., "Molecular Cloning: A Laboratory Manual", 4th ed., Cold Spring Harbor Laboratory Press (2012); Bernard R. Glick, Jack J. Pasternak and Cheryl L. Patten, "Molecular Biotechnology: principles and applications of recombinant DNA", 4th ed., Washington, D.C: ASM Press (2009); Evans Jr., T.C. and Xu M.-Q., "Heterologous gene expression in *E. coli*", 1st ed., Humana Press (2011).

The *argJ* gene may encode the monofunctional ornithine acetyltransferase protein ArgJ (KEGG, Kyoto Encyclopedia of Genes and

Genomes, entry No. MJ_0186; UniProtKB/Swiss-Prot, Protein Knowledgebase, entry No. Q57645). The *argJ* gene (GenBank accession No. NC_000909.1; nucleotide positions: 184215 to 185423, complement; Gene ID: 1451033) may be located between the *MJ_0187* gene on the same strand and the *MJ_0185* gene on the opposite strand on the chromosome of *M. jannaschii* DSM 2661. The nucleotide sequence of the *argJ* gene and the amino acid sequence of the monofunctional ArgJ protein encoded by the *argJ* gene may be shown in SEQ ID NO: 29 and SEQ ID NO: 30, respectively.

The *argJ* gene encodes the bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein ArgJ (KEGG, entry No. CTN_1181; UniProtKB/Swiss-Prot, entry No. Q9Z4S1). The *argJ* gene (GenBank accession No. NC_011978.1; nucleotide positions: 1146678 to 1147871, complement; Gene ID: 7377498) is located between the *argC* and *CTN_1180* genes on the chromosome of *T. neapolitana* DSM 4359. The nucleotide sequence of the *argJ* gene and the amino acid sequence of the bifunctional ArgJ protein encoded by the *argJ* gene are shown in SEQ ID NO: 1 and SEQ ID NO: 2, respectively.

The *argE* gene encodes the N-acetylornithine deacetylase protein ArgE (KEGG, entry No. b3957). The *argE* gene (GenBank accession No. NC_000913.2; nucleotide positions: 4151719 to 4152870, complement; Gene ID: 948456) is located between the *ppc* gene on the same strand and the *argC* gene on the opposite strand on the chromosome of *E. coli* strain K-12. The nucleotide sequence of the *argE* gene and the amino acid sequence of the ArgE protein encoded by the *argE* gene are shown in SEQ ID NO: 3 and SEQ ID NO: 4, respectively.

The *argA* gene encodes the amino acid N-acetyltransferase protein ArgA (KEGG, entry No. b2818). The *argA* gene (GenBank accession No. NC_000913.2; nucleotide positions: 2947264 to 2948595; Gene ID: 947289) is located between the *amiC* and *recD* genes, both on the opposite strand, on the chromosome of *E. coli* strain K-12. The nucleotide sequence of the *argA* gene and the amino acid sequence of the ArgA protein encoded by the *argA* gene are shown in SEQ ID NO: 5 and SEQ ID NO: 6, respectively.

The *argR* gene encodes the DNA-binding transcriptional dual regulator, L-arginine-binding, ArgR (KEGG, entry No. b3237). The *argR* gene (GenBank accession No. NC_000913.2; nucleotide positions: 3382725 to 3383195; Gene ID: 947861) is located between the *yhcN* gene on the same strand and the *mdh* gene on the opposite strand on the chromosome of *E. coli* strain K-12. The nucleotide sequence of the *argR* gene and the amino acid sequence of the ArgR protein encoded by the *argR* gene are shown in SEQ ID NO: 7 and SEQ ID NO: 8, respectively.

Since there may be some differences in DNA sequences between the genera or strains of the family *Methanocaldococcaceae*, *Thermotogaceae* and *Enterobacteriaceae*, the *argJ*, *argE*, *argA* and *argR* genes are not limited to the genes shown in SEQ ID NOs: 1, 3, 5, 7 and 29, but may include genes which are variant nucleotide sequences of or homologous to SEQ ID NOs: 1, 3, 5, 7 and 29, and which encode variants of the ArgJ, ArgE, ArgA and ArgR proteins.

The phrase "a variant protein" can mean a protein which has one or several changes in the sequence compared with SEQ ID NOs: 2, 4, 6, 8 and 30, whether they are substitutions, deletions, insertions, and/or additions of one or several amino acid residues, but still maintain an activity similar to that of the ArgJ, ArgE, ArgA and ArgR proteins, respectively, or the three-dimensional structure of the ArgJ, ArgE, ArgA and ArgR proteins is not significantly changed relative to the wild-type or non-modified proteins. The number of changes in the variant protein depends on the position or the type of amino acid residues in the three dimensional structure of the protein. It can be, but is not strictly limited to, 1 to 30, in another example 1 to 15, in another example 1 to 10, and in another example 1 to 5, in SEQ ID NOs: 2, 4, 6, 8 and 30.

The exemplary substitution, deletion, insertion, and/or addition of one or several amino acid residues can be a conservative mutation(s) so that the activity and features of the variant protein are maintained, and are similar to those of the ArgJ, ArgE, ArgA and ArgR proteins. The representative conservative mutation is a conservative substitution. The conservative substitution can be, but is not limited to, a substitution, wherein

substitution takes place mutually among Phe, Trp and Tyr, if the substitution site is an aromatic amino acid; among Ala, Leu, Ile and Val, if the substitution site is a hydrophobic amino acid; between Glu, Asp, Gln, Asn, Ser, His and Thr, if the substitution site is a hydrophilic amino acid; between Gln and Asn, if the substitution site is a polar amino acid; among Lys, Arg and His, if the substitution site is a basic amino acid; between Asp and Glu, if the substitution site is an acidic amino acid; and between Ser and Thr, if the substitution site is an amino acid having hydroxyl group. 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 Asn, Glu or Gln for Asp, substitution of Ser or Ala for Cys, substitution Asn, Glu, Lys, His, Asp or Arg for Gln, substitution Asn, Gln, Lys or Asp for Glu, substitution of Pro for Gly, substitution 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 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.

The exemplary substitution, deletion, insertion, and/or addition of one or several amino acid residues can also be a non-conservative mutation(s) provided that the mutation(s) is/are compensated for by one or more secondary mutations in the different position(s) of amino acids sequence so that the activity and features of the variant protein are maintained, and are similar to those of the ArgJ, ArgE, ArgA and ArgR proteins.

To evaluate the degree of protein or DNA homology, several calculation methods can be used, such as BLAST search, FASTA search and ClustalW method. The BLAST (Basic Local Alignment Search Tool, www.ncbi.nlm.nih.gov/BLAST/) search is the heuristic search algorithm employed by the programs blastp, blastn, blastx, megablast, tblastn, and tblastx; these programs ascribe significance to their findings using the statistical methods of Samuel K. and Altschul S.F. ("Methods for assessing the statistical significance of molecular sequence features by using general

scoring schemes” *Proc. Natl. Acad. Sci. USA*, 1990, 87:2264-2268; “Applications and statistics for multiple high-scoring segments in molecular sequences”. *Proc. Natl. Acad. Sci. USA*, 1993, 90:5873-5877). The computer program BLAST calculates three parameters: score, identity and similarity. The FASTA search method is described by Pearson W.R. (“Rapid and sensitive sequence comparison with FASTP and FASTA”, *Methods Enzymol.*, 1990, 183:63-98). The ClustalW method is described by Thompson J.D. et al. (“CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice”, *Nucleic Acids Res.*, 1994, 22:4673-4680).

Moreover, the *argJ*, *argE*, *argA* and *argR* genes can be variant nucleotide sequences. The phrase “a variant nucleotide sequence” can mean a nucleotide sequence which encodes “a variant protein” using any synonymous amino acid codons according to the standard genetic code table (see, e.g., Lewin B., *Genes VIII*, 2004, Pearson Education, Inc., Upper Saddle River, NJ 07458). The phrase “a variant nucleotide sequence” can also mean, but not be limited to, a nucleotide sequence which hybridizes under stringent conditions with the nucleotide sequence complementary to the sequence shown in SEQ ID NOs: 1, 3, 5, 7 and 29, or a probe which can be prepared from the nucleotide sequence under stringent conditions provided that it encodes functional protein. “Stringent conditions” include those under which a specific hybrid, for example, a hybrid having homology of not less than 70%, not less than 80%, not less than 90%, not less than 95%, not less than 96%, not less than 97%, not less than 98%, or not less than 99% is formed, and a non-specific hybrid, for example, a hybrid having homology lower than the above is not formed. For example, stringent conditions can be exemplified by washing one time or more, or in another example, two or three times, at a salt concentration of 1×SSC (standard sodium citrate or standard sodium chloride), 0.1% SDS (sodium dodecyl sulphate), or in another example, 0.1×SSC, 0.1% SDS at 60°C or 65°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 Amersham Hybond™-N+ positively charged nylon

membrane (GE Healthcare) under stringent conditions is 15 minutes. The washing step can be performed 2 to 3 times. As the probe, a part of the sequence complementary to the sequences shown in SEQ ID NOs: 1, 3, 5, 7 and 29 may also be used. Such a probe can be produced by PCR using oligonucleotides as primers prepared on the basis of the sequences shown in SEQ ID NOs: 1, 3, 5, 7 and 29, and a DNA fragment containing the nucleotide sequence as a template. The length of the probe is recommended to be >50 bp; it can be suitably selected depending on the hybridization conditions, and is usually 100 bp to 1 kbp. For example, when a DNA fragment having a length of about 300 bp is used as the probe, the washing conditions after hybridization can be exemplified by 2×SSC, 0.1% SDS at 50°C, 60°C or 65°C.

As the genes encoding the ArgJ proteins of the species *M. jannaschii* and *T. neapolitana*, and the ArgE, ArgA and ArgR proteins of the species *E. coli* have already been elucidated (see above), the variant nucleotide sequences encoding variant proteins of the ArgJ, ArgE, ArgA and ArgR proteins can be obtained by PCR (polymerase chain reaction; refer to White T.J. et al., *Trends Genet.*, 1989, 5:185-189) utilizing primers prepared based on the nucleotide sequence of the *argJ*, *argE*, *argA* and *argR* genes, or chemically synthesized as full-length gene structure. Genes encoding the ArgJ, ArgE, ArgA and ArgR proteins or their variant proteins of other microorganisms can be obtained in a similar manner.

Activity of ornithine acetyltransferase (monofunctional ArgJ) (EC: 2.3.1.35; synonyms: glutamate N-acetyltransferase, N-acetyl-L-glutamate synthetase, etc.) means activity of catalyzing the following reaction: N2-acetyl-L-ornithine + L-glutamate $\leftrightarrow$ L-ornithine + N-acetyl-L-glutamate.

Activity of ornithine acetyltransferase/N-acetylglutamate synthase (bifunctional ArgJ) (EC: 2.3.1.35/2.3.1.1) means activity of catalyzing the following reactions: N2-acetyl-L-ornithine + L-glutamate $\leftrightarrow$ L-ornithine + N-acetyl-L-glutamate, and acetyl-CoA + L-glutamate $\leftrightarrow$ CoA + N-acetyl-L-glutamate.

Activity of N-acetylornithine deacetylase (ArgE) (EC: 3.5.1.16; synonyms: 2-N-acetyl-L-ornithine amidohydrolase, N-acetylornithinase)

means activity of catalyzing the following reaction: N2-acetyl-L-ornithine + H₂O ↔ acetate + L-ornithine.

Activity of amino acid N-acetyltransferase (ArgA) (EC: 2.3.1.1; synonyms: N-acetylglutamate synthase, N-acetyl-L-glutamate synthetase) means activity of catalyzing the following reaction: acetyl-CoA + L-glutamate ↔ CoA + N-acetyl-L-glutamate.

The phrase “operably linked to a gene” can mean that the regulatory region(s) is linked to the nucleotide sequence of the nucleic acid molecule or gene of interest in a manner which allows for expression (e.g., enhanced, increased, constitutive, basal, antiterminated, attenuated, decreased or repressed expression) of the nucleotide sequence, preferably expression of a gene product encoded by the nucleotide sequence.

The phrase “an enzyme having at least an ornithine acetyltransferase activity” can mean that the enzyme has the activity of ornithine acetyltransferase (monofunctional ArgJ) or activity of ornithine acetyltransferase/N-acetylglutamate synthase (bifunctional ArgJ) as described above.

The bacterium as described herein can be obtained by introduction of the aforementioned DNAs into a bacterium inherently having an ability to produce an L-amino acid. Alternatively, the bacterium as described herein can be obtained by imparting an ability to produce an L-amino acid to a bacterium already harboring the aforementioned DNAs.

The bacterium can have, in addition to the properties already mentioned, other specific properties such as various nutrient requirements, drug resistance, drug sensitivity, and drug dependence, without departing from the scope of the present invention.

2. Method for producing L-amino acid

The method for producing L-amino acid can include the steps of cultivating the bacterium in a culture medium to allow the L-amino acid to be produced, excreted, and accumulated in the culture medium, and collecting the L-amino acid from the culture medium and/or the bacterial cells.

The cultivation of the bacterium, and collection and purification of an L-amino acid or a salt thereof from the medium and the like may be performed in a manner similar to conventional fermentation methods wherein an L-amino acid is produced using a microorganism. The culture medium for an L-amino acid production may be a typical medium that contains a carbon source, a nitrogen source, inorganic ions, and other organic components as required. As the carbon source, saccharides such as glucose, lactose, galactose, fructose, arabinose, maltose, xylose, trehalose, ribose, and hydrolyzates of starches; alcohols such as glycerol, mannitol, and sorbitol; organic acids such as gluconic acid, fumaric acid, citric acid, malic acid, and succinic acid; and the like can be used. As the nitrogen source, inorganic ammonium salts such as ammonium sulfate, ammonium chloride, and ammonium phosphate; organic nitrogen such as of soy bean hydrolyzates; ammonia gas; aqueous ammonia; and the like can be used. Vitamins such as vitamin B₁, required substances, for example, organic nutrients such as nucleic acids such as adenine and RNA, or yeast extract, and the like may be present in appropriate, even if trace, amounts. Other than these, small amounts of calcium phosphate, magnesium sulfate, iron ions, manganese ions, and the like may be added, if necessary.

Cultivation can be performed under aerobic conditions for 16 to 96 hours, or for 48 to 72 hours; the culture temperature during cultivation is controlled within 30 to 45°C, or within 30 to 37°C; and the pH is adjusted between 5 and 8, or between 6.5 and 7.2. The pH can be adjusted by using an inorganic or organic acidic or alkaline substance, as well as ammonia gas. Usually, a 1 to 5-day cultivation leads to the accumulation of the target L-amino acid in the liquid medium.

After cultivation, solids such as cells and cell debris can be removed from the liquid medium by centrifugation or membrane filtration, and then the target L-amino acid or a salt thereof can be recovered from the fermentation liquor by any combination of conventional techniques such as concentration, ion-exchange chromatography, and crystallization.

Examples

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

Example 1.

Construction of the mutant *E. coli* MG1655 strain having deleted the *argE* gene and harboring the pJ-T plasmid carrying the *argJ* gene from *T. neapolitana*

Firstly, the mutant *E. coli* MG1655 strain having deleted the *argR* gene was constructed using known gene-knockout method (Datsenko K.A. and Wanner B.L., *Proc. Natl. Acad. Sci. USA*, 2000, 97(12):6640-6645) (Example 1.1). Secondly, the *argE* gene was deleted using the same method (Example 1.2). The chloramphenicol-resistance gene (*cat*, Cm^R-marker) was used to mark the $\Delta argR$ mutation, and the kanamycin-resistance gene (*kan*, Km^R-marker) was used to mark the $\Delta argE$ mutation. Cells were cured from the Cm^R-marker as described (Datsenko K.A. and Wanner B.L., *Proc. Natl. Acad. Sci. USA*, 2000, 97(12):6640-6645). The pJ-T plasmid containing the *argJ* gene from *T. neapolitana* (U.S. Patent No. 6,897,048) was transferred to the obtained mutant *E. coli* MG1655 $\Delta argR$ and $\Delta argR\Delta argE::Km$ strains by electroporation. The electroporation was performed by "Bio-Rad" electroporator (U.S.A.) (No. 165-2098, version 2-89) according to the manufacturer's instructions. Thus the mutant *E. coli* MG1655 $\Delta argR$ /pJ-T and $\Delta argR\Delta argE::Km$ /pJ-T strains were obtained.

Example 1.1.

Deletion of the *argR* gene

The *E. coli* MG1655 $\Delta argR$ strain was constructed by the λ Red-mediated integration method initially developed by Datsenko K.A. and Wanner B.L. (*Proc. Natl. Acad. Sci. USA*, 2000, 97(12):6640-6645). The DNA fragment containing the chloramphenicol resistance marker (Cm^R) was obtained by PCR using primers P1 (SEQ ID NO: 9) and P2 (SEQ ID NO: 10), and the pMW118-*attL*-Cm-*attR* plasmid as the template (WO2005010175 A1). Primer

P1 contains a region complementary to the region located at the 5'-end of the *argR* gene and a region complementary to the *attR* region. Primer P2 contains a region complementary to the region located at the 3'-end of the *argR* gene and a region complementary to the *attL* region. The conditions for PCR were as follows: initial denaturation for 3 min at 95°C; profile for the initial 2 cycles: 1 min at 95°C, 30 sec at 50°C, 40 sec at 72°C; profile for the final 25 cycles: 30 sec at 95°C, 30 sec at 54°C, 40 sec at 72°C; final elongation for 5 min at 72°C. The obtained PCR product (about 1.6 kbp) was purified by electrophoresis in agarose gel and used for electroporation of the *E. coli* MG1655 strain containing the pKD46 plasmid having a temperature-sensitive replication origin. The pKD46 plasmid (Datsenko K.A. and Wanner B.L., *Proc. Natl. Acad. Sci. USA*, 2000, 97(12):6640-6645) includes a 2,154 nucleotide DNA fragment of phage λ (nucleotide positions from 31088 to 33241, GenBank accession No.: J02459), and contains genes of the λ Red homologous recombination system (γ , β , *exo* genes) 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 *E. coli* MG1655 strain (ATCC 47076). The *E. coli* MG1655 strain containing the recombinant plasmid pKD46 can be obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, USA (Accession No. CGSC7669).

Electrocompetent cells were prepared as follows: the *E. coli* MG1655/pKD46 strain was grown at 30°C overnight in LB medium (Luria-Bertani broth, also referred to as lysogenic broth; Sambrook J. and Russell D.W., *Molecular Cloning: A Laboratory Manual* (3rd ed.), Cold Spring Harbor Laboratory Press, 2001) containing ampicillin (150 mg/L), then the culture was diluted 100 times with 5 mL of SOB medium (Sambrook J. and Russell D.W., *Molecular Cloning: A Laboratory Manual* (3rd ed.), Cold Spring Harbor Laboratory Press, 2001) containing ampicillin (150 mg/L) and L-arabinose (1 mM). The cells were grown with aeration (250 rpm) at 30°C to OD₆₀₀ of ≈ 0.6 . Electrocompetent cells were made by concentrating 100-fold and washing three times with ice-cold deionized H₂O. Electroporation was performed using 70 μ L of cells and ≈ 100 ng of the PCR product. Cells after electroporation

were incubated with 1 mL of SOC medium (Sambrook J. and Russell D.W., Molecular Cloning: A Laboratory Manual (3rd ed.), Cold Spring Harbor Laboratory Press, 2001) at 37°C for 2.5 hours, plated onto L-agar containing chloramphenicol (25 mg/L) and grown at 37°C to select Cm^R-recombinants. To eliminate the pKD46 plasmid, two passages on L-agar containing chloramphenicol (25 mg/L) at 42°C were performed, and the obtained colonies were tested for sensitivity to ampicillin. The Cm^R-marker was eliminated using the pMW-Int/Xis helper plasmid (WO2005010175 A1) which was electroporated into the selected plasmid-less integrants using the procedure as described above for electroporation of the PCR-generated fragment. After electroporation, the cells were plated on L-agar containing 0.5% glucose and ampicillin (150 mg/L) and incubated at 37°C overnight to induce synthesis of the Int/Xis proteins. The grown clones were replica-plated on L-agar with and without chloramphenicol to select the Cm^S (chloramphenicol sensitive) variants. Thus the *E. coli* MG1655Δ*argR* strain was obtained.

Example 1.2.

Deletion of the *argE* gene

The *E. coli* MG1655Δ*argR* strain having deleted the *argE* gene was constructed by the λRed-mediated integration method initially developed by Datsenko K.A. and Wanner B.L. (*Proc. Natl. Acad. Sci. USA*, 2000, 97(12):6640-6645). The DNA fragment containing the Km^R-marker was obtained by PCR using primers P3 (SEQ ID NO: 11) and P4 (SEQ ID NO: 12), and the pMW118-*attL*-Km-*attR* plasmid as the template (Katashkina Zh.I. et al., *Mol. Biol.* (Mosk.), 2005, 39(5):823-831). The *E. coli* MG1655Δ*argR*/pKD46 strain was used for electroporation by the obtained DNA fragment (Example 1.1). The antibiotic resistant cells were selected using kanamycin (25 mg/L), and obtained Km^R-recombinants were cured from the pKD46 plasmid as described in Example 1.1. Thus the *E. coli* MG1655Δ*argR*Δ*argE*::Km was obtained.

Example 2.

Production of L-arginine by the modified *E. coli* MG1655 strain having deleted *argE* gene and harboring the pJ-T plasmid carrying the *argJ* gene from *T. neapolitana*

Eight independent colonies of each of the obtained strains (*E. coli* MG1655 Δ *argR*/pJ-T and Δ *argR* Δ *argE::Km*/pJ-T) were chosen for evaluation of L-arginine production. Strains obtained were grown on an L-agar plate at 37°C overnight, then a transfer loop of each of the obtained cultures was inoculated into 2 mL of fermentation medium in a 20 x 200 mm test-tube and cultivated at 32°C for 72 hours on a rotary shaker (220-230 rpm).

The fermentation medium contained (g/L):

Glucose	50
(NH ₄) ₂ SO ₄	35
K ₂ HPO ₄	2.0
MgSO ₄ ×7H ₂ O	1.0
Thiamine hydrochloride	0.002
Yeast extract	1.0 – 5.0
CaCO ₃	20

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. Antibiotic was introduced into the medium after sterilization.

The amount of L-arginine which had accumulated in the medium was determined by TLC or paper chromatography using the following mobile phase: butanol : acetic acid : water = 4 : 1 : 1 (v/v). A solution (2%) of ninhydrin in acetone was used as a visualizing reagent. A spot containing L-arginine was cut off, L-arginine was eluted in 0.5 % water solution of CdCl₂ and the amount of L-arginine was estimated spectrophotometrically at 540 nm. The results of 8 independent test-tube fermentations (as average values) are shown in Table 1. As it can be seen from the Table 1, the modified *E. coli* MG1655 Δ *argR*/pJ-T strain caused the higher amount of accumulation of L-

arginine as compared with the parent *E. coli* MG1655 Δ *argR* Δ *argE*::Km/pJ-T strain.

Table 1.

Effect of the Δ *argE* mutation on L-arginine production by the *E. coli* strain harboring the pJ-T plasmid carrying the *argJ* gene from *T. neapolitana*.

Strain	OD ₅₄₀	L-Arginine, g/L
MG1655 Δ <i>argR</i> /pJ-T	14.6 $\pm$ 0.7	1.8 $\pm$ 0.1
MG1655 Δ <i>argR</i> Δ <i>argE</i> ::Km/pJ-T	16.9 $\pm$ 0.6	2.8 $\pm$ 0.3

Example 3.

Construction of the mutant *E. coli* MG1655 strain having deleted the *argE* gene and the *argJ* gene from *T. neapolitana* inserted into the chromosome

The *argJ* gene from *T. neapolitana* was incorporated into the chromosome of the *E. coli* MG1655 Δ *argR* strain to avoid the effect of instability of the pJ-T plasmid carrying the *argJ* gene. To enhance the expression of the *argJ* gene, the effective artificial promoter P_{nlp8 ϕ 10} was constructed and placed before the *argJ* gene (Example 3.1).

To avoid the effect of the natural arginine biosynthesis pathway on L-arginine production, the *argA* gene was deleted from the chromosome (Example 3.1).

The *E. coli* MG1655 Δ *argA* Δ *argR* Δ *artP*-J::P_{nlp8 ϕ 10}*argJ* strain was used for further genetic manipulation. The Δ *argE* mutation was marked by the kanamycin resistance gene (*kan*) (Example 1.2) and transferred into the chromosome of the *E. coli* MG1655 Δ *argA* Δ *argR* Δ *artP*-J::P_{nlp8 ϕ 10}*argJ* strain using the well-known method of general P1-transduction (Miller J.H., Experiments in molecular genetics, Cold Spring Harbor Laboratory Press, 1972). The colonies of Km-resistant transductants were selected on L-agar

containing kanamycin (25 mg/L) (Example 1). Thus the *E. coli* MG1655 Δ *argA* Δ *argR* Δ *artP-J::P_{nlp8 ϕ 10}argJ* Δ *argE::Km* strain was obtained.

Example 3.1.

Construction of the *E. coli* MG1655 Δ *argA* Δ *argR* Δ *artP-J::P_{nlp8 ϕ 10}argJ* strain

The *E. coli* MG1655 having deleted the *argA* gene marked with *cat*-gene was constructed by the λ Red-mediated integration method initially developed by Datsenko K.A. and Wanner B.L. (*Proc. Natl. Acad. Sci. USA*, 2000, 97(12):6640-6645) (Example 1.1). The DNA fragment containing the Cm^R-marker was obtained by PCR using primers P5 (SEQ ID NO: 13) and P6 (SEQ ID NO: 14), and the pMW118-*attL*-Cm-*attR* plasmid as the template (WO2005010175 A1). The *E. coli* MG1655 Δ *argR*/pKD46 strain was used for electroporation by the obtained DNA fragment. The chloramphenicol resistance marker (Cm^R) was eliminated using the pMW-Int/Xis helper plasmid (WO2005010175 A1) as described in Example 1.1. Thus the *E. coli* MG1655 Δ *argA* Δ *argR* was obtained.

The DNA fragment containing a promoter of the *nlpD* gene from *E. coli* was obtained by PCR using primers P7 (SEQ ID NO: 15) and P8 (SEQ ID NO: 16), chromosomal DNA of *E. coli* MG1655 as the template. The conditions for PCR were as follows: initial denaturation for 3 min at 95°C; profile for the initial 2 cycles: 1 min at 95°C, 30 sec at 50°C, 40 sec at 72°C; profile for the final 25 cycles: 20 sec at 94°C, 20 sec at 55°C, 15 sec at 72°C; final elongation for 5 min at 72°C. The amplified DNA fragment (about 0.2 kbp) was purified by electrophoresis in agarose gel and treated with endonucleases *PaeI* and *SaII* (Fermentas) (Sambrook J., Fritsch E.F. and Maniatis T., "Molecular Cloning: A Laboratory Manual", 2nd ed., Cold Spring Harbor Laboratory Press (1989); instructions of manufacturer). The obtained DNA fragment was ligated with the pMIV-5JS plasmid (Russian patent application No. 2006132818 and EP1942183), which had been previously treated with endonucleases *PaeI* and *SaII*, using the manufacturer's instructions. The ligation mixture was incubated at 4°C overnight and then was used to transform the *E. coli* MG1655 strain (ATCC 47076) by

electroporation as described in Example 1. The resulting transformants were plated on L-agar containing ampicillin (50 mg/L), and the plates were incubated at 37°C overnight until individual colonies were visible. Plasmids were isolated from the obtained transformants and analyzed by the restriction analysis using *PaeI* and *SaII* nucleases. Thus the pMIV-PnlpD plasmid containing the native P_{nlpD} promoter of the *nlpD* gene from *E. coli* was obtained.

The randomization of the -10 region of the P_{nlpD} promoter and the selection of the P_{nlp8} promoter were performed. The 3'-end of P_{nlpD} was obtained by PCR amplification using primers P7 (SEQ ID NO: 15) and P9 (SEQ ID NO: 17), and the pMIV-PnlpD plasmid as the template. Primer P9 has random nucleotides, which are depicted in SEQ ID NO: 17 as "n", where n is A, G, C, or T. The conditions for PCR were as follows: initial denaturation for 3 min at 95°C; profile for the initial 2 cycles: 1 min at 95°C, 30 sec at 50°C, 40 sec at 72°C; profile for the final 25 cycles: 20 sec at 94°C, 20 sec at 60°C, 15 sec at 72°C; final elongation for 5 min at 72°C. The 5'-end of P_{nlpD} was obtained by PCR amplification using primers P8 (SEQ ID NO: 16) and P10 (SEQ ID NO: 18), and the pMIV-PnlpD plasmid as the template. Primer P10 has random nucleotides, which are depicted in SEQ ID NO: 18 as "n", where n is A, G, C, or T. The conditions for PCR were as follows: initial denaturation for 3 min at 95°C; profile for the initial 2 cycles: 1 min at 95°C, 30 sec at 50°C, 40 sec at 72°C; profile for the final 25 cycles: 20 sec at 94°C, 20 sec at 60°C, 15 sec at 72°C; final elongation for 5 min at 72°C.

Both amplified DNA fragments were purified by electrophoresis in agarose gel, treated with endonuclease *BglII* (Fermentas) and ligated in the equimolar ratio (Sambrook J., Fritsch E.F. and Maniatis T., "Molecular Cloning: A Laboratory Manual", 2nd ed., Cold Spring Harbor Laboratory Press, 1989). The ligation mixture was incubated at 4°C overnight. The obtained DNA fragment was PCR amplified using primers P7 (SEQ ID NO: 15) and P8 (SEQ ID NO: 16), and the obtained DNA fragment as the template. The conditions for PCR were as follows: initial denaturation for 3 min at 95°C; profile for the initial 2 cycles: 1 min at 95°C, 30 sec at 50°C, 40 sec at 72°C;

profile for the final 12 cycles: 20 sec at 94°C, 20 sec at 60°C, 15 sec at 72°C; final elongation for 5 min at 72°C. The amplified DNA fragment (about 0.2 kbp) was purified by electrophoresis in agarose gel.

The purified DNA fragment was treated with Klenow fragment (Fermentas) and ligated in equimolar ratio with the pMW118- $\lambda attL$ -Km^R- $\lambda attR$ plasmid (Russian patent application No. 2006134574), which had been previously treated with endonuclease *Xba*I (Fermentas) followed by treatment with Klenow fragment. The ligation mixture was incubated at 4°C overnight and then was used to transform the *E. coli* MG1655 by electroporation as described in Example 1. The resulting transformants were plated on L-agar containing kanamycin (20 mg/L), and the plates were incubated at 37°C overnight until individual colonies were visible. Plasmids were isolated from the obtained transformants and analyzed by the restriction analysis using *Pst*I and *Hind*III nucleases (Fermentas) (Sambrook J., Fritsch E.F. and Maniatis T., "Molecular Cloning: A Laboratory Manual", 2nd ed., Cold Spring Harbor Laboratory Press (1989); instructions of manufacture). Thus the pMW-Km-Pnlp8 plasmid containing the P_{nlp8} promoter was obtained.

The DNA fragment containing the *argJ* gene was obtained by PCR using primers P11 (SEQ ID NO: 19) and P12 (SEQ ID NO: 20), and the plasmid DNA pJ-T (U.S. Patent No. 6,897,048) as the template. The conditions for PCR were as follows: initial denaturation for 1 min at 95°C; profile for 25 cycles: 1 min at 95°C, 30 sec at 55°C, 1 min at 72°C; final elongation for 2 min at 72°C. The amplified DNA fragment (about 1.2 kbp) was purified by electrophoresis in agarose gel.

The DNA fragment containing the $\lambda attL$ -Km- $\lambda attR$ -P_{nlp8} ϕ ₁₀ cassette was obtained by PCR using primers P13 (SEQ ID NO: 21) and P14 (SEQ ID NO: 22), and the plasmid pMW-Km-Pnlp8 as the template. The conditions for PCR were as follows: initial denaturation for 1 min at 95°C; profile for 25 cycles: 1 min at 95°C, 30 sec at 55°C, 1min 40 sec at 72°C; final elongation for 5 min at 72°C. The amplified DNA fragment (about 1.7 kbp) (Figure 1) was purified by electrophoresis in agarose gel. Primers P11 (SEQ ID NO: 19) and P14 (SEQ ID NO: 22) contain overlapping regions.

The obtained DNA fragments (Figure 1) with overlapping regions were PCR amplified using primers P12 (SEQ ID NO: 20) and P13 (SEQ ID NO: 21), and the obtained DNA fragments as the templates. The conditions for PCR were as follows: initial denaturation for 2 min at 95°C; profile for 30 cycles: 30 sec at 94°C, 30 sec at 55°C, 2 min at 72°C; final elongation for 5 min at 72°C. The amplified DNA fragment (about 2.9 kbp) was purified by electrophoresis in agarose gel.

The obtained DNA fragment contains the Km^R-marker and the *argJ* gene under control of the P_{nlp8φ10} promoter. This fragment was integrated in place of the *artPIQMJ* gene cluster into the chromosome of the *E. coli* MG1655Δ*argA*Δ*argR* strain by the λRed-mediated integration method as described above. Thus the *E. coli* MG1655Δ*argA*Δ*argR*Δ*artP-J::P_{nlp8φ10}argJ* was obtained.

Example 4.

Production of L-arginine by the modified *E. coli* MG1655 strain having deleted the *argE* gene and the *argJ* gene from *T. neapolitana* inserted into the chromosome

Six independent colonies of each of the obtained strains (*E. coli* MG1655Δ*argA*Δ*argR*Δ*artP-J::P_{nlp8φ10}argJ* and MG1655Δ*argA*Δ*argR*Δ*artP-J::P_{nlp8φ10}argJ*Δ*argE::Km*) were chosen for evaluation of L-arginine production. The L-arginine production was evaluated as described in Example 2. The results of 6 independent test-tube fermentations (as average values) are shown in Table 2. As it can be seen from the Table 2, the modified *E. coli* MG1655Δ*argA*Δ*argR*Δ*artP-J::P_{nlp8φ10}argJ*Δ*argE::Km* strain caused the higher amount of accumulation of L-arginine as compared with the parent *E. coli* MG1655Δ*argA*Δ*argR*Δ*artP-J::P_{nlp8φ10}argJ* strain.

Table 2.

Effect of the $\Delta argE$ mutation on L-arginine production by the *E. coli* strain having the *argJ* gene inserted into the chromosome.

Strain	OD ₅₄₀	L-Arginine, g/L
MG1655 $\Delta argA\Delta argR\Delta artP$ -J::P _{nlp8ϕ10} <i>argJ</i>	24.7 $\pm$ 0.5	0.9 $\pm$ 0.1
MG1655 $\Delta argA\Delta argR\Delta artP$ -J::P _{nlp8ϕ10} <i>argJ</i> $\Delta argE$::Km	23.0 $\pm$ 1.3	3.0 $\pm$ 0.6

Example 5.

Construction of the mutant *E. coli* MG1655 strain having attenuated the *argE* gene and the *argJ* gene from *T. neapolitana* inserted into the chromosome

The *E. coli* MG1655 $\Delta argA\Delta argR\Delta artP$ -J::P_{nlp8 ϕ 10}*argJ* strain was used as parent strain to construct the strain having the *argE* gene with attenuated expression. Firstly, the Cm^R-marker was introduced into intergenic region between *ppc* and *argE* genes of MG1655 strain by the λ Red-mediated integration method (Example 1.1). The DNA fragment containing the Cm^R-marker encoded by the *cat* gene was obtained by PCR using primers P15 (SEQ ID NO: 23) and P16 (SEQ ID NO: 24), and the pMW118-*attL*-Cm-*attR* plasmid as the template (WO2005010175 A1). Primer P15 contains a region complementary to the region located downstream to the *argE* gene and a region complementary to the *attR* region. Primer P16 contains a region complementary to the region located upstream to the *ppc* gene and a region complementary to the *attL* region. The conditions for PCR were as follows: initial denaturation for 3 min at 95°C; profile for the initial 2 cycles: 1 min at 95°C, 30 sec at 50°C, 40 sec at 72°C; profile for the final 25 cycles: 30 sec at 95°C, 30 sec at 54°C, 40 sec at 72°C; final elongation for 5 min at 72°C. The PCR product (about 1.6 kbp) was purified by electrophoresis in agarose gel and used for electroporation of the *E. coli* MG1655/pKD46 strain as described in Example 1.1. After electrotransformation of the *E. coli*

MG1655/pKD46 strain by the obtained DNA fragment having *cat* gene, several colonies were selected on L-agar plates containing chloramphenicol (20 mg/L). Electrocompetent cells were prepared, electroporation was performed, and Cm^R-recombinants were selected as described in Example 1.1. Thus the *E. coli* MG1655-Cm-*argE* strain was obtained.

Secondly, the L76K replacement in ArgE was obtained. The first DNA fragment containing the Cm^R-marker and the distal part of *argE* encoding mutation L76K was obtained by PCR using primers P17 (SEQ ID NO: 25) and P18 (SEQ ID NO: 26) containing overlapping regions, and the chromosomal DNA of the *E. coli* MG1655-Cm-*argE* strain as the template. The conditions for PCR were as follows: initial denaturation for 1 min at 95°C; profile for 25 cycles: 1 min at 95°C, 30 sec at 55°C, 2 min at 72°C; final elongation for 5 min at 72°C. The amplified first DNA fragment (about 2.8 kbp) (Figure 2) was purified by electrophoresis in agarose gel.

The second DNA fragment containing the proximal part of *argE* encoding mutation L76K was obtained by PCR using primers P19 (SEQ ID NO: 27) and P20 (SEQ ID NO: 28), and the chromosomal DNA of the *E. coli* MG1655-Cm-*argE* strain as the template. The conditions for PCR were as follows: initial denaturation for 1 min at 95°C; profile for 25 cycles: 1 min at 95°C, 30 sec at 55°C, 30 sec at 72°C; final elongation for 5 min at 72°C. The amplified second DNA fragment (about 0.3 kbp) (Figure 2) was purified by electrophoresis in agarose gel.

The third DNA fragment was obtained by PCR using primers P17 (SEQ ID NO: 25) and P20 (SEQ ID NO: 28), and obtained the first and second DNA fragments with overlapping regions (Figure 2) as the template. The conditions for PCR were as follows: initial denaturation for 2 min at 95°C; profile for 30 cycles: 30 sec at 94°C, 30 sec at 55°C, 2 min 20 sec at 72°C; final elongation for 5 min at 72°C. The amplified third DNA fragment (about 3.1 kbp) was purified by electrophoresis in agarose gel. Thus the third DNA fragment containing the Cm^R-marker and the *argE* gene with replacements T226A and T227A in nucleotide sequence of SEQ ID NO: 3, which result in the L76K

mutation, was obtained. The mutant *argE* gene was named the *argEm24* gene.

Finally, the third DNA fragment was integrated instead of the native *argE* gene into the chromosome of the *E. coli* MG1655 Δ *argA* Δ *argR* Δ *artP-J::P_{nlp8}10argJ* strain by the λ Red-mediated integration method as described above. Thus the *E. coli* MG1655 Δ *argA* Δ *argR* Δ *artP-J::P_{nlp8}10argJargEm24::Cm* strain was obtained.

Example 6.

Production of L-arginine by the modified *E. coli* MG1655 strain having attenuated the *argE* gene and the *argJ* gene from *T. neapolitana* inserted into the chromosome

Six independent colonies of each of the obtained strains (*E. coli* MG1655 Δ *argA* Δ *argR* Δ *artP-J::P_{nlp8}10argJargEm24::Cm* and MG1655 Δ *argA* Δ *argR* Δ *artP-J::P_{nlp8}10argJ*) were chosen for evaluation of L-arginine production. The L-arginine production was evaluated as described in Example 2. The results of 6 independent test-tube fermentations (as average values) are shown in Table 3. As it can be seen from the Table 3, the modified *E. coli* MG1655 Δ *argA* Δ *argR* Δ *artP-J::P_{nlp8}10argJargEm24::Cm* strain caused the higher amount of accumulation of L-arginine as compared with the parent *E. coli* MG1655 Δ *argA* Δ *argR* Δ *artP-J::P_{nlp8}10argJ* strain.

Table 3.

Effect of the *argEm24* mutation on L-arginine production by the *E. coli* strain having the *argJ* gene inserted into the chromosome.

Strain	OD ₅₄₀	L-Arginine, g/L
MG1655 Δ <i>argA</i> Δ <i>argR</i> Δ <i>artP-J::P_{nlp8}10argJ</i>	24.7 $\pm$ 0.5	0.9 $\pm$ 0.1
MG1655 Δ <i>argA</i> Δ <i>argR</i> Δ <i>artP-J::P_{nlp8}10argJargEm24::Cm</i>	24.5 $\pm$ 3.4	9.3 $\pm$ 0.9

Example 7.

Enzymatic activity of the mutant ArgE^{L76K} protein

Enzymatic activity of the control and mutant ArgE^{L76K} proteins encoded by the mutant *argEm24* gene was measured as described in Takahara K. et al. *FEBS J.*, 2005, 272:5353-5364 using crude proteins extracts obtained after cells lysis by sonication. The *E. coli* MG1655, MG1655 Δ *argE*, and MG1655*argEm24* strains were used as protein sources to evaluate attenuation level from the L76K mutation in ArgE. The *E. coli* MG1655 Δ *argE* and MG1655*argEm24* strains were obtained as described in Auxiliary example 1. As it can be seen from Table 4, the ArgE^{L76K} protein showed specific activity of less than about 1% as compared with the non-modified ArgE protein.

Table 4.

Effect of the *argEm24* mutation on N-acetylornithine deacetylase (ArgE) activity (in nmol/mg min) in the *E. coli* MG1655 strain. The values are mean $\pm$ SD (n = 4), where SD represents standard deviation.

Strain	Specific activity of ArgE, nmol/min mg
MG1655 (control)	750
MG1655 Δ <i>argE</i>	<0.5
MG1655 <i>argEm24</i>	3.0

Auxiliary example 1.

The *E. coli* MG1655 Δ *argE*::Km was obtained by the procedure described in Example 1.2 for the *E. coli* MG1655 Δ *argR* Δ *argE*::Km strain. The Km^R-marker was eliminated using the pMW-Int/Xis helper plasmid (WO2005010175 A1) as described in Example 1.1 for Cm^R-marker. Thus the *E. coli* MG1655 Δ *argE* strain was obtained.

The *E. coli* MG1655*argEm24*::Cm strain was obtained as described in

Example 5 for the *E. coli* MG1655 Δ *argA* Δ *argR* Δ *artP-J::P_{nlp8 ϕ 10}argJ argEm24::Cm* strain. The Cm^R-marker was eliminated using the pMW-Int/Xis helper plasmid (WO2005010175 A1) as described in Example 1.1. Thus the *E. coli* MG1655 Δ *argEm24* strain was obtained.

Explanation of Sequence Listing

- 1: nucleotide sequence of *Thermotoga neapolitana argJ* gene
- 2: amino acid sequece of *Thermotoga neapolitana* ArgJ protein
- 3: nucleotide sequence of *Escherichia coli argE* gene
- 4: amino acid sequece of *Escherichia coli* ArgE protein
- 5: nucleotide sequence of *Escherichia coli argA* gene
- 6: amino acid sequece of *Escherichia coli* ArgA protein
- 7: nucleotide sequence of *Escherichia coli argR* gene
- 8: amino acid sequece of *Escherichia coli* ArgR protein
- 9-28: primers P1 to P20, respectively
- 29: nucleotide sequence of *Methanocaldococcus jannaschii argJ* gene
- 30: amino acid sequece of *Methanocaldococcus jannaschii* ArgJ protein

CLAIMS

1. An L-arginine-producing bacterium of the family *Enterobacteriaceae* having a recombinant DNA comprising the *argJ* gene encoding an enzyme having at least an ornithine acetyltransferase activity, wherein said bacterium has been modified to contain the downregulated N-acetylornithine deacetylase activity.
2. The bacterium according to claim 1, wherein said *argJ* gene is derived from a microorganism having an enzyme having the ornithine acetyltransferase or ornithine acetyltransferase/N-acetylglutamate synthase activity.
3. The bacterium according to claims 1 or 2, wherein said *argJ* gene is derived from the microorganism belonging to the family selected from the group consisting of the families *Thermotogaceae*, *Bacillaceae* and *Methanocaldococcaceae*.
4. The bacterium according to claim 3, wherein said *argJ* gene is derived from the species *Thermotoga neapolitana*.
5. The bacterium according to any one of claims 1 to 4, wherein said *argJ* gene encodes a protein selected from the group consisting of:
 - (A) a protein having the amino acid sequence of SEQ ID NO: 2 which has ornithine acetyltransferase/N-acetylglutamate synthase activity; and
 - (B) a variant protein having the amino acid sequence of SEQ ID NO: 2, but which includes substitution, deletion, insertion, and/or addition of one or several amino acid residues and has ornithine acetyltransferase/N-acetylglutamate synthase activity according to the amino acid sequence of SEQ ID NO: 2.
6. The bacterium according to any one of claims 1 to 5, wherein said N-acetylornithine deacetylase is a protein selected from the group consisting of:
 - (D) a protein having the amino acid sequence of SEQ ID NO: 4; and
 - (E) a variant protein having the amino acid sequence of SEQ ID NO: 4, but which includes substitution, deletion, insertion, and/or addition of one or several amino acid residues and has N-acetylornithine

deacetylase activity according to the amino acid sequence of SEQ ID NO: 4.

7. The bacterium according to any one of claims 1 to 6, wherein said activity of N-acetylornithine deacetylase is decreased.
8. The bacterium according to any one of claims 1 to 6, wherein said activity of N-acetylornithine deacetylase is abolished.
9. The bacterium according to any one of claims 1 to 8, wherein said bacterium belongs to the genus *Escherichia*.
10. The bacterium according to claim 9, wherein said bacterium belongs to the species *Escherichia coli*.
11. The bacterium according to any one of claims 1 to 8, wherein said bacterium belongs to the genus *Pantoea*.
12. The bacterium according to claim 11, wherein said bacterium belongs to the species *Pantoea ananatis*.
13. A method for producing L-arginine or a salt thereof comprising:
 - (i) cultivating the bacterium according to any one of claims 1 to 12 in a culture medium to accumulate L-arginine or the salt thereof in the bacterium or culture medium, or both; and, if necessary,
 - (ii) collecting L-arginine or the salt thereof from the bacterium or culture medium.

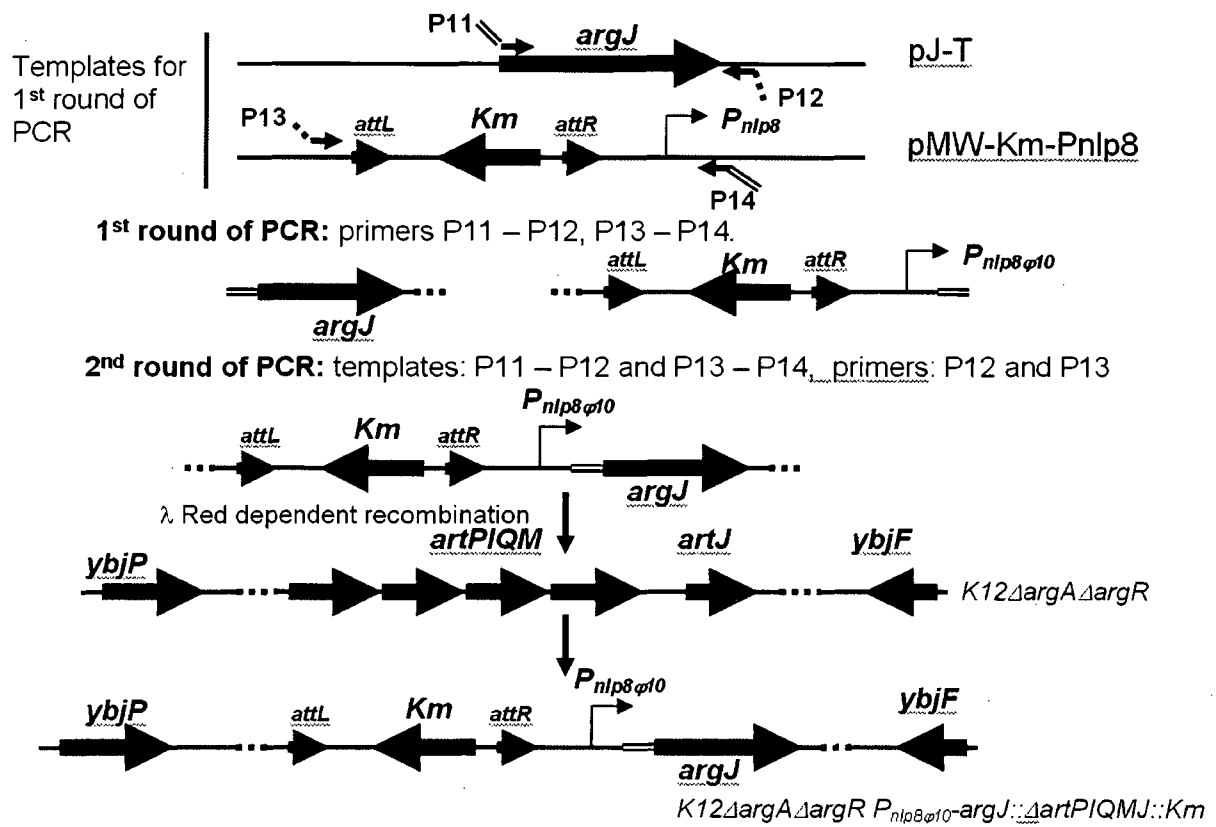


Fig. 1

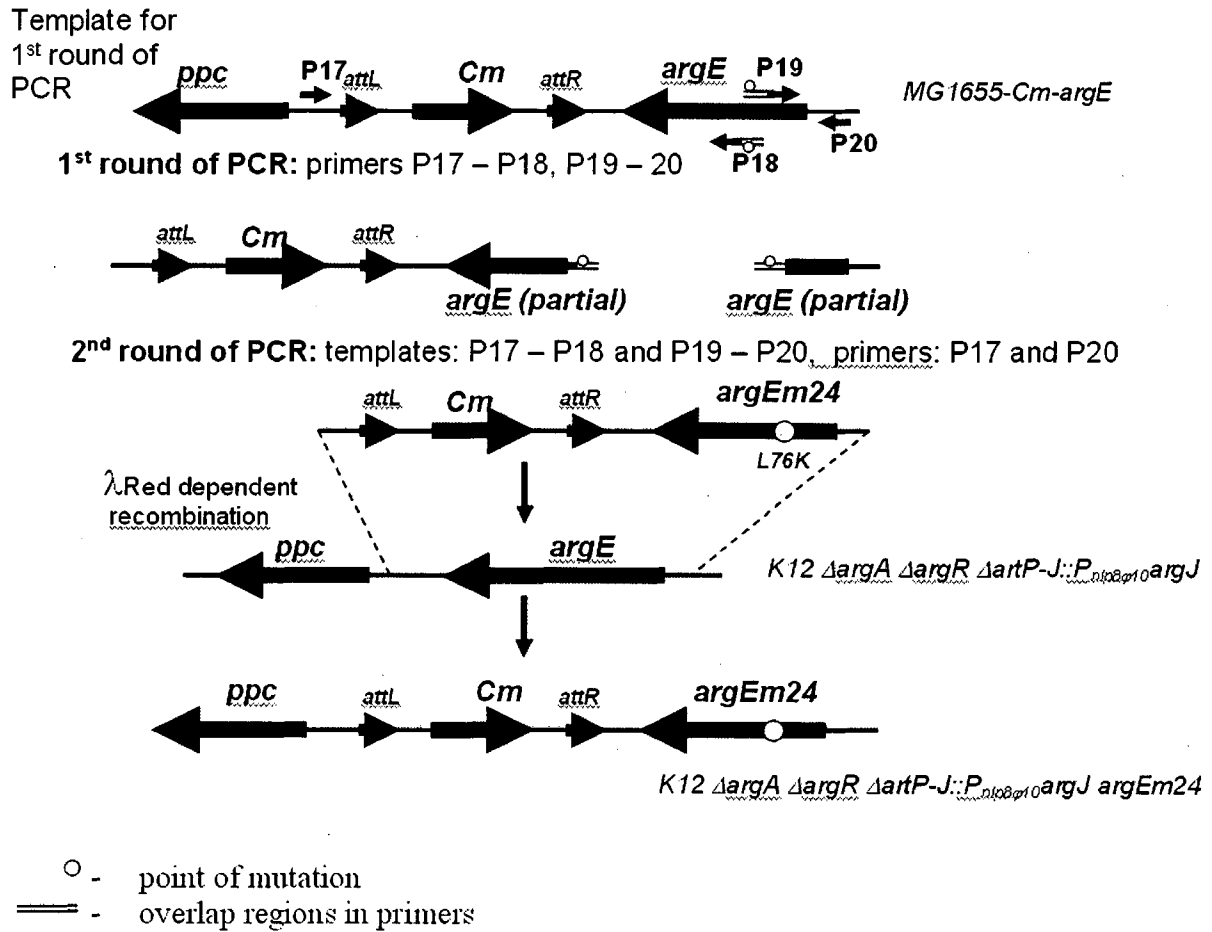


Fig. 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2013/072341

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N1/21 C12N9/10 C12N9/80 C12P13/10
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N C12P

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

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

EPO-Internal, BIOSIS, CHEM ABS Data, Sequence Search, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 201 758 A1 (AJINOMOTO KK [JP]) 2 May 2002 (2002-05-02) paragraph [0004] - paragraph [0010]; example 2 paragraph [0017] - paragraph [0035] paragraph [0045] - paragraph [0048] ----- -/--	1-13



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 application or patent 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

7 November 2013

Date of mailing of the international search report

25/11/2013

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INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2013/072341

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE UniProt [Online] 23 April 2003 (2003-04-23), "RecName: Full=Arginine biosynthesis bifunctional protein ArgJ; Includes: RecName: Full=Glutamate N-acetyltransferase; EC=2.3.1.35; AltName: Full=Ornithine acetyltransferase; Short=OATase; AltName: Full=Ornithine transacetylase;", XP002715800, retrieved from EBI accession no. UNIPROT:Q9Z4S1 Database accession no. Q9Z4S1 the whole document	5
A	----- DATABASE UniProt [Online] 16 November 2011 (2011-11-16), "RecName: Full=Acetylornithine deacetylase; Short=A0; Short=Acetylornithinase; EC=3.5.1.16; AltName: Full=N-acetylornithinase;", XP002715801, retrieved from EBI accession no. UNIPROT:G2BGN4 Database accession no. G2BGN4 the whole document -----	6

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/JP2013/072341

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			DE 60038560 T2	25-06-2009
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			JP 2002315571 A	29-10-2002
			US 2002098555 A1	25-07-2002
