

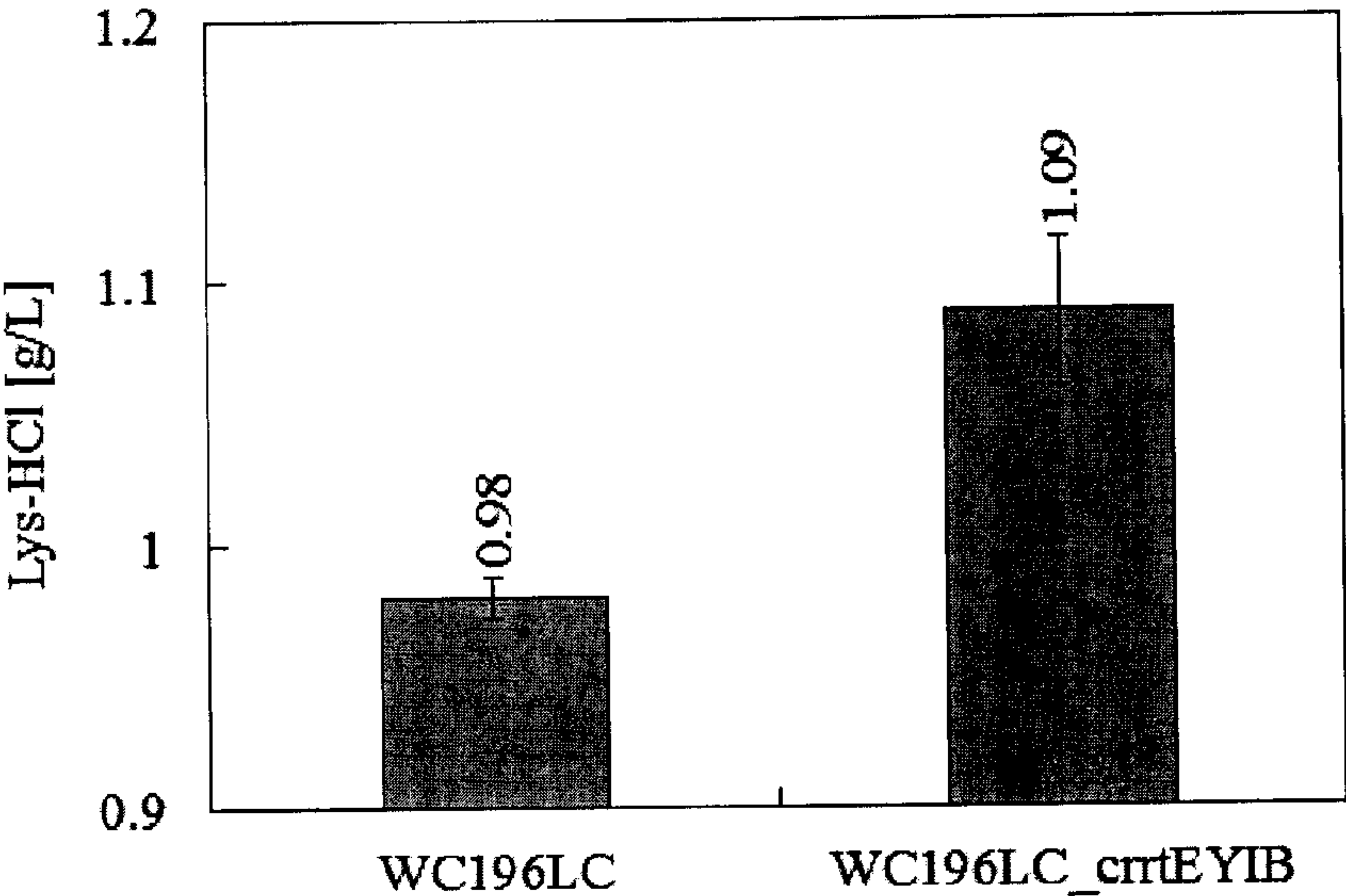


(12) **DEMANDE DE BREVET CANADIEN**
CANADIAN PATENT APPLICATION
(13) **A1**

(86) Date de dépôt PCT/PCT Filing Date: 2017/08/10 (87) Date publication PCT/PCT Publication Date: 2018/02/15 (85) Entrée phase nationale/National Entry: 2019/02/07 (86) N° demande PCT/PCT Application No.: JP 2017/029050 (87) N° publication PCT/PCT Publication No.: 2018/030507 (30) Priorité/Priority: 2016/08/10 (JP2016-157800)	(51) Cl.Int./Int.Cl. <i>C12N 15/09</i> (2006.01), <i>C12N 1/21</i> (2006.01), <i>C12P 13/04</i> (2006.01), <i>C12P 13/08</i> (2006.01) (71) Demandeur/Applicant: AJINOMOTO CO., INC., JP (72) Inventeurs/Inventors: YAMADA, KAZUTERU, JP; ZHANG, YE, JP; ABE, KENJI, JP; IWANAGA, NAOKI, JP; TAKESHITA, RYO, JP; UEHARA, YURI, JP; HIKICHI, CHIKA, JP;
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(54) Titre : PROCEDE DE PRODUCTION D'ACIDE L-AMINE
(54) Title: PRODUCTION METHOD FOR L-AMINO ACID

[Fig. 1]



(57) **Abrégé/Abstract:**
The present invention provides a production method for an L-amino acid such as L-lysine. An L-amino acid is produced by culturing, in a culture medium, bacteria having L-amino acid producing ability, bacteria that have been modified to have enhanced carotenoid biosynthesis enzyme activity, and collecting the L-amino acid from the culture medium.

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ABSTRACT

A method for producing an L-amino acid such as L-lysine is provided.

An L-amino acid is produced by culturing a bacterium having an L-amino acid-producing ability, which has been modified so that the activity of a carotenoid biosynthesis enzyme is increased, in a medium, and collecting the L-amino acid from the medium.

DESCRIPTION

PRODUCTION METHOD FOR L-AMINO ACID

Technical Field

[0001]

The present invention relates to a method for producing an L-amino acid such as L-lysine by fermentation using a bacterium. L-Amino acids are industrially useful as raw materials of seasonings, feed additives, and so forth.

Background Art

[0002]

L-Amino acids are industrially produced by, for example, fermentation using microorganisms such as bacteria having an L-amino acid-producing ability (Non-patent document 1). As such microorganisms, for example, strains isolated from the nature and mutant strains thereof have been used. Also, an L-amino acid-producing ability of microorganisms can be improved by using recombinant DNA techniques.

[0003]

Carotenoids are compounds having a basic structure of $C_{40}H_{56}$ consisting of 8 isoprene units, and typically exhibit a color of orange, red, or the like. Carotenoids are biosynthesized by various organisms such as plants, algae, and bacteria. There has not been reported the relationship between biosynthesis of carotenoids and production of L-amino acids.

[0004]

There has been reported a method for producing salmons having moderate red-orange colored flesh by cultivating salmons with feeding of a carotenoid (Patent document 1).

Prior art references

Patent documents

[0005]

Patent document 1: JP patent No. 5706056

Non-patent documents

[0006]

Non-patent document 1: Akashi, K. et al., Amino Acid Fermentation. Japan Scientific Societies Press, p.195 to 215, 1986.

Summary of the Invention

Object to be Achieved by the Invention

[0007]

An object of the present invention is to develop a novel technique for improving an L-amino acid-producing ability of a bacterium, and thereby provide a method for efficiently producing an L-amino acid.

Means for Achieving the Object

[0008]

In order to achieve the aforementioned object, the inventors of the present invention conducted various researches. As a result, they found that an L-amino acid-producing ability of a bacterium can be improved by modifying the bacterium so that the expression of gene(s) encoding carotenoid biosynthesis enzyme(s) is increased, and accomplished the present invention.

[0009]

The present invention can be thus embodied, for example, as follows.

[1] A method for producing an L-amino acid, the method comprising:
culturing a bacterium having an L-amino acid-producing ability in a medium to accumulate an L-amino acid in the medium; and
collecting the L-amino acid from the medium,
wherein the bacterium has been modified so that the activity of a carotenoid biosynthesis enzyme is increased as compared with a non-modified strain.

[2] The method mentioned above, wherein the carotenoid biosynthesis enzyme consists of one or more kinds of enzymes selected from geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, lycopene beta-cyclase, carotene ketolase, and carotene hydroxylase.

[3] The method mentioned above, wherein at least the activity or activities of one or more kinds of enzymes selected from geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and lycopene beta-cyclase is/are increased.

[4] The method mentioned above, wherein at least the activities of geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and lycopene beta-cyclase are increased.

[5] The method mentioned above, wherein the activity or activities of carotene ketolase and/or carotene hydroxylase is/are further increased.

[6] The method mentioned above, wherein the geranylgeranyl pyrophosphate synthase is a protein defined in (a), (b), or (c) mentioned below:

- (a) a protein comprising the amino acid sequence of SEQ ID NO: 12 or 24;
- (b) a protein comprising the amino acid sequence of SEQ ID NO: 12 or 24, but which includes substitution, deletion, insertion, and/or addition of 1 to 10 amino acid residues, and having geranylgeranyl pyrophosphate synthase activity;
- (c) a protein comprising an amino acid sequence showing an identity of 90% or higher to the amino acid sequence of SEQ ID NO: 12 or 24, and having geranylgeranyl pyrophosphate synthase activity.

[7] The method mentioned above, wherein the phytoene synthase is a protein defined in (a), (b), or (c) mentioned below:

- (a) a protein comprising the amino acid sequence of SEQ ID NO: 14 or 26;
- (b) a protein comprising the amino acid sequence of SEQ ID NO: 14 or 26, but which includes substitution, deletion, insertion, and/or addition of 1 to 10 amino acid residues, and having phytoene synthase activity;
- (c) a protein comprising an amino acid sequence showing an identity of 90% or higher to the amino acid sequence of SEQ ID NO: 14 or 26, and having phytoene synthase activity.

[8] The method mentioned above, wherein the phytoene desaturase is a protein defined in (a), (b), or (c) mentioned below:

- (a) a protein comprising the amino acid sequence of SEQ ID NO: 16 or 28;
- (b) a protein comprising the amino acid sequence of SEQ ID NO: 16 or 28, but which includes substitution, deletion, insertion, and/or addition of 1 to 10 amino acid residues, and having phytoene desaturase activity;
- (c) a protein comprising an amino acid sequence showing an identity of 90% or higher to the amino acid sequence of SEQ ID NO: 16 or 28, and having phytoene desaturase activity.

[9] The method mentioned above, wherein the lycopene beta-cyclase is a protein defined in (a), (b), or (c) mentioned below:

- (a) a protein comprising the amino acid sequence of SEQ ID NO: 18;
- (b) a protein comprising the amino acid sequence of SEQ ID NO: 18, but which includes substitution, deletion, insertion, and/or addition of 1 to 10 amino acid residues, and having lycopene beta-cyclase activity;
- (c) a protein comprising an amino acid sequence showing an identity of 90% or higher to the amino acid sequence of SEQ ID NO: 18, and having lycopene beta-cyclase activity.

[10] The method mentioned above, wherein the carotene ketolase is a protein defined in (a), (b), or (c) mentioned below:

- (a) a protein comprising the amino acid sequence of SEQ ID NO: 20, 30, or 61;
- (b) a protein comprising the amino acid sequence of SEQ ID NO: 20, 30, or 61, but which includes substitution, deletion, insertion, and/or addition of 1 to 10 amino acid residues, and having carotene ketolase activity;
- (c) a protein comprising an amino acid sequence showing an identity of 90% or higher to the amino acid sequence of SEQ ID NO: 20, 30, or 61, and having carotene ketolase activity.

[11] The method mentioned above, wherein the carotene hydroxylase is a protein defined in (a), (b), or (c) mentioned below:

- (a) a protein comprising the amino acid sequence of SEQ ID NO: 22, 58, 59, or 60;
- (b) a protein comprising the amino acid sequence of SEQ ID NO: 22, 58, 59, or 60, but which includes substitution, deletion, insertion, and/or addition of 1 to 10 amino acid

residues, and having carotene hydroxylase activity;

(c) a protein comprising an amino acid sequence showing an identity of 90% or higher to the amino acid sequence of SEQ ID NO: 22, 58, 59, or 60, and having carotene hydroxylase activity.

[12] The method mentioned above, wherein the activity of the carotenoid biosynthesis enzyme is increased by increasing the expression of a gene encoding the carotenoid biosynthesis enzyme.

[13] The method mentioned above, wherein the expression of the gene is increased by increasing the copy number of the gene and/or modifying an expression control sequence of the gene.

[14] The method mentioned above, wherein the bacterium is a bacterium belonging to the family *Enterobacteriaceae* or a coryneform bacterium.

[15] The method mentioned above, wherein the bacterium is a bacterium belonging to the genus *Pantoea* or *Escherichia*.

[16] The method mentioned above, wherein the bacterium is *Pantoea ananatis* or *Escherichia coli*.

[17] The method mentioned above, wherein the bacterium is a bacterium belonging to the genus *Corynebacterium*.

[18] The method mentioned above, wherein the bacterium is *Corynebacterium glutamicum*.

[19] The method mentioned above, wherein the L-amino acid consists of an L-amino acid of aspartate family and/or an L-amino acid of glutamate family.

[20] The method mentioned above, wherein the L-amino acid consists of one or more kinds of L-amino acids selected from L-lysine, L-threonine, and L-glutamic acid.

[21] The method mentioned above, wherein the bacterium has a carotenoid-producing ability, and a carotenoid is accumulated in cells of the bacterium by the culturing.

[22] The method mentioned above, wherein the cells contain the carotenoid in an amount of 10 µg/g-DCW or more.

[23] The method mentioned above, wherein the carotenoid consists of one or more kinds of carotenoids selected from beta-carotene, zeaxanthin, canthaxanthin, and astaxanthine.

[24] The method mentioned above, which further comprises collecting the cells.

[25] A method for producing bacterial cells containing a carotenoid, the method comprising:

culturing a bacterium having an L-amino acid-producing ability and a carotenoid-producing ability in a medium to accumulate a carotenoid in cells of the bacterium; and

collecting the cells,

wherein the bacterium has been modified so that the activity of a carotenoid biosynthesis enzyme is increased as compared with a non-modified strain.

[26] The method mentioned above, wherein the cells contain the carotenoid in an amount of 10 µg/g-DCW or more.

[27] The method mentioned above, wherein the carotenoid consists of one or more kinds of carotenoids selected from beta-carotene, zeaxanthin, canthaxanthin, and astaxanthine.

[28] Cells of a bacterium,
wherein the cells contain a carotenoid,
wherein the bacterium having an L-amino acid-producing ability and a

carotenoid-producing ability, and

wherein the bacterium has been modified so that the activity of a carotenoid biosynthesis enzyme is increased as compared with a non-modified strain.

[29] The cells mentioned above, wherein the cells contain the carotenoid in an amount of 10 µg/g-DCW or more.

[30] The cells mentioned above, wherein the carotenoid consists of one or more kinds of carotenoids selected from beta-carotene, zeaxanthin, canthaxanthin, and astaxanthine.

[31] A composition for culturing an aquatic organism, the composition containing the cells mentioned above,

wherein the aquatic organism is salmon, shrimp, or seabream.

[32] A feed for an aquatic organism, the feed containing the cells mentioned above, wherein the aquatic organism is salmon, shrimp, or seabream.

[33] A method for culturing an aquatic organism, the method comprising:
feeding cells produced by the method mentioned above, the cells mentioned above, the composition mentioned above, or the feed mentioned above to an aquatic organism,

wherein the aquatic organism is salmon, shrimp, or seabream.

Brief Description of Drawings

[0010]

[FIG. 1] A diagram showing results of L-lysine production culture using an *E. coli* strain having an enhanced expression of carotenoid biosynthesis enzyme genes (*crtEYIB*) (n = 6).

[FIG. 2] A diagram showing results of L-lysine production culture using *E. coli* strains having an enhanced expression of carotenoid biosynthesis enzyme genes (*crtEYIB* and *crtZ*) (n = 3):

Control, WC196LC;

crtZ(Pa), WC196LC_crtEYIB(ML)_ΔyhfK::P4071_crtZ(Pa);
 crtZ(Br), WC196LC_crtEYIB(ML)_ΔyhfK::P4071_crtZ(Br);
 crtZ(Pc), WC196LC_crtEYIB(ML)_ΔyhfK::P4071_crtZ(Pc);
 crtZ(Al), WC196LC_crtEYIB(ML)_ΔyhfK::P4071_crtZ(Al).

[FIG. 3] A diagram showing results of L-lysine production culture using *E. coli* strains having an enhanced expression of carotenoid biosynthesis enzyme genes (*crtEYIB*, *crtZ*, and *crtW*) (n = 3):

pMIV5JS, WC196LC/pMIV5JS;
 pMIV_Pnlp8_crtW, WC196LC_crtEYIB(ML)_ΔyhfK::P4071_crtZ(Pa)/pMIV-Pnlp8-crtW;
 pMW219, WC196LC/pMW219;
 pMW_Ptac_crtW,
 WC196LC_crtEYIB(ML)_ΔyhfK::P4071_crtZ(Pa)/pMW_Ptac_crtW.

[FIG. 4] A diagram showing results of L-threonine production culture using an *E. coli* strain having an enhanced expression of carotenoid biosynthesis enzyme genes (*crtEYIB*):

pMW119, VKPM B-3996/pMW119 (n = 4);
 pMW119-crtEYIB, VKPM B-3996/pMW119-crtEYIB (n = 10).

[FIG. 5] A diagram showing quantification results of carotenoids in a *C. glutamicum* strain having an enhanced expression of carotenoid biosynthesis enzyme genes (*crtY* and *crtZ*) (n = 3). The concentrations of substances corresponding to peaks detected by HPLC other than zeaxanthin (Zx) were estimated on the basis of the peak area of a Zx standard solution, and the sum total of them was indicated as "Others".

[FIG. 6] A diagram showing results of L-lysine production culture using a *C. glutamicum* strain having an enhanced expression of carotenoid biosynthesis enzyme genes (*crtY* and *crtZ*) (n = 4).

[FIG. 7] A diagram showing quantification results of carotenoids in a *P. ananatis* strain having an enhanced expression of a carotenoid biosynthesis enzyme gene (*crtE*) (n = 3). Standard solutions of astaxanthin (Ax), canthaxanthin (Cx), zeaxanthin (Zx), and beta-carotene (b-CR) were used for HPLC, and the concentrations of these substances were calculated on the basis of the respective peak areas. The concentrations of substances corresponding to detected peaks other than these substances were estimated on the basis of the peak area of the Ax standard solution, and

the sum total of them was indicated as "Others".

[FIG. 8] A diagram showing results of L-glutamic acid production culture using a *P. ananatis* strain having an enhanced expression of a carotenoid biosynthesis enzyme gene (*crtE*).

[FIG. 9] A diagram showing quantification results of carotenoids in a *P. ananatis* strain having an enhanced expression of carotenoid biosynthesis enzyme genes (*crtE* and *crtW*) (n = 3). Standard solutions of astaxanthin (Ax), canthaxanthin (Cx), zeaxanthin (Zx), and beta-carotene (b-CR) were used for HPLC, and the concentrations of these substances were calculated on the basis of the respective peak areas. The concentrations of substances corresponding to detected peaks other than these substances were estimated on the basis of the peak area of the Ax standard solution, and the sum total of them was indicated as "Others".

[FIG. 10] A diagram showing results of L-glutamic acid production culture using a *P. ananatis* strain having an enhanced expression of carotenoid biosynthesis enzyme genes (*crtE* and *crtW*).

Modes for Carrying out the Invention

[0011]

Hereinafter, the present invention will be explained in detail.

[0012]

<1> Bacterium of the present invention

The bacterium of the present invention is a bacterium having an L-amino acid-producing ability, which has been modified so that the activity of a carotenoid biosynthesis enzyme is increased.

[0013]

<1-1> Bacterium having L-amino acid-producing ability

In the present invention, the term "bacterium having an L-amino acid-producing ability" refers to a bacterium having an ability to generate and accumulate an objective L-amino acid in a medium in such a degree that the L-amino acid can be collected, when the bacterium is cultured in the medium. The bacterium having an L-amino acid-producing ability may be a bacterium that is able to accumulate an objective L-amino acid in a medium in an amount larger than that obtainable with a non-modified strain. The term "non-modified strain" refers to a control strain that has not been

modified so that the activity of a carotenoid biosynthesis enzyme is increased. That is, examples of the non-modified strain include a wild-type strain and parental strain. The bacterium having an L-amino acid-producing ability may be a bacterium that is able to accumulate an objective L-amino acid in a medium in an amount of preferably 0.5 g/L or more, more preferably 1.0 g/L or more.

[0014]

Examples of the L-amino acid include basic amino acids such as L-lysine, L-ornithine, L-arginine, L-histidine, and L-citrulline; aliphatic amino acids such as L-isoleucine, L-alanine, L-valine, L-leucine, and glycine; amino acids which are hydroxy-monoaminocarboxylic acids such as L-threonine and L-serine; cyclic amino acids such as L-proline; aromatic amino acids such as L-phenylalanine, L-tyrosine, and L-tryptophan; sulfur-containing amino acids such as L-cysteine, L-cystine, and L-methionine; acidic amino acids such as L-glutamic acid and L-aspartic acid; and amino acids having an amide group in the side chain such as L-glutamine and L-asparagine. Particular examples of the L-amino acid include L-amino acid of glutamate family and L-amino acid of aspartate family. The term "L-amino acid of glutamate family" collectively refers to L-glutamic acid and L-amino acids that are biosynthesized via L-glutamic acid as an intermediate. Examples of the L-amino acids that are biosynthesized via L-glutamic acid as an intermediate include L-glutamine, L-proline, L-arginine, L-citrulline, and L-ornithine. The term "L-amino acid of aspartate family" collectively refers to L-aspartic acid and L-amino acids that are biosynthesized via L-aspartic acid as an intermediate. Examples of the L-amino acids that are biosynthesized via L-aspartic acid as an intermediate include L-lysine, L-threonine, L-isoleucine, and L-methionine. More particular examples of the L-amino acid include L-lysine, L-threonine, and L-glutamic acid. The bacterium of the present invention may have an ability to produce a single kind of L-amino acid, or two or more kinds of L-amino acids.

[0015]

In the present invention, the term "amino acid" refers to an L-amino acid, unless otherwise stated. In the present invention, the term "L-amino acid" refers to an L-amino acid in a free form, a salt thereof, or a mixture thereof, unless otherwise stated. Examples of salt will be described later.

[0016]

Examples of the bacterium include bacteria belonging to the family *Enterobacteriaceae* and coryneform bacteria.

[0017]

Examples of bacteria belonging to the family *Enterobacteriaceae* include bacteria belonging to the genus *Escherichia*, *Enterobacter*, *Pantoea*, *Klebsiella*, *Serratia*, *Erwinia*, *Photobacterium*, *Providencia*, *Salmonella*, *Morganella*, or the like. Specifically, bacteria classified into the family *Enterobacteriaceae* according to the taxonomy used in the NCBI (National Center for Biotechnology Information) database (<http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=91347>) can be used.

[0018]

The *Escherichia* bacteria are not particularly limited, and examples thereof include those classified into the genus *Escherichia* according to the taxonomy known to those skilled in the field of microbiology. Examples of the *Escherichia* bacteria include, for example, those described in the work of Neidhardt et al. (Backmann B.J., 1996, Derivations and Genotypes of some mutant derivatives of *Escherichia coli* K-12, pp.2460-2488, Table 1, In F.D. Neidhardt (ed.), *Escherichia coli* and *Salmonella* Cellular and Molecular Biology/Second Edition, American Society for Microbiology Press, Washington, D.C.). Examples of the *Escherichia* bacteria include, for example, *Escherichia coli*. Specific examples of *Escherichia coli* include, for example, *Escherichia coli* K-12 strains such as W3110 strain (ATCC 27325) and MG1655 strain (ATCC 47076); *Escherichia coli* K5 strain (ATCC 23506); *Escherichia coli* B strains such as BL21 (DE3) strain; and derivative strains thereof.

[0019]

The *Enterobacter* bacteria are not particularly limited, and examples include those classified into the genus *Enterobacter* according to the taxonomy known to those skilled in the field of microbiology. Examples of the *Enterobacter* bacterium include, for example, *Enterobacter agglomerans* and *Enterobacter aerogenes*. Specific examples of *Enterobacter agglomerans* include, for example, the *Enterobacter agglomerans* ATCC 12287 strain. Specific examples of *Enterobacter aerogenes* include, for example, the *Enterobacter aerogenes* ATCC 13048 strain, NBRC 12010 strain (Biotechnol. Bioeng., 2007, Mar. 27;98(2):340-348), and AJ110637 strain (FERM BP-10955). Examples of the *Enterobacter* bacteria also include, for example, the strains described in European Patent Application Laid-open (EP-A) No. 0952221. In

addition, *Enterobacter agglomerans* also include some strains classified as *Pantoea agglomerans*.

[0020]

The *Pantoea* bacteria are not particularly limited, and examples include those classified into the genus *Pantoea* according to the taxonomy known to those skilled in the field of microbiology. Examples of the *Pantoea* bacteria include, for example, *Pantoea ananatis*, *Pantoea stewartii*, *Pantoea agglomerans*, and *Pantoea citrea*. Specific examples of *Pantoea ananatis* include, for example, the *Pantoea ananatis* LMG20103 strain, AJ13355 strain (FERM BP-6614), AJ13356 strain (FERM BP-6615), AJ13601 strain (FERM BP-7207), SC17 strain (FERM BP-11091), SC17(0) strain (VKPM B-9246), and SC17sucA strain (FERM BP-8646). Some of *Enterobacter* bacteria and *Erwinia* bacteria were reclassified into the genus *Pantoea* (Int. J. Syst. Bacteriol., 39, 337-345 (1989); Int. J. Syst. Bacteriol., 43, 162-173 (1993)). For example, some strains of *Enterobacter agglomerans* were recently reclassified into *Pantoea agglomerans*, *Pantoea ananatis*, *Pantoea stewartii*, or the like on the basis of nucleotide sequence analysis of 16S rRNA etc. (Int. J. Syst. Bacteriol., 39, 337-345 (1989)). In the present invention, the *Pantoea* bacteria include those reclassified into the genus *Pantoea* as described above.

[0021]

Examples of the *Erwinia* bacteria include *Erwinia amylovora* and *Erwinia carotovora*. Examples of the *Klebsiella* bacteria include *Klebsiella planticola*.

[0022]

Examples of the coryneform bacteria include bacteria belonging to the genus *Corynebacterium*, *Brevibacterium*, *Microbacterium*, or the like.

[0023]

Specific examples of the coryneform bacteria include the following species.

Corynebacterium acetoacidophilum

Corynebacterium acetoglutamicum

Corynebacterium alkanolyticum

Corynebacterium callunae

Corynebacterium crenatum

Corynebacterium glutamicum

Corynebacterium lilium

Corynebacterium melassecola
Corynebacterium thermoaminogenes (*Corynebacterium efficiens*)
Corynebacterium herculis
Brevibacterium divaricatum (*Corynebacterium glutamicum*)
Brevibacterium flavum (*Corynebacterium glutamicum*)
Brevibacterium immariophilum
Brevibacterium lactofermentum (*Corynebacterium glutamicum*)
Brevibacterium roseum
Brevibacterium saccharolyticum
Brevibacterium thiogenitalis
Corynebacterium ammoniagenes (*Corynebacterium stationis*)
Brevibacterium album
Brevibacterium cerinum
Microbacterium ammoniaphilum

[0024]

Specific examples of the coryneform bacteria include the following strains.

Corynebacterium acetoacidophilum ATCC 13870
Corynebacterium acetoglutamicum ATCC 15806
Corynebacterium alkanolyticum ATCC 21511
Corynebacterium callunae ATCC 15991
Corynebacterium crenatum AS1.542
Corynebacterium glutamicum ATCC 13020, ATCC 13032, ATCC 13060,
 ATCC 13869, FERM BP-734
Corynebacterium lilium ATCC 15990
Corynebacterium melassecola ATCC 17965
Corynebacterium efficiens (*Corynebacterium thermoaminogenes*) AJ12340
 (FERM BP-1539)
Corynebacterium herculis ATCC 13868
Brevibacterium divaricatum (*Corynebacterium glutamicum*) ATCC 14020
Brevibacterium flavum (*Corynebacterium glutamicum*) ATCC 13826, ATCC
 14067, AJ12418 (FERM BP-2205)
Brevibacterium immariophilum ATCC 14068
Brevibacterium lactofermentum (*Corynebacterium glutamicum*) ATCC 13869

Brevibacterium roseum ATCC 13825

Brevibacterium saccharolyticum ATCC 14066

Brevibacterium thiogenitalis ATCC 19240

Corynebacterium ammoniagenes (*Corynebacterium stationis*) ATCC 6871,
ATCC 6872

Brevibacterium album ATCC 15111

Brevibacterium cerinum ATCC 15112

Microbacterium ammoniaphilum ATCC 15354

[0025]

The *Corynebacterium* bacteria include bacteria that had previously been classified into the genus *Brevibacterium*, but are presently united into the genus *Corynebacterium* (Int. J. Syst. Bacteriol., 41, 255 (1991)). Moreover, *Corynebacterium stationis* includes bacteria that had previously been classified as *Corynebacterium ammoniagenes*, but are presently re-classified into *Corynebacterium stationis* on the basis of nucleotide sequence analysis of 16S rRNA etc. (Int. J. Syst. Evol. Microbiol., 60, 874-879 (2010)).

[0026]

These strains are available from, for example, the American Type Culture Collection (Address: 12301 Parklawn Drive, Rockville, Maryland 20852, P.O. Box 1549, Manassas, VA 20108, United States of America). That is, registration numbers are given to the respective strains, and the strains can be ordered by using these registration numbers (refer to <http://www.atcc.org/>). The registration numbers of the strains are listed in the catalogue of the American Type Culture Collection. These strains can also be obtained from, for example, the depositories at which the strains were deposited.

[0027]

The bacterium of the present invention may be a bacterium inherently having an L-amino acid-producing ability, or may be a bacterium modified so that it has an L-amino acid-producing ability. The bacterium having an L-amino acid-producing ability can be obtained by imparting an L-amino acid-producing ability to such a bacterium as mentioned above, or by enhancing an L-amino acid-producing ability of such a bacterium as mentioned above.

[0028]

To impart or enhance an L-amino acid-producing ability, methods conventionally employed in the breeding of amino acid-producing strains of coryneform bacteria, *Escherichia* bacteria, and so forth (refer to "Amino Acid Fermentation", Gakkai Shuppan Center (Ltd.), 1st Edition, published May 30, 1986, pp.77-100) can be used. Examples of such methods include, for example, acquiring an auxotrophic mutant strain, acquiring an L-amino acid analogue-resistant strain, acquiring a metabolic regulation mutant strain, and constructing a recombinant strain in which the activity of an L-amino acid biosynthetic enzyme is enhanced. In the breeding of L-amino acid-producing bacteria, one of the above-described properties such as auxotrophy, analogue resistance, and metabolic regulation mutation may be imparted alone, or two or three or more of such properties may be imparted in combination. Also, in the breeding of L-amino acid-producing bacteria, the activity of one of L-amino acid biosynthetic enzymes may be enhanced alone, or the activities of two or three or more of such enzymes may be enhanced in combination. Furthermore, imparting property(s) such as auxotrophy, analogue resistance, and metabolic regulation mutation can be combined with enhancing the activity(s) of biosynthetic enzyme(s).

[0029]

An auxotrophic mutant strain, analogue-resistant strain, or metabolic regulation mutant strain having an L-amino acid-producing ability can be obtained by subjecting a parental strain or wild-type strain to a usual mutagenesis treatment, and then selecting a strain exhibiting autotrophy, analogue resistance, or a metabolic regulation mutation, and having an L-amino acid-producing ability from the obtained mutant strains. Examples of the usual mutagenesis treatment include irradiation of X-ray or ultraviolet and a treatment with a mutation agent such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), and methyl methanesulfonate (MMS).

[0030]

An L-amino acid-producing ability can also be imparted or enhanced by enhancing the activity of an enzyme involved in biosynthesis of an objective L-amino acid. An enzyme activity can be enhanced by, for example, modifying a bacterium so that the expression of a gene encoding the enzyme is enhanced. Methods for enhancing gene expression are described in WO00/18935, EP1010755A, and so forth. The detailed procedures for enhancing enzyme activity will be described later.

[0031]

Furthermore, an L-amino acid-producing ability can also be imparted or enhanced by reducing the activity of an enzyme that catalyzes a reaction branching away from the biosynthetic pathway of an objective L-amino acid to generate a compound other than the objective L-amino acid. The "enzyme that catalyzes a reaction branching away from the biosynthetic pathway of an objective L-amino acid to generate a compound other than the objective L-amino acid" referred to herein includes an enzyme involved in decomposition of the objective amino acid. The method for reducing an enzyme activity will be described later.

[0032]

Hereinafter, L-amino acid-producing bacteria and methods for imparting or enhancing an L-amino acid-producing ability will be specifically exemplified. All of the properties of the L-amino acid-producing bacteria and modifications for imparting or enhancing an L-amino acid-producing ability may be used independently or in any appropriate combination.

[0033]

<L-Glutamic acid-producing bacteria>

Examples of methods for imparting or enhancing L-glutamic acid-producing ability include, for example, a method of modifying a bacterium so that the bacterium has an increased activity or activities of one or more kinds of enzymes selected from the L-glutamic acid biosynthesis enzymes. Examples of such enzymes include, but not particularly limited to, glutamate dehydrogenase (*gdhA*), glutamine synthetase (*glnA*), glutamate synthase (*gltBD*), isocitrate dehydrogenase (*icdA*), aconitate hydratase (*acnA*, *acnB*), citrate synthase (*gltA*), methylcitrate synthase (*prpC*), pyruvate carboxylase (*pyc*), pyruvate dehydrogenase (*aceEF*, *lpdA*), pyruvate kinase (*pykA*, *pykF*), phosphoenolpyruvate synthase (*ppsA*), enolase (*eno*), phosphoglyceromutase (*pgmA*, *pgmI*), phosphoglycerate kinase (*pgk*), glyceraldehyde-3-phosphate dehydrogenase (*gapA*), triose phosphate isomerase (*tpiA*), fructose biphosphate aldolase (*fbp*), glucose phosphate isomerase (*pgi*), 6-phosphogluconate dehydratase (*edd*), 2-keto-3-deoxy-6-phosphogluconate aldolase (*eda*), and transhydrogenase. Shown in the parentheses after the names of the enzymes are examples of genes encoding the enzymes (the same shall apply to the same occasions hereinafter). It is preferable to enhance the activity or activities of one or more kinds of enzymes selected from, for example, glutamate dehydrogenase, citrate synthase, phosphoenol pyruvate carboxylase, and methylcitrate

synthase, among these enzymes.

[0034]

Examples of strains belonging to the family *Enterobacteriaceae* and modified so that the expression of the citrate synthase gene, phosphoenolpyruvate carboxylase gene, and/or glutamate dehydrogenase gene are increased include those disclosed in EP1078989A, EP955368A, and EP952221A. Furthermore, examples of strains belonging to the family *Enterobacteriaceae* and modified so that the expression of a gene of the Entner-Doudoroff pathway (*edd*, *eda*) is increased include those disclosed in EP1352966B. Examples of coryneform bacteria modified so that the expression of the glutamate synthetase gene (*gltBD*) is increased include those disclosed in WO99/07853.

[0035]

Examples of methods for imparting or enhancing L-glutamic acid-producing ability also include, for example, a method of modifying a bacterium so that the bacterium has a reduced activity or activities of one or more kinds of enzymes selected from the enzymes that catalyze a reaction branching away from the biosynthesis pathway of L-glutamic acid to generate a compound other than L-glutamic acid. Examples of such enzymes include, but not particularly limited to, isocitrate lyase (*aceA*), α -ketoglutarate dehydrogenase (*sucA*, *odhA*), acetolactate synthase (*ilvI*), formate acetyltransferase (*pfl*), lactate dehydrogenase (*ldh*), alcohol dehydrogenase (*adh*), glutamate decarboxylase (*gadAB*), and succinate dehydrogenase (*sdhABCD*). It is preferable to reduce or delete, for example, the α -ketoglutarate dehydrogenase activity, among these enzymes.

[0036]

Escherichia bacteria having a reduced α -ketoglutarate dehydrogenase activity or deficient in the α -ketoglutarate dehydrogenase activity, and methods for obtaining them are described in U.S. Patent Nos. 5,378,616 and 5,573,945. Furthermore, methods for reducing or deleting the α -ketoglutarate dehydrogenase activity of *Enterobacteriaceae* bacteria such as *Pantoea* bacteria, *Enterobacter* bacteria, *Klebsiella* bacteria, and *Erwinia* bacteria are disclosed in U.S. Patent Nos. 6,197,559, 6,682,912, 6,331,419, and 8,129,151, and WO2008/075483. Specific examples of *Escherichia* bacteria having a reduced α -ketoglutarate dehydrogenase activity or deficient in the α -ketoglutarate dehydrogenase activity include the following strains.

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

E. coli AJ12624 (FERM BP-3853)

E. coli AJ12628 (FERM BP-3854)

E. coli AJ12949 (FERM BP-4881)

[0037]

E. coli W3110sucA::Km^r is a strain obtained by disrupting the *sucA* gene encoding α -ketoglutarate dehydrogenase of *E. coli* W3110. This strain is completely deficient in the α -ketoglutarate dehydrogenase activity.

[0038]

Coryneform bacteria in which the α -ketoglutarate dehydrogenase activity is reduced or eliminated, and methods for obtaining those are disclosed in WO2008/075483. Specific examples of coryneform bacteria in which the α -ketoglutarate dehydrogenase activity is reduced or eliminated include, for example, the following strains.

Corynebacterium glutamicum (*Brevibacterium lactofermentum*) L30-2 strain (Japanese Patent Laid-open (Kokai) No. 2006-340603)

Corynebacterium glutamicum (*Brevibacterium lactofermentum*) Δ S strain (WO95/34672)

Corynebacterium glutamicum (*Brevibacterium lactofermentum*) AJ12821 (FERM BP-4172, French Patent No. 9401748)

Corynebacterium glutamicum (*Brevibacterium flavum*) AJ12822 (FERM BP-4173, French Patent No. 9401748)

Corynebacterium glutamicum AJ12823 (FERM BP-4174, French Patent No. 9401748)

[0039]

Examples of L-glutamic acid-producing bacteria and parental strains for deriving them also include *Pantoea* bacteria, such as the *Pantoea ananatis* AJ13355 strain (FERM BP-6614), *Pantoea ananatis* SC17 strain (FERM BP-11091), and *Pantoea ananatis* SC17(0) strain (VKPM B-9246). The AJ13355 strain is a strain isolated from soil in Iwata-shi, Shizuoka-ken, Japan as a strain that can proliferate in a low pH medium containing L-glutamic acid and a carbon source. The SC17 strain is a strain selected as a low phlegm-producing mutant strain from the AJ13355 strain (U.S. Patent No. 6,596,517). The SC17 strain was deposited at the independent administrative agency, National Institute of Advanced Industrial Science and

Technology, International Patent Organism Depository (currently independent administrative agency, National Institute of Technology and Evaluation, International Patent Organism Depository, #120, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken, 292-0818, Japan) on February 4, 2009, and assigned an accession number of FERM BP-11091. The AJ13355 strain was deposited at the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology (currently, independent administrative agency, National Institute of Technology and Evaluation, International Patent Organism Depository, #120, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken, 292-0818, Japan) on February 19, 1998 and assigned an accession number of FERM P-16644. Then, the deposit was converted to an international deposit under the provisions of Budapest Treaty on January 11, 1999, and assigned an accession number of FERM BP-6614.

[0040]

Furthermore, examples of L-glutamic acid-producing bacteria and parental strains for deriving them also include *Pantoea* bacteria having a reduced α -ketoglutarate dehydrogenase activity or deficient in the α -ketoglutarate dehydrogenase activity. Examples of such strains include the AJ13356 strain (U.S. Patent No. 6,331,419), which is an α -ketoglutarate dehydrogenase E1 subunit (*sucA*) gene-deficient strain of the AJ13355 strain, and the SC17*sucA* strain (U.S. Patent No. 6,596,517), which is a *sucA* gene-deficient strain of the SC17 strain. The AJ13356 strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (currently, independent administrative agency, National Institute of Technology and Evaluation, International Patent Organism Depository, #120, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken, 292-0818, Japan) on February 19, 1998, and assigned an accession number of FERM P-16645. Then, the deposit was converted into an international deposit under the provisions of the Budapest Treaty on January 11, 1999, and assigned an accession number of FERM BP-6616. The SC17*sucA* strain was assigned a private number of AJ417, and deposited at the independent administrative agency, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository (currently, independent administrative agency, National Institute of Technology and Evaluation, International Patent Organism Depository, #120, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken, 292-0818, Japan) on February 26, 2004, under an accession number of FERM BP-8646.

[0041]

The AJ13355 strain was identified as *Enterobacter agglomerans* when it was isolated, but it was recently reclassified as *Pantoea ananatis* on the basis of nucleotide sequencing of 16S rRNA and so forth. Therefore, although the AJ13355 and AJ13356 strains are deposited at the aforementioned depository as *Enterobacter agglomerans*, they are referred to as *Pantoea ananatis* in this specification.

[0042]

Furthermore, examples of L-glutamic acid-producing bacteria and parental strains for deriving them also include *Pantoea* bacteria such as the *Pantoea ananatis* SC17sucA/RSFCPG+pSTVCB strain, *Pantoea ananatis* AJ13601 strain, *Pantoea ananatis* NP106 strain, and *Pantoea ananatis* NA1 strain. The SC17sucA/RSFCPG+pSTVCB strain was obtained by introducing the plasmid RSFCPG containing the citrate synthase gene (*gltA*), phosphoenolpyruvate carboxylase gene (*ppc*), and glutamate dehydrogenase gene (*gdhA*) derived from *Escherichia coli*, and the plasmid pSTVCB containing the citrate synthase gene (*gltA*) derived from *Brevibacterium lactofermentum*, into the SC17sucA strain. The AJ13601 strain is a strain selected from the SC17sucA/RSFCPG+pSTVCB strain as a strain resistant to a high concentration of L-glutamic acid at a low pH. The NP106 strain was obtained from the AJ13601 strain by curing the RSFCPG and pSTVCB plasmids. The NA1 strain was obtained from the NP106 strain by introducing the plasmid RSFPPG thereinto (WO2010/027045). The plasmid RSFPPG has a structure corresponding to the plasmid RSFCPG except that *gltA* gene thereof has been replaced with a methylcitrate synthase gene (*prpC*), and hence, contains *prpC* gene, *ppc* gene, and *gdhA* gene (WO2008/020654). The AJ13601 strain was deposited at the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology (currently, independent administrative agency, National Institute of Technology and Evaluation, International Patent Organism Depository, #120, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken, 292-0818, Japan) on August 18, 1999, and assigned an accession number FERM P-17516. Then, the deposit was converted to an international deposit under the provisions of the Budapest Treaty on July 6, 2000, and assigned an accession number FERM BP-7207.

[0043]

Examples of L-glutamic acid-producing bacteria and parental strains for

deriving them also include strains in which both the α -ketoglutarate dehydrogenase (*sucA*) activity and the succinate dehydrogenase (*sdh*) activity are reduced or deleted (Japanese Patent Laid-open (Kokai) No. 2010-041920). Specific examples of such strains include, for example, a *sucAsdhA* double-deficient strain of the *Pantoea ananatis* NA1 strain and an *odhAsdhA* double-deficient strain of the *Corynebacterium glutamicum* ATCC 14067 strain, i.e. the *Corynebacterium glutamicum* 8L3G Δ SDH strain (Japanese Patent Laid-open (Kokai) No. 2010-041920).

[0044]

Examples of L-glutamic acid-producing bacteria and parental strains for deriving them also include auxotrophic mutant strains. Specific examples of auxotrophic mutant strains include, for example, *E. coli* VL334thrC⁺ (VKPM B-8961, EP1172433). *E. coli* VL334 (VKPM B-1641) is an L-isoleucine and L-threonine auxotrophic strain having mutations in the *thrC* and *ilvA* genes (U.S. Patent No. 4,278,765). *E. coli* VL334thrC⁺ is an L-isoleucine-auxotrophic L-glutamic acid-producing bacterium obtained by introducing a wild-type allele of the *thrC* gene into the VL334 strain. The wild-type allele of the *thrC* gene was introduced by the method of general transduction using a bacteriophage P1 grown on the wild-type *E. coli* K-12 strain (VKPM B-7) cells.

[0045]

Examples of L-glutamic acid-producing bacteria and parental strains for deriving them also include strains having resistance to an aspartic acid analogue. Such strains can also be deficient in the α -ketoglutarate dehydrogenase activity. Specific examples of strains having resistance to an aspartic acid analogue and deficient in the α -ketoglutarate dehydrogenase activity include, for example, *E. coli* AJ13199 (FERM BP-5807, U.S. Patent No. 5,908,768), *E. coli* FFRM P-12379, which additionally has a lowered L-glutamic acid-decomposing ability (U.S. Patent No. 5,393,671), and *E. coli* AJ13138 (FERM BP-5565, U.S. Patent No. 6,110,714).

[0046]

Examples of methods for imparting or enhancing L-glutamic acid-producing ability also include, for example, a method of enhancing the expression of an L-glutamic acid secretion gene, such as *yhfK* gene (WO2005/085419) or *ybjL* gene (WO2008/133161).

[0047]

Furthermore, examples of methods for imparting or enhancing L-glutamic acid-producing ability to or in coryneform bacteria also include methods of imparting resistance to an organic acid analogue, respiratory inhibitor, or the like, and methods of imparting sensitivity to a cell wall synthesis inhibitor. Specific examples of such methods include, for example, the method of imparting monofluoroacetic acid resistance (Japanese Patent Laid-open (Kokai) No. 50-113209), the method of imparting adenine resistance or thymine resistance (Japanese Patent Laid-open (Kokai) No. 57-065198), the method of attenuating urease (Japanese Patent Laid-open (Kokai) No. 52-038088), the method of imparting malonic acid resistance (Japanese Patent Laid-open (Kokai) No. 52-038088), the method of imparting resistance to benzopyrones or naphthoquinones (Japanese Patent Laid-open (Kokai) No. 56-1889), the method of imparting HOQNO resistance (Japanese Patent Laid-open (Kokai) No. 56-140895), the method of imparting α -ketomalonic acid resistance (Japanese Patent Laid-open (Kokai) No. 57-2689), the method of imparting guanidine resistance (Japanese Patent Laid-open (Kokai) No. 56-35981), the method of imparting sensitivity to penicillin (Japanese Patent Laid-open (Kokai) No. 4-88994), and so forth.

[0048]

Specific examples of such resistant or sensitive bacteria include the following strains.

Corynebacterium glutamicum (*Brevibacterium flavum*) AJ3949 (FERM BP-2632, Japanese Patent Laid-open (Kokai) No. 50-113209)

Corynebacterium glutamicum AJ11628 (FERM P-5736, Japanese Patent Laid-open (Kokai) No. 57-065198)

Corynebacterium glutamicum (*Brevibacterium flavum*) AJ11355 (FERM P-5007, Japanese Patent Laid-open (Kokai) No. 56-1889)

Corynebacterium glutamicum AJ11368 (FERM P-5020, Japanese Patent Laid-open (Kokai) No. 56-1889)

Corynebacterium glutamicum (*Brevibacterium flavum*) AJ11217 (FERM P-4318, Japanese Patent Laid-open (Kokai) No. 57-2689)

Corynebacterium glutamicum AJ11218 (FERM P-4319, Japanese Patent Laid-open (Kokai) No. 57-2689)

Corynebacterium glutamicum (*Brevibacterium flavum*) AJ11564 (FERM BP-5472, Japanese Patent Laid-open (Kokai) No. 56-140895)

Corynebacterium glutamicum (*Brevibacterium flavum*) AJ11439 (FERM BP-5136, Japanese Patent Laid-open (Kokai) No. 56-35981)

Corynebacterium glutamicum H7684 (FERM BP-3004, Japanese Patent Laid-open (Kokai) No. 04-88994)

Corynebacterium glutamicum (*Brevibacterium lactofermentum*) AJ11426 (FERM P-5123, Japanese Patent Laid-open (Kokai) No. 56-048890)

Corynebacterium glutamicum AJ11440 (FERM P-5137, Japanese Patent Laid-open (Kokai) No. 56-048890)

Corynebacterium glutamicum (*Brevibacterium lactofermentum*) AJ11796 (FERM P-6402, Japanese Patent Laid-open (Kokai) No. 58-158192)

[0049]

Furthermore, examples of methods for imparting or enhancing L-glutamic acid-producing ability to or in coryneform bacteria also include a method of enhancing the expression of *yggB* gene and a method of introducing a mutant *yggB* gene having a mutation in the coding region (WO2006/070944). That is, the bacterium of the present invention may have been modified so that the expression of *yggB* gene is increased, or may have been modified so as to harbor (have) a mutant *yggB* gene.

[0050]

The *yggB* gene is a gene encoding a mechanosensitive channel. Examples of the *yggB* gene include *yggB* genes of coryneform bacteria. Specific examples of the *yggB* genes of coryneform bacteria include, for example, *yggB* genes of *Corynebacterium glutamicum* ATCC13869, *Corynebacterium glutamicum* ATCC13032, *Corynebacterium glutamicum* ATCC14967, and *Corynebacterium melassecola* ATCC17965 (WO2006/070944). The *yggB* gene of *Corynebacterium glutamicum* ATCC 13032 corresponds to the sequence complementary to the sequence of the nucleotide numbers 1,336,091 to 1,337,692 in the genome sequence registered as Genbank Accession No. NC_003450 in the NCBI database, and is also called *NCgl1221*. The YggB protein encoded by the *yggB* gene of *Corynebacterium glutamicum* ATCC 13032 is registered as GenBank accession No. NP_600492. In addition, the nucleotide sequence of the *yggB* gene of *Corynebacterium glutamicum* 2256 (ATCC 13869) and the amino acid sequence of the YggB protein encoded by the gene are shown in SEQ ID NOS: 7 and 8, respectively.

[0051]

In the present invention, a *yggB* gene having the "specific mutation" described later is also referred to as "mutant *yggB* gene", and a protein encoded thereby is also referred to as "mutant YggB protein". Furthermore, in the present invention, a *yggB* gene not having the "specific mutation" described later is also referred to as "wild-type *yggB* gene", and a protein encoded thereby is also referred to as "wild-type YggB protein". Incidentally, as for the YggB protein, change of the amino acid sequence caused by the "specific mutation" in the *yggB* gene is also referred to as "specific mutation". The term "wild-type" referred to herein is used for convenience to distinguish the "wild-type" *yggB* gene or YggB protein from the "mutant" *yggB* gene or YggB protein, and the "wild-type" *yggB* gene or YggB protein is not limited to those obtained as natural substances, so long as it does not have the "specific mutation". Examples of the wild-type YggB protein include the YggB proteins exemplified above, such as YggB protein having the amino acid sequence of SEQ ID NO: 8. Examples of the wild-type YggB protein also include conservative variants (variants in which the original function thereof is maintained) of the YggB proteins exemplified above, provided that the conservative variants do not have the "specific mutation". The "original function" regarding the YggB protein may be, for example, a function as a mechanosensitive channel or a property that an increased expression thereof in a coryneform bacterium provides an improved L-glutamic acid-producing ability of the coryneform bacterium.

[0052]

The "specific mutation" is not particularly limited, so long as it changes the amino acid sequence of the YggB protein such as those described above to thereby improve an L-glutamic acid-producing ability of a coryneform bacterium. Examples of the "specific mutation" include mutation on the C-terminus side and mutation in a transmembrane region (WO2006/070944). The "specific mutation" may also be a combination of these.

[0053]

(1) Mutation on C-terminus side

The mutation on the C-terminus side is a mutation introduced into the region of the wild-type *yggB* gene coding for the amino acid residues of the positions 419 to 533 of the wild-type YggB protein. The mutation on the C-terminus side may be introduced at one or more sites in the region. The type of change of the amino acid

sequence induced by the mutation on the C-terminus side is not particularly limited. The mutation on the C-terminus side may be a mutation causing amino acid substitution (missense mutation), insertion of amino acid residue, deletion of amino acid residue, introduction of stop codon (nonsense mutation), frame shift mutation, or a combination of these. The mutation on the C-terminus side is preferably, for example, a mutation for inserting a nucleotide sequence such as an insertion sequence (henceforth also referred to as "IS") or transposon.

[0054]

(1-1) Insertion of nucleotide sequence

Examples of the mutation on the C-terminus side include, for example, a mutation that inserts a nucleotide sequence at the site coding for the valine residue at the position 419 of the wild-type YggB protein (2A-1 type mutation). The 2A-1 type mutation may be, for example, a mutation that causes deletion or substitution for a part or all of the amino acid residues of the positions 419 to 533 of the wild-type YggB protein. Specific examples of the mutant *yggB* gene having the 2A-1 type mutation include, for example, the *yggB* gene including IS inserted into the next of "G" at the position 1255 in SEQ ID NO: 7, and thereby coding for a mutant YggB protein having a full length of 423 amino residues, which is shorter than that of the original wild-type YggB protein (SEQ ID NO: 8). The nucleotide sequence of this mutant *yggB* gene (V419::IS) and the amino acid sequence of the mutant YggB protein encoded by the gene are shown in SEQ ID NOS: 9 and 10, respectively. In the SEQ ID NO: 9, the positions 1 to 1269 correspond to CDS for this mutant YggB protein (V419::IS). Specific examples of the L-glutamic acid-producing bacterium having the mutant *yggB* gene (V419::IS) include, for example, the *C. glutamicum* 2256 Δ sucA Δ ldhA *yggB** strain (WO2014/185430).

[0055]

(1-2) Substitution for proline residues

Examples of the mutation on the C-terminus side also include, for example, a mutation that replaces a proline residue present at the positions 419 to 533 of the wild-type YggB protein with another amino acid residue. Examples of such a proline residue include the proline residues at the positions 424, 437, 453, 457, 462, 469, 484, 489, 497, 515, 529, and 533 of the wild-type YggB protein. It is especially preferable to replace the proline residue(s) of the position(s) 424 and/or 437 with other amino acid

residue(s). The "other amino acid" is not particularly limited so long as it is a naturally occurring amino acid other than proline. Examples of the "other amino acid" include Lys, Glu, Thr, Val, Leu, Ile, Ser, Asp, Asn, Gln, Arg, Cys, Met, Phe, Trp, Tyr, Gly, Ala, and His. For example, the proline residue at the position 424 may be replaced with preferably a residue of hydrophobic amino acid (Ala, Gly, Val, Leu, or Ile), more preferably a residue of branched chain amino acid (Leu, Val, or Ile). Furthermore, for example, the proline residue at the position 437 may be replaced preferably with a residue of an amino acid having hydroxyl group in the side chain (Thr, Ser, or Tyr), more preferably with a Ser residue.

[0056]

(2) Mutation in transmembrane region

The YggB protein is estimated to have five transmembrane regions. The transmembrane regions correspond to the amino acid residues of the positions 1 to 23 (first transmembrane region), the positions 25 to 47 (second transmembrane region), the positions 62 to 84 (third transmembrane region), the positions 86 to 108 (fourth transmembrane region), and the positions 110 to 132 (fifth transmembrane region) of the wild-type YggB protein. The mutation in a transmembrane region is a mutation in the regions coding for these transmembrane regions of the wild-type *yggB* gene. The mutation in transmembrane region may be introduced into one or more sites in the regions. The mutation in transmembrane region is preferably a mutation that induces substitution, deletion, addition, insertion, or inversion of one or several amino acid residues, but does not include any frame shift mutation or nonsense mutation. The number meant by the term "one or several" is preferably 1 to 20, more preferably 1 to 10, still more preferably 1 to 5, particularly preferably 1 to 3. Examples of the mutation in a transmembrane region include a mutation that inserts one or several amino acid residues, such as Cys-Ser-Leu, between the leucine residue at the position 14 and the tryptophan residue at the position 15; a mutation that replaces the alanine residue at the position 100 with another amino acid residue, such as a residue of an amino acid having hydroxyl group in the side chain (i.e. Thr, Ser, or Tyr), preferably Thr residue; a mutation that replaces the alanine residue at the position 111 with another amino acid residue such as a residue of an amino acid having hydroxyl group in the side chain (i.e. Thr, Ser, or Tyr), preferably Thr residue; in the wild-type YggB protein

[0057]

In the present invention, an "amino acid residue at the position X of the wild-type YggB protein" means the amino acid residue corresponding to that of the position X in SEQ ID NO: 8, unless otherwise stated. The "position X" in an amino acid sequence is the X-th position counted from the N-terminus of the amino acid sequence, and the amino acid residue of the N-terminus is the amino acid residue of the position 1. That is, the aforementioned positions of amino acid residues indicate relative positions, and the absolute positions thereof may shift due to deletion, insertion, addition, or the like of an amino acid residue or residues. For example, the "amino acid residue at the position 419 of the wild-type YggB protein" means the amino acid residue corresponding to that of the position 419 in SEQ ID NO: 8, and when one amino acid residue is deleted at a position on the N-terminus side of the position 419, the 418th amino acid residue from the N-terminus is "the amino acid residue at the position 419 of the wild-type YggB protein". Furthermore, when one amino acid residue is inserted a position on the N-terminus side of the position 419, the 420th amino acid residue from the N-terminus is "the amino acid residue at the position 419 of the wild-type YggB protein". Specifically, for example, amino acid residues of the positions 419 to 529 of the YggB protein of *Corynebacterium glutamicum* ATCC14967 correspond to amino acid residues of the positions 419 to 533 of the wild-type YggB protein.

[0058]

Which amino acid residue is "the amino acid residue corresponding to that of the position X in SEQ ID NO: 8" in the amino acid sequence of an arbitrary YggB protein can be determined by alignment between the amino acid sequence of the arbitrary YggB protein and the amino acid sequence of SEQ ID NO: 8. The alignment can be performed by, for example, using known gene analysis software. Specific examples of such software include DNASIS produced by Hitachi Solutions, GENETYX produced by Genetyx, and so forth (Elizabeth C. Tyler et al., Computers and Biomedical Research, 24 (1) 72-96, 1991; Barton GJ et al., Journal of Molecular Biology, 198 (2), 327-37, 1987).

[0059]

A mutant *yggB* gene can be obtained by modifying a wild-type *yggB* gene so as to have the aforementioned "specific mutation". The modification of DNA can be performed by a known method. For example, an objective mutation can be introduced into an objective site of DNA by the site-specific mutation method. Specific examples

of the site-specific mutation method include, for example, a method of using PCR (Higuchi, R., 61, in PCR Technology, Erlich, H.A. Eds., Stockton Press, 1989; Carter P., Meth. In Enzymol., 154, 382, 1987), and a method of using a phage (Kramer, W. and Frits, H.J., Meth. in Enzymol., 154, 350, 1987; Kunkel, T.A. et al., Meth. in Enzymol., 154, 367, 1987). Furthermore, a mutant *yggB* gene can also be obtained by chemical synthesis.

[0060]

Such modification of a bacterium that the bacterium has a mutant *yggB* gene can be attained by introducing the mutant *yggB* gene into the bacterium. Such modification of a bacterium that the bacterium has a mutant *yggB* gene can also be attained by introducing a mutation into the *yggB* gene of the bacterium through natural mutation or a treatment with a mutagen.

[0061]

The methods for imparting or enhancing L-glutamic acid-producing ability can also be effective for imparting or enhancing an ability to produce L-amino acids that are biosynthesized via L-glutamic acid as an intermediate, such as L-glutamine, L-proline, L-arginine, L-citrulline, and L-ornithine. Hence, a bacterium having an ability to produce any of these L-amino acids that are biosynthesized via L-glutamic acid may have, as required, such a property possessed by an L-glutamic acid-producing bacterium as mentioned above. For example, a bacterium having an ability to produce any of these L-amino acids that are biosynthesized via L-glutamic acid may have been modified so that the activity of α -ketoglutarate dehydrogenase and/or succinate dehydrogenase is reduced.

[0062]

<L-Glutamine-producing bacteria>

Examples of the method for imparting or enhancing L-glutamine-producing ability include, for example, a method of modifying a bacterium so that the activity or activities of one or more kinds of enzymes selected from the L-glutamine biosynthesis enzymes are enhanced. Examples of such enzymes include, but not particularly limited to, glutamate dehydrogenase (*gdhA*) and glutamine synthetase (*glnA*). The glutamine synthetase activity can also be enhanced by disruption of the glutamine adenylyltransferase gene (*glnE*) or disruption of the PII control protein gene (*glnB*) (EP1229121).

[0063]

Examples of the method for imparting or enhancing L-glutamine-producing ability also include, for example, a method of modifying a bacterium so that the activity or activities of one or more kinds of enzymes that catalyze a reaction branching away from the biosynthesis pathway of L-glutamine to generate a compound other than L-glutamine are reduced. Examples of such enzymes include, but not particularly limited to, glutaminase.

[0064]

Specific examples of L-glutamine-producing bacteria and parent strains for deriving them include, for example, coryneform bacteria in which the activity or activities of glutamate dehydrogenase (*gdhA*) and/or glutamine synthetase (*glnA*) (EP1229121, EP1424398) are enhanced, and coryneform bacteria in which the glutaminase activity (Japanese Patent Laid-open (Kokai) No. 2004-187684) is reduced. Examples of L-glutamine-producing bacteria and parental strains for deriving them include a strain belonging to the genus *Escherichia* and having a mutant glutamine synthetase in which the tyrosine residue of the position 397 of glutamine synthetase has been replaced with another amino acid residue (US2003-0148474A).

[0065]

Examples of the methods for imparting or enhancing L-glutamine-producing ability to or in coryneform bacteria also include the method of imparting 6-diazo-5-oxonorleucine resistance (Japanese Patent Laid-open (Kokai) No. 3-232497), the method of imparting purine analogue resistance and methionine sulfoxide resistance (Japanese Patent Laid-open (Kokai) No. 61-202694), and the method of imparting α -ketomalonic acid resistance (Japanese Patent Laid-open (Kokai) No. 56-151495). Specific examples of coryneform bacteria having L-glutamine-producing ability include, for example, the following strains.

Corynebacterium glutamicum (*Brevibacterium flavum*) AJ11573 (FERM P-5492, Japanese Patent Laid-open (Kokai) No. 56-151495)

Corynebacterium glutamicum (*Brevibacterium flavum*) AJ11576 (FERM BP-10381, Japanese Patent Laid-open (Kokai) No. 56-151495)

Corynebacterium glutamicum (*Brevibacterium flavum*) AJ12212 (FERM P-8123, Japanese Patent Laid-open (Kokai) No. 61-202694)

[0066]

<L-Proline-producing bacteria>

Examples of methods for imparting or enhancing L-proline-producing ability include, for example, a method of modifying a bacterium so that the bacterium has an increased activity or activities of one or more kinds of enzymes selected from the L-proline biosynthesis enzymes. Examples of such enzymes include glutamate-5-kinase (*proB*), γ -glutamylphosphate reductase, and proline-5-carboxylate reductase (*putA*). For enhancing the activity of such an enzyme, for example, the *proB* gene encoding a glutamate-5-kinase desensitized to feedback inhibition by L-proline (German Patent No. 3127361) can be preferably used.

[0067]

Examples of methods for imparting or enhancing L-proline-producing ability also include, for example, a method of modifying a bacterium so that the bacterium has a reduced activity of an enzyme involved in decomposition of L-proline. Examples of such an enzyme include proline dehydrogenase and ornithine aminotransferase.

[0068]

Specific examples of L-proline-producing bacteria and parental strains for deriving them include, for example, *E. coli* NRRL B-12403 and NRRL B-12404 (British Patent No. 2075056), *E. coli* VKPM B-8012 (Russian Patent Application No. 2000124295), *E. coli* plasmid mutant strains described in German Patent No. 3127361, *E. coli* plasmid mutant strains described by Bloom F.R. et al. (The 15th Miami winter symposium, 1983, p. 34), *E. coli* 702 strain (VKPM B-8011), which is a 3,4-dehydroxyproline and azetidine-2-carboxylate resistant strain, and *E. coli* 702ilvA strain (VKPM B-8012), which is an *ilvA* gene-deficient strain of the 702 strain (EP1172433).

[0069]

<L-Threonine-producing bacteria>

Examples of methods for imparting or enhancing L-threonine-producing ability include, for example, a method of modifying a bacterium so that the bacterium has an increased activity or activities of one or more kinds of enzymes selected from the L-threonine biosynthesis enzymes. Examples of such enzymes include, but not particularly limited to, aspartokinase III (*lysC*), aspartate semialdehyde dehydrogenase (*asd*), aspartokinase I (*thrA*), homoserine kinase (*thrB*), threonine synthase (*thrC*), and aspartate aminotransferase (aspartate transaminase) (*aspC*). Among these enzymes, it is preferable to enhance activity or activities of one or more kinds of enzymes selected

from aspartokinase III, aspartate semialdehyde dehydrogenase, aspartokinase I, homoserine kinase, aspartate aminotransferase, and threonine synthase. Any of the genes encoding the L-threonine biosynthesis enzymes can be introduced into a bacterium having a reduced ability to decompose threonine. Examples of such a strain in which threonine decomposition is suppressed include, for example, the *E. coli* TDH6 strain, which is deficient in the threonine dehydrogenase activity (Japanese Patent Laid-open (Kokai) No. 2001-346578).

[0070]

The activities of the L-threonine biosynthesis enzymes are inhibited by the endproduct, L-threonine. Therefore, for constructing L-threonine-producing strains, it is preferred that the genes of the L-threonine biosynthesis enzymes are modified so that the enzymes are desensitized to feedback inhibition by L-threonine. The aforementioned *thrA*, *thrB*, and *thrC* genes constitute the threonine operon, which forms an attenuator structure. The expression of the threonine operon is inhibited by isoleucine and threonine in the culture broth and also suppressed by attenuation. Therefore, expression of the threonine operon can be enhanced by removing the leader sequence or the attenuator in the attenuation region (Lynn, S.P., Burton, W.S., Donohue, T.J., Gould, R.M., Gumpert, R.L, and Gardner, J.F., J. Mol. Biol. 194:59-69 (1987); WO02/26993; WO2005/049808; and WO2003/097839).

[0071]

The native promoter of the threonine operon is present upstream of the threonine operon, and can be replaced with a non-native promoter (WO98/04715). Also, the threonine operon may be constructed so that the threonine biosynthesis genes are expressed under control of the repressor and promoter of λ -phage (EP0593792B). Furthermore, a bacterium modified so that it is desensitized to feedback inhibition by L-threonine can also be obtained by selecting a strain resistant to α -amino- β -hydroxyisovaleric acid (AHV), which is an L-threonine analogue.

[0072]

It is preferred that the expression amount of the threonine operon that is modified so as to be desensitized to feedback inhibition by L-threonine as described above is increased in a host by increasing the copy number thereof or by ligating it to a potent promoter. The copy number can be increased by introducing a plasmid containing the threonine operon into a host. The copy number can also be increased

by transferring the threonine operon to the genome of a host using a transposon, Mu-phage, or the like.

[0073]

Examples of methods for imparting or enhancing L-threonine-producing ability also include, for example, a method of imparting L-threonine resistance to a host, and a method of imparting L-homoserine resistance to a host. Such resistance can be imparted by, for example, enhancing the expression of a gene that imparts L-threonine resistance or a gene that imparts L-homoserine resistance. Examples of the genes that impart the above-mentioned resistance include the *rhtA* gene (Res. Microbiol. 154:123-135 (2003)), *rhtB* gene (EP0994190A), *rhtC* gene (EP1013765A), *yfiK* gene, and *yeaS* gene (EP1016710A). As for methods for imparting L-threonine resistance to a host, those described in EP0994190A and WO90/04636 can be referred to.

[0074]

Specific examples of L-threonine-producing bacteria and parental strains for deriving them include, for example, *E. coli* TDH-6/pVIC40 (VKPM B-3996, U.S. Patent Nos. 5,175,107 and 5,705,371), *E. coli* 472T23/pYN7 (ATCC 98081, U.S. Patent No. 5,631,157), *E. coli* NRRL-21593 (U.S. Patent No. 5,939,307), *E. coli* FERM BP-3756 (U.S. Patent No. 5,474,918), *E. coli* FERM BP-3519 and FERM BP-3520 (U.S. Patent No. 5,376,538), *E. coli* MG442 (Gusyatiner et al., Genetika (in Russian), 14, 947-956 (1978)), *E. coli* VL643 and VL2055 (EP1149911A), and *E. coli* VKPM B-5318 (EP0593792B).

[0075]

The VKPM B-3996 strain is a strain obtained by introducing the plasmid pVIC40 into the TDH-6 strain. The TDH-6 strain has sucrose-assimilating ability and is deficient in the *thrC* gene, and the *ilvA* gene thereof has a leaky mutation. The TDH-6 strain also has a mutation in the *rhtA* gene, which imparts resistance to high concentration of threonine or homoserine. The plasmid pVIC40 is a plasmid obtained by inserting the *thrA*BC* operon containing a mutant *thrA* gene encoding an aspartokinase-homoserine dehydrogenase I resistant to feedback inhibition by threonine and the wild-type *thrBC* genes into an RSF1010-derived vector (U.S. Patent No. 5,705,371). This mutant *thrA* gene encodes an aspartokinase-homoserine dehydrogenase I which is substantially desensitized to feedback inhibition by threonine. The B-3996 strain was deposited on November 19, 1987 at the All-Union Scientific

Center of Antibiotics (Nagatinskaya Street 3-A, 117105 Moscow, Russia) under the accession number RIA 1867. This strain was also deposited at the Russian National Collection of Industrial Microorganisms (VKPM, FGUP GosNII Genetika, 1 Dorozhny proezd., 1 Moscow 117545, Russia) on April 7, 1987 under the accession number VKPM B-3996.

[0076]

The VKPM B-5318 strain is prototrophic with regard to isoleucine, and harbors the plasmid pPRT614, which corresponds to the plasmid pVIC40 of which the regulatory region of the threonine operon is replaced with the temperature-sensitive λ -phage CI repressor and PR promoter. The VKPM B-5318 strain was deposited at the Russian National Collection of Industrial Microorganisms (VKPM, FGUP GosNII Genetika, 1 Dorozhny proezd., 1 Moscow 117545, Russia) on May 3, 1990 under the accession number of VKPM B-5318.

[0077]

The *thrA* gene which encodes aspartokinase-homoserine dehydrogenase I of *E. coli* has been elucidated (nucleotide numbers 337 to 2799, GenBank accession NC_000913.2, gi: 49175990). The *thrA* gene is located between the *thrL* and *thrB* genes on the chromosome of *E. coli* K-12. The *thrB* gene which encodes homoserine kinase of *Escherichia coli* has been elucidated (nucleotide numbers 2801 to 3733, GenBank accession NC_000913.2, gi: 49175990). The *thrB* gene is located between the *thrA* and *thrC* genes on the chromosome of *E. coli* K-12. The *thrC* gene which encodes threonine synthase of *E. coli* has been elucidated (nucleotide numbers 3734 to 5020, GenBank accession NC_000913.2, gi: 49175990). The *thrC* gene is located between the *thrB* gene and the *yaaX* open reading frame on the chromosome of *E. coli* K-12. The *thrA*BC* operon containing a mutant *thrA* gene which encodes an aspartokinase-homoserine dehydrogenase I resistant to feedback inhibition by threonine and the wild-type *thrBC* genes can be obtained from the well-known plasmid pVIC40, which is present in the threonine-producing *E. coli* strain VKPM B-3996 (U.S. Patent No. 5,705,371).

[0078]

The *rhtA* gene of *E. coli* is located at 18 min on the *E. coli* chromosome close to the *glnHPQ* operon, which encodes components of the glutamine transport system. The *rhtA* gene is identical to ORF1 (*ybiF* gene, nucleotide numbers 764 to 1651,

GenBank accession number AAA218541, gi:440181) and locates between the *pexB* and *ompX* genes. The unit expressing a protein encoded by the ORF1 has been designated *rhtA* gene (rht: resistance to homoserine and threonine). It has also been revealed that the *rhtA23* mutation that imparts resistance to high concentration of threonine or homoserine is an A-for-G substitution at position -1 with respect to the ATG start codon (ABSTRACTS of the 17th International Congress of Biochemistry and Molecular Biology in conjugation with Annual Meeting of the American Society for Biochemistry and Molecular Biology, San Francisco, California August 24-29, 1997, abstract No. 457; EP1013765A).

[0079]

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

[0080]

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

[0081]

Furthermore, examples of coryneform bacteria having L-threonine-producing ability include, for example, *Corynebacterium acetoacidophilum* AJ12318 (FERM BP-1172, U.S. Patent No. 5,188,949).

[0082]

<L-Lysine-producing bacteria>

Examples of methods for imparting or enhancing L-lysine-producing ability include, for example, a method of modifying a bacterium so that the bacterium has an increased activity or activities of one or more kinds of enzymes selected from the L-lysine biosynthesis enzymes. Examples of such enzymes include, but not particularly limited to, dihydrodipicolinate synthase (*dapA*), aspartokinase III (*lysC*), dihydrodipicolinate reductase (*dapB*), diaminopimelate decarboxylase (*lysA*),

diaminopimelate dehydrogenase (*ddh*) (U.S. Patent No. 6,040,160), phosphoenolpyruvate carboxylase (*ppc*), aspartate semialdehyde dehydrogenase (*asd*), aspartate aminotransferase (aspartate transaminase) (*aspC*), diaminopimelate epimerase (*dapF*), tetrahydrodipicolinate succinylase (*dapD*), succinyl diaminopimelate deacylase (*dapE*), and aspartase (*aspA*) (EP1253195A). It is preferable to enhance the activity or activities of one or more kinds of enzymes selected from, for example, dihydrodipicolinate reductase, diaminopimelate decarboxylase, diaminopimelate dehydrogenase, phosphoenolpyruvate carboxylase, aspartate aminotransferase, diaminopimelate epimerase, aspartate semialdehyde dehydrogenase, tetrahydrodipicolinate succinylase, and succinyl diaminopimelate deacylase, among these enzymes. Furthermore, L-lysine-producing bacteria and parental strains for deriving them can express an increased level of the gene involved in energy efficiency (*cyo*) (EP1170376A), the gene encoding nicotinamide nucleotide transhydrogenase (*pntAB*) (U.S. Patent No. 5,830,716), the *ybjE* gene (WO2005/073390), or combinations of these. Since aspartokinase III (*lysC*) is subject to feedback inhibition by L-lysine, a mutant *lysC* gene encoding an aspartokinase III desensitized to feedback inhibition by L-lysine (U.S. Patent No. 5,932,453) may be used for enhancing the activity of this enzyme. Examples of the aspartokinase III desensitized to feedback inhibition by L-lysine include aspartokinase III derived from *Escherichia coli* and having one or more mutations selected from a mutation for replacing the methionine residue at position 318 with an isoleucine residue; a mutation for replacing the glycine residue at position 323 with an aspartic acid residue; and a mutation for replacing the threonine residue at position 352 with an isoleucine residue (U.S. Patent Nos. 5,661,012 and 6,040,160). Furthermore, since dihydrodipicolinate synthase (*dapA*) is subject to feedback inhibition by L-lysine, a mutant *dapA* gene encoding a dihydrodipicolinate synthase desensitized to feedback inhibition by L-lysine may be used for enhancing the activity of this enzyme. Examples of the dihydrodipicolinate synthase desensitized to feedback inhibition by L-lysine include dihydrodipicolinate synthase derived from *Escherichia coli* and having a mutation for replacing the histidine residue at position 118 with a tyrosine residue (U.S. Patent No. 6,040,160).

[0083]

Examples of methods for imparting or enhancing L-lysine-producing ability also include, for example, a method of modifying a bacterium so that the bacterium has

a reduced activity or activities of one or more kinds of enzymes selected from the enzymes that catalyze a reaction branching away from the biosynthetic pathway of L-lysine to generate a compound other than L-lysine. Examples of such enzymes include, but not particularly limited to, homoserine dehydrogenase, lysine decarboxylase (U.S. Patent No. 5,827,698), and malic enzyme (WO2005/010175).

[0084]

Furthermore, examples of methods for imparting or enhancing L-lysine-producing ability to or in coryneform bacteria also include a method of modifying the bacteria so that the activity of a lysine excretion system (*lysE*) is increased (WO97/23597). The *lysE* gene of *Corynebacterium glutamicum* ATCC 13032 corresponds to the sequence complementary to the sequence of the nucleotide numbers 1,329,712 to 1,330,413 in the genome sequence registered as Genbank Accession No. NC_006958 (VERSION NC_006958.1 GI:62388892) in the NCBI database. The LysE protein of *Corynebacterium glutamicum* ATCC 13032 is registered as GenBank accession No. YP_225551 (YP_225551.1 GI:62390149).

[0085]

Examples of L-lysine-producing bacteria and parental strains for deriving them also include mutant strains having resistance to an L-lysine analogue. L-Lysine analogues inhibit the growth of bacteria such as bacteria of the family *Enterobacteriaceae* and coryneform bacteria, but this inhibition is fully or partially released when L-lysine is present in the medium. Examples of these L-lysine analogues include, but not particularly limited to, oxalysine, lysine hydroxamate, S-(2-aminoethyl)-L-cysteine (AEC), γ -methyllysine, and α -chlorocaprolactam. Mutant strains having resistance to these lysine analogues can be obtained by subjecting a bacterium to a conventional artificial mutagenesis treatment.

[0086]

Specific examples of L-lysine-producing bacteria and parental strains for deriving them include *E. coli* AJ11442 (FERM BP-1543, NRRL B-12185, U.S. Patent No. 4,346,170) and *E. coli* VL611. In these strains, aspartokinase is desensitized to feedback inhibition by L-lysine.

[0087]

Specific examples of L-lysine-producing bacteria and parental strains for deriving them also include the *E. coli* WC196 strain. The WC196 strain was bred by

imparting AEC resistance to the W3110 strain, which was derived from *E. coli* K-12 (U.S. Patent No. 5,827,698). The WC196 strain was designated *E. coli* AJ13069 and deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (currently, independent administrative agency, National Institute of Technology and Evaluation, International Patent Organism Depositary, #120, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken, 292-0818, Japan) on December 6, 1994 and assigned an accession number of FERM P-14690. Then, the deposit was converted to an international deposit under the provisions of the Budapest Treaty on September 29, 1995, and assigned an accession number of FERM BP-5252 (U.S. Patent No. 5,827,698).

[0088]

Preferred examples of L-lysine-producing bacteria include *E. coli* WC196 Δ cadA Δ ldc and *E. coli* WC196 Δ cadA Δ ldc/pCABD2 (WO2010/061890). The *E. coli* WC196 Δ cadA Δ ldc is a strain constructed from the WC196 strain by disrupting the *cadA* and *ldcC* genes encoding lysine decarboxylase. The WC196 Δ cadA Δ ldc/pCABD2 strain was constructed by introducing the plasmid pCABD2 containing lysine biosynthesis enzyme genes (U.S. Patent No. 6,040,160) into the WC196 Δ cadA Δ ldc strain. The WC196 Δ cadA Δ ldc strain, designated as AJ110692, was deposited at the independent administrative agency, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary (currently, independent administrative agency, National Institute of Technology and Evaluation, International Patent Organism Depositary, #120, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken, 292-0818, Japan) on October 7, 2008 as an international deposit, and assigned an accession number of FERM BP-11027. The plasmid pCABD2 contains a mutant *dapA* gene derived from *Escherichia coli* and encoding a dihydrodipicolinate synthase (DDPS) having a mutation for desensitization to feedback inhibition by L-lysine (H118Y), a mutant *lysC* gene derived from *Escherichia coli* and encoding aspartokinase III having a mutation for desensitization to feedback inhibition by L-lysine (T352I), the *dapB* gene derived from *Escherichia coli* and encoding dihydrodipicolinate reductase, and the *ddh* gene derived from *Brevibacterium lactofermentum* and encoding diaminopimelate dehydrogenase.

[0089]

Preferred examples of L-lysine-producing bacteria also include *E. coli* AJIK01

(NITE BP-01520). The AJIK01 strain was designated *E. coli* AJ111046, and deposited at the independent administrative agency, National Institute of Technology and Evaluation, International Patent Organism Depository (#122, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken, 292-0818, Japan) on January 29, 2013. Then, it was converted to an international deposit under the provisions of the Budapest Treaty on May 15, 2014, and assigned an accession number of NITE BP-01520.

[0090]

Examples of coryneform bacteria having L-lysine-producing ability include, for example, the AEC-resistant mutant strains (*Corynebacterium glutamicum* (*Brevibacterium lactofermentum* AJ11082) (NRRL B-11470) strain etc., Japanese Patent Publication (Kokoku) Nos. 56-1914, 56-1915, 57-14157, 57-14158, 57-30474, 58-10075, 59-4993, 61-35840, 62-24074, 62-36673, 5-11958, 7-112437, and 7-112438); mutant strains requiring an amino acid such as L-homoserine for their growth (Japanese Patent Publication Nos. 48-28078 and 56-6499); mutant strains showing resistance to AEC and further requiring an amino acid such as L-leucine, L-homoserine, L-proline, L-serine, L-arginine, L-alanine, and L-valine (U.S. Patent Nos. 3,708,395 and 3,825,472); mutant strains showing resistance to DL- α -amino- ϵ -caprolactam, α -amino-lauryllactam, aspartic acid analogue, sulfa drug, quinoid, and N-lauroylleucine; mutant strains showing resistance to an oxaloacetate decarboxylase inhibitor or a respiratory chain enzyme inhibitor (Japanese Patent Laid-open (Kokai) Nos. 50-53588, 50-31093, 52-102498, 53-9394, 53-86089, 55-9783, 55-9759, 56-32995, 56-39778, Japanese Patent Publication Nos. 53-43591 and 53-1833); mutant strains requiring inositol or acetic acid (Japanese Patent Laid-open (Kokai) Nos. 55-9784 and 56-8692); mutant strains that are susceptible to fluoropyruvic acid or a temperature of 34°C or higher (Japanese Patent Laid-open (Kokai) Nos. 55-9783 and 53-86090); and mutant strains showing resistance to ethylene glycol (U.S. Patent No. 4,411,997).

[0091]

<L-Arginine-producing bacteria>

Examples of methods for imparting or enhancing L-arginine-producing ability include, for example, a method of modifying a bacterium so that the bacterium has an increased activity or activities of one or more kinds of enzymes selected from the L-arginine biosynthesis enzymes. Examples of such enzymes include, but not particularly limited to, N-acetylglutamate synthase (*argA*), N-acetylglutamate kinase

(*argB*), N-acetylglutamyl phosphate reductase (*argC*), acetylornithine transaminase (*argD*), acetylornithine deacetylase (*argE*), ornithine carbamoyl transferase (*argF*, *argI*), argininosuccinate synthetase (*argG*), argininosuccinate lyase (*argH*), ornithine acetyl transferase (*argJ*), and carbamoyl phosphate synthetase (*carAB*). As the N-acetylglutamate synthase gene (*argA*), for example, a gene encoding a mutant N-acetylglutamate synthase desensitized to feedback inhibition by L-arginine by substitution for the amino acid residues corresponding to the positions 15 to 19 of the wild type enzyme (EP1170361A) can preferably be used.

[0092]

Specific examples of L-arginine-producing bacteria and parental strains for deriving them include, for example, the *E. coli* 237 strain (VKPM B-7925, US2002-058315A1), derivative strains thereof introduced with the *argA* gene encoding a mutant N-acetyl glutamate synthase (Russian Patent Application No. 2001112869, EP1170361A1), *E. coli* 382 strain derived from the 237 strain and having an improved acetic acid-assimilating ability (VKPM B-7926, EP1170358A1), and *E. coli* 382ilvA+ strain, which is a strain obtained from the 382 strain by introducing the wild-type *ilvA* gene from *E. coli* K-12 strain thereto. The *E. coli* strain 237 was deposited at the Russian National Collection of Industrial Microorganisms (VKPM, FGUP GosNII Genetika, 1 Dorozhny proezd., 1 Moscow 117545, Russia) on April 10, 2000 under an accession number of VKPM B-7925, and the deposit was converted to an international deposit under the provisions of Budapest Treaty on May 18, 2001. The *E. coli* 382 strain was deposited at the Russian National Collection of Industrial Microorganisms (VKPM, FGUP GosNII Genetika, 1 Dorozhny proezd., 1 Moscow 117545, Russia) on April 10, 2000 under accession number of VKPM B-7926.

[0093]

Examples of L-arginine-producing bacteria and parental strains for deriving them also include strains having resistance to amino acid analogues, and so forth. Examples of such strains include *E. coli* mutant strains having resistance to α -methylmethionine, p-fluorophenylalanine, D-arginine, arginine hydroxamate, S-(2-aminoethyl)-cysteine, α -methylserine, β -2-thienylalanine, or sulfaguanidine (Japanese Patent Laid-open (Kokai) No. 56-106598).

[0094]

Examples of L-arginine-producing bacteria and parent strains for deriving them

also include such coryneform bacteria as a strain deficient in ArgR, which is an arginine repressor (US2002-0045223A), and a strain in which glutamine synthetase activity is increased (US2005-0014236A).

[0095]

Examples of L-arginine-producing bacteria and parent strains for deriving them also include mutant strains of coryneform bacteria, the mutant strains having resistance to an amino acid analogue or the like. Examples of such strains include, for example, strains having resistance to 2-thiazolealanine and further exhibiting auxotrophy for L-histidine, L-proline, L-threonine, L-isoleucine, L-methionine, or L-tryptophan (Japanese Patent Laid-open (Kokai) No. 54-44096); strains resistant to ketomalonic acid, fluoromalonic acid, or monofluoroacetic acid (Japanese Patent Laid-open (Kokai) No. 57-18989); strains resistant to argininol (Japanese Patent Publication No. 62-24075); strains resistant to X-guanidine (X represents an aliphatic chain or a derivative thereof, Japanese Patent Laid-open (Kokai) No. 2-186995); and strains resistant to arginine hydroxamate and 6-azauracil (Japanese Patent Laid-open (Kokai) No. 57-150381). Specific examples of coryneform bacteria having L-arginine-producing ability include the following strains.

Corynebacterium glutamicum (*Brevibacterium flavum*) AJ11169 (FERM BP-6892)

Corynebacterium glutamicum (*Brevibacterium lactofermentum*) AJ12092 (FERM BP-6906)

Corynebacterium glutamicum (*Brevibacterium flavum*) AJ11336 (FERM BP-6893)

Corynebacterium glutamicum (*Brevibacterium flavum*) AJ11345 (FERM BP-6894)

Corynebacterium glutamicum (*Brevibacterium lactofermentum*) AJ12430 (FERM BP-2228)

[0096]

<L-Citrulline-producing bacteria and L-ornithine-producing bacteria>

L-citrulline and L-ornithine are intermediates of the biosynthetic pathway of L-arginine. Hence, examples of methods for imparting or enhancing an ability to produce L-citrulline and/or L-ornithine include, for example, a method of modifying a bacterium so that the bacterium has an increased activity or activities of one or more

kinds of enzymes selected from the L-arginine biosynthesis enzymes. Examples of such enzymes include, but not particularly limited to, N-acetylglutamate synthase (*argA*), N-acetylglutamate kinase (*argB*), N-acetylglutamyl phosphate reductase (*argC*), acetylornithine transaminase (*argD*), acetylornithine deacetylase (*argE*), ornithine carbamoyl transferase (*argF*, *argI*), ornithine acetyl transferase (*argJ*), and carbamoyl phosphate synthetase (*carAB*), for L-citrulline. Furthermore, Examples of such enzymes include, but not particularly limited to, N-acetylglutamate synthase (*argA*), N-acetylglutamate kinase (*argB*), N-acetylglutamyl phosphate reductase (*argC*), acetylornithine transaminase (*argD*), acetylornithine deacetylase (*argE*), and ornithine acetyl transferase (*argJ*), for L-ornithine.

[0097]

An L-citrulline-producing bacterium can be easily obtained from, for example, an L-arginine bacterium such as the *E. coli* 382 strain (VKPM B-7926) by decreasing the activity of argininosuccinate synthetase encoded by *argG* gene. Also, an L-ornithine-producing bacterium can be easily obtained from, for example, an L-arginine bacterium such as the *E. coli* 382 strain (VKPM B-7926) by decreasing the activity of ornithine carbamoyl transferase encoded by *argF* and *argI* genes.

[0098]

Specific examples of L-citrulline-producing bacteria and parental strains for deriving them include, for example, strains belonging to the genus *Escherichia*, such as the *E. coli* strains 237/pMADS11, 237/pMADS12, and 237/pMADS13, which have a mutant N-acetylglutamate synthase (Russian patent No. 2,215,783, U.S. Patent No. 6,790,647, and EP1170361B1), *E. coli* strains 333 (VKPM B-8084) and 374 (VKPM B-8086), which have carbamoyl phosphate synthetase resistant to feedback inhibition (Russian patent No. 2,264,459), *E. coli* strains having an increased activity of α -ketoglutarate synthase and having a modified activity of ferredoxin NADP⁺ reductase, pyruvate synthase, and/or α -ketoglutarate dehydrogenase (EP2133417A); and the *P. ananatis* NA1sucAsdhA strain, which has a reduced activity of succinate dehydrogenase and α -ketoglutarate dehydrogenase (US2009-286290A1).

[0099]

<L-Histidine-producing bacteria>

Examples of methods for imparting or enhancing L-histidine-producing ability include, for example, a method of modifying a bacterium so that the bacterium has an

increased activity or activities of one or more kinds of enzymes selected from the L-histidine biosynthesis enzymes. Examples of such enzymes include, but not particularly limited to, ATP phosphoribosyltransferase (*hisG*), phosphoribosyl AMP cyclohydrolase (*hisI*), phosphoribosyl-ATP pyrophosphohydrolase (*hisI*), phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase (*hisA*), amidotransferase (*hisH*), histidinol phosphate aminotransferase (*hisC*), histidinol phosphatase (*hisB*), and histidinol dehydrogenase (*hisD*).

[0100]

Among these enzymes, the L-histidine biosynthesis enzymes encoded by *hisG* and *hisBHAFI* are known to be inhibited by L-histidine. Therefore, the ability to produce L-histidine can be imparted or enhanced by, for example, introducing a mutation for conferring resistance to feedback inhibition into the gene encoding ATP phosphoribosyltransferase (*hisG*) (Russian Patent Nos. 2,003,677 and 2,119,536).

[0101]

Specific examples of L-histidine-producing bacteria and parental strains for deriving them include, for example, strains belonging to the genus *Escherichia*, such as the *E. coli* 24 strain (VKPM B-5945, RU2003677), *E. coli* NRRL B-12116 to B-12121 (U.S. Patent No. 4,388,405), *E. coli* H-9342 (FERM BP-6675) and H-9343 (FERM BP-6676, U.S. Patent No. 6,344,347), *E. coli* H-9341 (FERM BP-6674, EP1085087), *E. coli* AI80/pFM201 (U.S. Patent No. 6,258,554), *E. coli* FERM P-5038 and FERM P-5048, which have been introduced with a vector carrying a DNA encoding an L-histidine-biosynthesis enzyme (Japanese Patent Laid-open (Kokai) No. 56-005099), *E. coli* strains introduced with a gene for amino acid transport (EP1016710A), and *E. coli* 80 strain, which has been imparted with resistance to sulfaguanidine, DL-1,2,4-triazole-3-alanine, and streptomycin (VKPM B-7270, Russian Patent No. 2119536).

[0102]

<L-Cysteine-producing bacteria>

Examples of methods for imparting or enhancing L-cysteine-producing ability include, for example, a method of modifying a bacterium so that the bacterium has an increased activity or activities of one or more kinds of enzymes selected from the L-cysteine biosynthesis enzymes. Examples of such enzymes include, but not particularly limited to, serine acetyltransferase (*cysE*) and 3-phosphoglycerate dehydrogenase (*serA*). The serine acetyltransferase activity can be enhanced by, for

example, introducing a mutant *cysE* gene encoding a mutant serine acetyltransferase resistant to feedback inhibition by cysteine into a bacterium. Such a mutant serine acetyltransferase is disclosed in, for example, Japanese Patent Laid-open (Kokai) No. 11-155571 and US2005-0112731A. Furthermore, the 3-phosphoglycerate dehydrogenase activity can be enhanced by, for example, introducing a mutant *serA* gene encoding a mutant 3-phosphoglycerate dehydrogenase resistant to feedback inhibition by serine into a bacterium. Such a mutant 3-phosphoglycerate dehydrogenase is disclosed in, for example, U.S. Patent No. 6,180,373.

[0103]

Furthermore, examples of methods for imparting or enhancing L-cysteine-producing ability also include, for example, a method of modifying a bacterium so that the bacterium has a reduced activity or activities of one or more kinds of enzymes selected from the enzymes that catalyze a reaction branching away from the biosynthesis pathway of L-cysteine to generate a compound other than L-cysteine. Examples of such enzymes include, for example, enzymes involved in decomposition of L-cysteine. Examples of the enzymes involved in decomposition of L-cysteine include, but not particularly limited to, cystathionine- β -lyase (*metC*, Japanese Patent Laid-open (Kokai) No. 11-155571; Chandra et al., Biochemistry, 21 (1982) 3064-3069), tryptophanase (*tnaA*, Japanese Patent Laid-open (Kokai) No. 2003-169668; Austin Newton et al., J. Biol. Chem., 240 (1965) 1211-1218), O-acetylserine sulfhydrylase B (*cysM*, Japanese Patent Laid-open (Kokai) No. 2005-245311), the *malY* gene product (Japanese Patent Laid-open (Kokai) No. 2005-245311), the *d0191* gene product of *Pantoea ananatis* (Japanese Patent Laid-open (Kokai) No. 2009-232844), and cysteine desulfhydrase (*aecD*, Japanese Patent Laid-open (Kokai) No. 2002-233384).

[0104]

Furthermore, examples of methods for imparting or enhancing L-cysteine-producing ability also include, for example, a method of enhancing the L-cysteine excretory system, and a method of enhancing the sulfate/thiosulfate transport system. Examples of proteins of the L-cysteine excretory system include the protein encoded by the *ydeD* gene (Japanese Patent Laid-open (Kokai) No. 2002-233384), the protein encoded by the *yfiK* gene (Japanese Patent Laid-open (Kokai) No. 2004-49237), the proteins encoded by the *emrAB*, *emrKY*, *yoiIH*, *acrEF*, *bcr*, and *cusA* genes (Japanese Patent Laid-open (Kokai) No. 2005-287333), and the protein encoded by the *yeaS* gene

(Japanese Patent Laid-open (Kokai) No. 2010-187552). Examples of the proteins of the sulfate/thiosulfate transport system include the proteins encoded by the *cysPTWAM* gene cluster.

[0105]

Specific examples of L-cysteine-producing bacteria and parental strains for deriving them include, for example, *E. coli* JM15 transformed with different *cysE* alleles encoding feedback-resistant serine acetyltransferases (U.S. Patent No. 6,218,168, Russian patent application 2003121601), *E. coli* W3110 having an over-expressed gene encoding a protein suitable for secretion of a cytotoxic substance (U.S. Patent No. 5,972,663), *E. coli* strains having a reduced cysteine desulfohydase activity (Japanese Patent Laid-open (Kokai) No. 11-155571), and *E. coli* W3110 having an increased activity of a positive transcriptional regulator for cysteine regulon encoded by the *cysB* gene (WO01/27307A1).

[0106]

Furthermore, examples of coryneform bacteria having L-cysteine-producing ability include coryneform bacteria having serine acetyltransferase desensitized to feedback inhibition by L-cysteine thereby to show enhanced intracellular serine acetyltransferase activity (Japanese Patent Laid-open (Kokai) No. 2002-233384).

[0107]

<L-Serine-producing bacteria>

Examples of methods for imparting or enhancing L-serine-producing ability include, for example, a method of modifying a bacterium so that the bacterium has an increased activity or activities of one or more kinds of enzymes selected from the L-serine biosynthesis enzymes. Examples of such enzymes include, but not particularly limited to, 3-phosphoglycerate dehydrogenase (*serA*), phosphoserine transaminase (*serC*), and phosphoserine phosphatase (*serB*) (Japanese Patent Laid-open (Kokai) No. 11-253187). 3-phosphoglycerate dehydrogenase activity can be increased by, for example, introducing a mutant *serA* gene encoding a mutant 3-phosphoglycerate dehydrogenase resistant to feedback inhibition by L-serine into a bacterium. The mutant 3-phosphoglycerate dehydrogenase is disclosed in, for example, U.S. Patent No. 6,180,373.

[0108]

Examples of L-serine-producing bacteria and parental strains for deriving them

include, for example, coryneform bacteria resistant to azaserine or β -(2-thienyl)-DL-alanine and deficient in L-serine decomposition ability (Japanese Patent Laid-open (Kokai) No. 10-248588). Specific examples of such coryneform bacteria include, for example, *Corynebacterium glutamicum* (*Brevibacterium flavum*) AJ13324 (FERM P-16128), which is resistant to azaserine and deficient in L-serine decomposition ability, and *Corynebacterium glutamicum* (*Brevibacterium flavum*) AJ13325 (FERM P-16129), which is resistant to β -(2-thienyl)-DL-alanine and deficient in L-serine decomposition ability (Japanese Patent Laid-open (Kokai) No. 10-248588).

[0109]

<L-Methionine-producing bacteria>

Examples of L-methionine-producing bacteria and parental strains for deriving them include L-threonine auxotrophic strains and mutant strains resistant to norleucine (Japanese Patent Laid-open (Kokai) No. 2000-139471). Examples of L-methionine-producing bacteria and parental strains for deriving them also include a strain containing a mutant homoserine transsuccinylase resistant to feedback inhibition by L-methionine (Japanese Patent Laid-open (Kokai) No. 2000-139471, US2009-0029424A). Since L-methionine is biosynthesized via L-cysteine as an intermediate, L-methionine-producing ability can also be improved by improving L-cysteine-producing ability (Japanese Patent Laid-open (Kokai) No. 2000-139471, US2008-0311632A).

[0110]

Specific examples of L-methionine-producing bacteria and parental strains for deriving them include, for example, *E. coli* AJ11539 (NRRL B-12399), *E. coli* AJ11540 (NRRL B-12400), *E. coli* AJ11541 (NRRL B-12401), *E. coli* AJ11542 (NRRL B-12402, British Patent No. 2075055), the *E. coli* 218 strain (VKPM B-8125, Russian Patent No. 2209248) and the 73 strain (VKPM B-8126, Russian Patent No. 2215782), which are resistant to norleucine, which is an analogue of L-methionine, and *E. coli* AJ13425 (FERMP-16808, Japanese Patent Laid-open (Kokai) No. 2000-139471). The AJ13425 strain is an L-threonine auxotrophic strain derived from the *E. coli* W3110, in which the methionine repressor is deleted, the intracellular S-adenosylmethionine synthetase activity is attenuated, and the intracellular homoserine transsuccinylase activity, cystathionine γ -synthase activity, and aspartokinase-homoserine dehydrogenase II activity are enhanced.

[0111]

<L-Leucine-producing bacteria>

Examples of methods for imparting or enhancing L-leucine-producing ability include, for example, a method of modifying a bacterium so that the bacterium has an increased activity or activities of one or more kinds of enzymes selected from the L-leucine biosynthesis enzymes. Examples of such enzymes include, but not particularly limited to, the enzymes encoded by the genes of the *leuABCD* operon. Furthermore, for enhancing the activity of such an enzyme, for example, the mutant *leuA* gene encoding an isopropyl maleate synthase desensitized to feedback inhibition by L-leucine (U.S. Patent No. 6,403,342) can be preferably used.

[0112]

Specific examples of L-leucine-producing bacteria and parental strains for deriving them include, for example, strains belonging to the genus *Escherichia*, such as *E. coli* strains resistant to leucine (for example, the 57 strain (VKPM B-7386, U.S. Patent No. 6,124,121)), *E. coli* strains resistant to an leucine analogue such as β -2-thienylalanine, 3-hydroxyleucine, 4-azaleucine, and 5,5,5-trifluoroleucine (Japanese Patent Publication (Kokoku) No. 62-34397 and Japanese Patent Laid-open (Kokai) No. 8-70879), *E. coli* strains obtained by a gene engineering technique described in WO96/06926, and *E. coli* H-9068 (Japanese Patent Laid-open (Kokai) No. 8-70879).

[0113]

Examples of coryneform bacteria having L-leucine-producing ability include, for example, *Corynebacterium glutamicum* (*Brevibacterium lactofermentum*) AJ3718 (FERM P-2516), which is resistant to 2-thiazole alanine and β -hydroxyleucine and auxotrophic for isoleucine and methionine.

[0114]

<L-Isoleucine-producing bacteria>

Examples of methods for imparting or enhancing L-isoleucine-producing ability include, for example, a method of modifying a bacterium so that the bacterium has increased activity or activities of one or more enzymes selected from the L-isoleucine biosynthesis enzymes. Examples of such enzymes include, but not particularly limited to, threonine deaminase and acetohydroxy acid synthase (Japanese Patent Laid-open (Kokai) No. 2-458, EP0356739A, U.S. Patent No. 5,998,178).

[0115]

Specific examples of L-isoleucine-producing bacteria and parental strains for

deriving them include, for example, *Escherichia* bacteria such as mutant strains having resistance to 6-dimethylaminopurine (Japanese Patent Laid-open (Kokai) No. 5-304969), mutant strains having resistance to an isoleucine analogue such as thiaisleucine and isoleucine hydroxamate, and mutant strains having resistance to such an isoleucine analogue and further having resistance to DL-ethionine and/or arginine hydroxamate (Japanese Patent Laid-open (Kokai) No. 5-130882).

[0116]

Examples of coryneform bacteria having L-isoleucine-producing ability include, for example, the coryneform bacterium in which *brnE* gene encoding a branched chain amino acid excretion protein is amplified (Japanese Patent Laid-open (Kokai) No. 2001-169788), the coryneform bacterium to which L-isoleucine-producing ability is imparted by protoplast fusion with an L-lysine-producing bacterium (Japanese Patent Laid-open (Kokai) No. 62-74293), the coryneform bacterium in which homoserine dehydrogenase is enhanced (Japanese Patent Laid-open (Kokai) No. 62-91193), the threonine hydroxamate resistant strain (Japanese Patent Laid-open (Kokai) No. 62-195293), the α -ketomalonate resistant strain (Japanese Patent Laid-open (Kokai) No. 61-15695), the methyllysine resistant strain (Japanese Patent Laid-open (Kokai) No. 61-15696), and *Corynebacterium glutamicum* (*Brevibacterium flavum*) AJ12149 (FERM BP-759, U.S. Patent No. 4,656,135).

[0117]

<L-Valine-producing bacteria>

Examples of methods for imparting or enhancing L-valine-producing ability include, for example, a method of modifying a bacterium so that the bacterium has an increased activity or activities of one or more kinds of enzymes selected from the L-valine biosynthesis enzymes. Examples of such enzymes include, but not particularly limited to, the enzymes encoded by the genes of the *ilvGMEDA* operon and the enzymes encoded by the *ilvBNC* operon. The *ilvBN* gene encodes acetohydroxy acid synthase, and the *ilvC* gene encodes isomeroreductase (WO00/50624). Expressions of the *ilvGMEDA* operon and the *ilvBNC* operon are suppressed (attenuated) by L-valine, L-isoleucine, and/or L-leucine. Therefore, for enhancing the activity of such an enzyme, it is preferred that the suppression of expression by the produced L-valine is released by removing or modifying a region required for the attenuation. Furthermore, the threonine deaminase encoded by the *ilvA* gene is an enzyme that catalyzes the

deamination reaction of L-threonine resulting 2-ketobutyric acid, which is the rate-limiting step of the L-isoleucine biosynthesis system. Therefore, for L-valine production, it is preferred that the *ilvA* gene is, for example, disrupted, and thereby the threonine deaminase activity is decreased.

[0118]

Examples of methods for imparting or enhancing L-valine-producing ability also include, for example, a method of modifying a bacterium so that the bacterium has a reduced activity or activities of one or more kinds of enzymes selected from the enzymes that catalyze a reaction branching away from the biosynthesis pathway of L-valine to generate a compound other than L-valine. Examples of such enzymes include, but not particularly limited to, threonine dehydratase involved in the L-leucine synthesis, and the enzymes involved in the D-pantothenic acid synthesis (WO00/50624).

[0119]

Specific examples of L-valine-producing bacteria and parental strains for deriving them include, for example, *E. coli* strains modified so as to overexpress the *ilvGMEDA* operon (U.S. Patent No. 5,998,178).

[0120]

Examples of L-valine-producing bacteria and parental strains for deriving them also include mutant strains having a mutation in amino-acyl t-RNA synthetase (U.S. Patent No. 5,658,766). Examples of such strains include, for example, *E. coli* VL1970, which has a mutation in the *ileS* gene encoding isoleucine t-RNA synthetase. *E. coli* VL1970 was deposited at the Russian National Collection of Industrial Microorganisms (VKPM, FGUP GosNII Genetika, 1 Dorozhny Proezd, 1 Moscow 117545, Russia) on June 24, 1988 under the accession number of VKPM B-4411. Examples of L-valine-producing bacteria and parental strains for deriving them also include mutant strains requiring lipoic acid for growth and/or lacking H⁺-ATPase (WO96/06926).

[0121]

Examples of L-valine-producing bacteria and parent strains for deriving them also include strains resistant to an amino acid analogue or the like. Examples of such strains include, for example, the coryneform bacterium strains which are auxotrophic for L-isoleucine and L-methionine, and resistant to D-ribose, purine ribonucleoside, or pyrimidine ribonucleoside, and have an ability to produce L-valine (FERM P-1841, FERM P-29) (Japanese Patent Publication No. 53-025034), coryneform bacterium

strains resistant to polyketides (FERM P-1763, FERM P-1764) (Japanese Patent Publication No. 06-065314), and coryneform bacterium strains resistant to L-valine in a medium containing acetic acid as the sole carbon source and sensitive to pyruvic acid analogues (fluoropyruvic acid etc.) in a medium containing glucose as the sole carbon source (FERM BP-3006, BP-3007) (Japanese Patent No. 3006929).

[0122]

<L-Alanine-producing bacteria>

Examples of L-alanine-producing bacteria and parent strains for deriving them include the coryneform bacteria deficient in the H⁺-ATPase (Appl. Microbiol. Biotechnol., 2001 Nov., 57(4):534-40) and coryneform bacteria in which the aspartate β-decarboxylase activity is enhanced (Japanese Patent Laid-open (Kokai) No. 07-163383).

[0123]

<L-Tryptophan-producing bacteria, L-phenylalanine-producing bacteria, and L-tyrosine-producing bacteria>

Examples of methods for imparting or enhancing L-tryptophan-producing ability, L-phenylalanine-producing ability, and/or L-tyrosine-producing ability include, for example, a method of modifying a bacterium so that the bacterium has an increased activity or activities of one or more enzymes selected from the L-tryptophan, L-phenylalanine, and/or L-tyrosine biosynthesis enzymes.

[0124]

Examples of enzymes common to the biosynthesis systems of these aromatic amino acids include, but not particularly limited to, 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (*aroG*), 3-dehydroquinate synthase (*aroB*), shikimate dehydrogenase (*aroE*), shikimate kinase (*aroL*), 5-enolpyruvylshikimate-3-phosphate synthase (*aroA*), and chorismate synthase (*aroC*) (EP763127B). The expressions of the genes encoding these enzymes are controlled by the tyrosine repressor (*tyrR*), and the activities of these enzymes may be enhanced by deleting the *tyrR* gene (EP763127B).

[0125]

Examples of the L-tryptophan biosynthesis enzymes include, but not particularly limited to, anthranilate synthase (*trpE*), tryptophan synthase (*trpAB*), and phosphoglycerate dehydrogenase (*serA*). For example, by introducing a DNA

containing the tryptophan operon, L-tryptophan-producing ability can be imparted or enhanced. Tryptophan synthase consists of α and β subunits encoded by the *trpA* and *trpB* genes, respectively. Since the anthranilate synthase is subject to feedback inhibition by L-tryptophan, a gene encoding this enzyme introduced with a mutation for desensitization to feedback inhibition may be used for enhancing the activity of that enzyme. Since the phosphoglycerate dehydrogenase is subject to feedback inhibition by L-serine, a gene encoding this enzyme introduced with a mutation for desensitization to feedback inhibition may be used for enhancing the activity of that enzyme. Furthermore, by increasing the expression of the operon (*ace* operon) consisting of the maleate synthase gene (*aceB*), isocitrate lyase gene (*aceA*), and isocitrate dehydrogenase kinase/phosphatase gene (*aceK*), L-tryptophan-producing ability may be imparted or enhanced (WO2005/103275).

[0126]

Examples of the L-phenylalanine biosynthesis enzymes include, but not particularly limited to, chorismate mutase and prephenate dehydratase. The chorismate mutase and prephenate dehydratase are encoded by the *pheA* gene as a bifunctional enzyme. Since the chorismate mutase and prephenate dehydratase are subject to feedback inhibition by L-phenylalanine, genes encoding these enzymes introduced with a mutation for desensitization to feedback inhibition may be used for enhancing the activities of these enzymes.

[0127]

Examples of the L-tyrosine biosynthesis enzymes include, but not particularly limited to, chorismate mutase and prephenate dehydrogenase. The chorismate mutase and prephenate dehydrogenase are encoded by the *tyrA* gene as a bifunctional enzyme. Since the chorismate mutase and prephenate dehydrogenase are subject to feedback inhibition by L-tyrosine, genes encoding these enzymes introduced with a mutation for desensitization to feedback inhibition may be used for enhancing the activities of these enzymes.

[0128]

The L-tryptophan, L-phenylalanine, and/or L-tyrosine-producing bacteria may be modified so that biosynthesis of an aromatic amino acid other than the objective aromatic amino acid is reduced. Furthermore, the L-tryptophan, L-phenylalanine, and/or L-tyrosine-producing bacteria may be modified so that a by-product uptake

system is enhanced. Examples of the by-product include aromatic amino acids other than the objective aromatic amino acid. Examples of the gene encoding such a by-product uptake system include, for example, *tnaB* and *mtr*, which are genes encoding the L-tryptophan uptake system, *pheP*, which is a gene encoding the L-phenylalanine uptake system, and *tyrP*, which is a gene encoding the L-tyrosine uptake system (EP1484410).

[0129]

Specific examples of L-tryptophan-producing bacteria and parental strains for deriving them include, for example, *E. coli* JP4735/pMU3028 (DSM10122) and JP6015/pMU91 (DSM10123), which have a mutant *trpS* gene encoding a partially inactivated tryptophanyl-tRNA synthetase (U.S. Patent No. 5,756,345), *E. coli* SV164, which has a *trpE* allele encoding an anthranilate synthase desensitized to feedback inhibition by tryptophan, *E. coli* SV164 (pGH5), which has a *serA* allele encoding a phosphoglycerate dehydrogenase desensitized to feedback inhibition by serine and a *trpE* allele encoding an anthranilate synthase desensitized to feedback inhibition by tryptophan (U.S. Patent No. 6,180,373), a strain introduced with a tryptophan operon containing a *trpE* allele encoding an anthranilate synthase desensitized to feedback inhibition by tryptophan (Japanese Patent Laid-open (Kokai) Nos. 57-71397 and 62-244382, U.S. Patent No. 4,371,614), *E. coli* AGX17(pGX44) (NRRL B-12263) and AGX6(pGX50)aroP (NRRL B-12264), which are deficient tryptophanase (U.S. Patent No. 4,371,614), *E. coli* AGX17/pGX50,pACKG4-pps, which has an increased phosphoenolpyruvate-producing ability (WO97/08333, U.S. Patent No. 6,319,696), and strains belonging to the genus *Escherichia* having an increased activity of the protein encoded by the *yedA* or *yddG* gene (US2003-0148473A1 and US2003-0157667A1).

[0130]

Examples of coryneform bacteria having L-tryptophan-producing ability include, for example, *Corynebacterium glutamicum* AJ12118 (FERM BP-478, Japanese Patent No. 1681002), which is resistant to sulfaguanidine, the strain introduced with the tryptophan operon (Japanese Patent Laid-open (Kokai) No. 63-240794), and the strain introduced with a gene encoding shikimate kinase derived from a coryneform bacterium (Japanese Patent No. 1994749).

[0131]

Specific examples of L-phenylalanine-producing bacteria and parental strains

for deriving them include, for example, *E. coli* AJ12739 (tyrA::Tn10, tyrR) (VKPM B-8197), which is deficient in the chorismate mutase-prephenate dehydrogenase and the tyrosine repressor (WO03/044191), *E. coli* HW1089 (ATCC 55371), which contains a mutant *pheA34* gene encoding a chorismate mutase-prephenate dehydratase desensitized to feedback inhibition (U.S. Patent No. 5,354,672), *E. coli* MWEC101-b (KR8903681), *E. coli* NRRL B-12141, NRRL B-12145, NRRL B-12146, and NRRL B-12147 (U.S. Patent No. 4,407,952). Specific examples of L-phenylalanine-producing bacteria and parental strains for deriving them also include, for example, *E. coli* K-12 <W3110(tyrA)/pPHAB> (FERM BP-3566), *E. coli* K-12 <W3110(tyrA)/pPHAD> (FERM BP-12659), *E. coli* K-12 <W3110(tyrA)/pPHATerm> (FERM BP-12662), and *E. coli* K-12 AJ12604 <W3110(tyrA)/pBR-aroG4, pACMAB> (FERM BP-3579), which contains a gene encoding a chorismate mutase-prephenate dehydratase desensitized to feedback inhibition (EP488424B1). Specific examples of L-phenylalanine-producing bacteria and parental strains for deriving them further include, for example, strains belonging to the genus *Escherichia* having an increased activity of the protein encoded by the *yedA* gene or the *yddG* gene (US2003-0148473A, US2003-0157667A, WO03/044192).

[0132]

Examples of coryneform bacteria having L-phenylalanine-producing ability include, for example, the *Corynebacterium glutamicum* strains BPS-13 (FERM BP-1777), K77 (FERM BP-2062), and K78 (FERM BP-2063) (EP331145A, Japanese Patent Laid-open (Kokai) No. 02-303495), of which phosphoenolpyruvate carboxylase or pyruvate kinase activity is reduced, and the tyrosine-auxotrophic strain (Japanese Patent Laid-open (Kokai) No. 05-049489).

[0133]

Examples of coryneform bacteria having L-tyrosine-producing ability include, for example, *Corynebacterium glutamicum* AJ11655 (FERM P-5836, Japanese Patent Publication No. 2-6517), and *Corynebacterium glutamicum* (*Brevibacterium lactofermentum*) AJ12081 (FERM P-7249, Japanese Patent Laid-open (Kokai) No. 60-70093).

[0134]

Furthermore, examples of methods for imparting or enhancing an L-amino acid-producing ability include, for example, a method of modifying a bacterium so that

the bacterium has an increased activity for secreting an L-amino acid from a bacterial cell. Such an activity for secreting an L-amino acid can be increased by, for example, increasing the expression of a gene encoding a protein responsible for secretion of the L-amino acid. Examples of genes encoding the proteins responsible for secretion of various amino acids include, for example, *b2682* gene (*ygaZ*), *b2683* gene (*ygaH*), *b1242* gene (*yche*), and *b3434* gene (*yhgN*) (Japanese Patent Laid-open (Kokai) No. 2002-300874).

[0135]

Furthermore, examples of methods for imparting or enhancing an L-amino acid-producing ability also include, for example, a method of modifying a bacterium so that the bacterium has an increased activity or activities of one or more proteins selected from proteins involved in the glycometabolism and proteins involved in the energy metabolism.

[0136]

Examples of the proteins involved in the glycometabolism include proteins involved in uptake of saccharides and the glycolysis system enzymes. Examples of genes encoding a protein involved in the glycometabolism include glucose-6-phosphate isomerase gene (*pgi*, WO01/02542), pyruvate carboxylase gene (*pyc*, WO99/18228, EP1092776A), phosphoglucomutase gene (*pgm*, WO03/04598), fructose biphosphate aldolase gene (*pfkB*, *fbp*, WO03/04664), transaldolase gene (*talB*, WO03/008611), fumarase gene (*fum*, WO01/02545), non-PTS sucrose uptake gene (*csc*, EP1149911A), and sucrose assimilation gene (*scrAB* operon, U.S. Patent No. 7,179,623).

[0137]

Examples of genes encoding the proteins involved in the energy metabolism include the transhydrogenase gene (*pntAB*, U.S. Patent No. 5,830,716) and cytochrome bo-type oxidase gene (*cyoB*, EP1070376A).

[0138]

Furthermore, examples of methods for imparting or enhancing an ability to produce useful substances such as L-amino acids include, for example, a method of modifying a bacterium so that the activity of phosphoketolase is increased (WO2006/016705). Hence, the bacterium of the present invention may have been modified so that the activity of phosphoketolase is increased. This method may be

effective particularly for imparting or enhancing an ability to produce an L-amino acid of glutamate family such as L-glutamic acid. Examples of phosphoketolase include D-xylulose-5-phosphate phosphoketolase and fructose-6-phosphate phosphoketolase. Either one of the D-xylulose-5-phosphate phosphoketolase activity and the fructose-6-phosphate phosphoketolase activity may be enhanced, or both may be enhanced.

[0139]

The term "D-xylulose-5-phosphate phosphoketolase activity" refers to an activity for converting xylulose-5-phosphate into glyceraldehyde-3-phosphate and acetyl phosphate with consuming phosphoric acid to release one molecule of H₂O. This activity can be measured by the method described by Goldberg, M. et al. (Methods Enzymol., 9, 515-520, 1996) or the method described by L. Meile (J. Bacteriol., 183:2929-2936, 2001). Examples of D-xylulose-5-phosphate phosphoketolase include those of bacteria belonging to the genera *Acetobacter*, *Bifidobacterium*, *Lactobacillus*, *Thiobacillus*, *Streptococcus*, *Methylococcus*, *Butyrivibrio*, and *Fibrobacter*, and yeast belonging to the genera *Candida*, *Rhodotorula*, *Rhodospiridium*, *Pichia*, *Yarrowia*, *Hansenula*, *Kluyveromyces*, *Saccharomyces*, *Trichosporon*, and *Wingea*. Specific examples of D-xylulose-5-phosphate phosphoketolase and genes encoding them are disclosed in WO2006/016705.

[0140]

The term "fructose-6-phosphate phosphoketolase activity" refers to an activity for converting fructose-6-phosphate into erythrose-4-phosphate and acetyl phosphate with consuming phosphoric acid to release one molecule of H₂O. This activity can be measured by the method described by Racker, E. (Methods Enzymol., 5, 276-280, 1962) or the method described by L. Meile (J. Bacteriol., 183:2929-2936, 2001). Examples of fructose-6-phosphate phosphoketolase include those of bacteria belonging to the genera *Acetobacter*, *Bifidobacterium*, *Chlorobium*, *Brucella*, *Methylococcus*, and *Gardnerella*, and yeast belonging to the genera *Rhodotorula*, *Candida*, and *Saccharomyces*. Specific examples of fructose-6-phosphate phosphoketolase and genes encoding them are disclosed in WO2006/016705.

[0141]

Both the D-xylulose-5-phosphate phosphoketolase activity and the fructose-6-phosphate phosphoketolase activity may also be retained by a single enzyme (i.e. D-xylulose-5-phosphate phosphoketolase/fructose-6-phosphate phosphoketolase).

[0142]

The genes and proteins used for breeding L-amino acid-producing bacteria may have, for example, the nucleotide sequences and amino acid sequences of known genes and proteins, such as those exemplified above, respectively. Also, the genes and proteins used for breeding L-amino acid-producing bacteria may be conservative variants of known genes and proteins, such as those exemplified above, respectively. Specifically, for example, the genes used for breeding L-amino acid-producing bacteria may each be a gene encoding a protein having an amino acid sequence of a known protein, but including substitution, deletion, insertion, or addition of one or several amino acid residues at one or several positions, so long as the original function thereof is maintained. For the conservative variants of genes and proteins, the descriptions concerning conservative variants of the carotenoid biosynthesis enzyme gene and carotenoid biosynthesis enzyme mentioned later can be applied, *mutatis mutandis*.

[0143]

Furthermore, the bacterium of the present invention may have a carotenoid-producing ability. In the present invention, the term "bacterium having a carotenoid-producing ability" refers to a bacterium having an ability to generate and accumulate an objective carotenoid in cells of the bacterium, when the bacterium is cultured in a medium. The bacterium having a carotenoid-producing ability may be a bacterium that is able to accumulate an objective carotenoid in cells of the bacterium in an amount larger than that obtainable with a non-modified strain. The term "non-modified strain" refers to a control strain that has not been modified so that the activity of a carotenoid biosynthesis enzyme is increased. That is, examples of the non-modified strain include a wild-type strain and parental strain. The bacterium having a carotenoid-producing ability may be a bacterium that is able to accumulate an objective carotenoid in cells of the bacterium in an amount of 10 µg/g-DCW or more, preferably 50 µg/g-DCW or more, more preferably 100 µg/g-DCW or more, further preferably 200 µg/g-DCW or more, particularly preferably 500 µg/g-DCW or more. The amount of an objective carotenoid accumulated in cells of the bacterium having a carotenoid-producing ability may also be, for example, 100 mg/g-DCW or less, 50 mg/g-DCW or less, 10 mg/g-DCW or less, 5 mg/g-DCW or less, or 1 mg/g-DCW or less. The amount of an objective carotenoid accumulated in cells of the bacterium having a carotenoid-producing ability may also be within a range defined by any combination of

the ranges exemplified above. The term "DCW" refers to dry cell weight. Examples of the carotenoid include carotenes and xanthophylls. Specific examples of the carotenoid include, for example, phytoene, phytofluene, zeta-carotene, neurosporene, lycopene, beta-carotene, beta-cryptoxanthin, echinenone, 3-hydroxyechinenone, 3'-hydroxyechinenone, zeaxanthin, canthaxanthin, adonixanthin, adonirubin, and astaxanthine. Particular examples of the carotenoid include beta-carotene, zeaxanthin, canthaxanthin, and astaxanthine. More particular examples of the carotenoid include astaxanthine. The bacterium of the present invention may have an ability to produce a single kind of carotenoid, or two or more kinds of carotenoids. The accumulation amount of carotenoid exemplified above may represent, for example, the amount of total carotenoids, or the amount of selected one or more carotenoids. The bacterium of the present invention may be a bacterium inherently having a carotenoid-producing ability, or may be a bacterium modified so that it has a carotenoid-producing ability. The bacterium having a carotenoid-producing ability can be obtained by imparting a carotenoid-producing ability to such a bacterium as mentioned above, or by enhancing a carotenoid-producing ability of such a bacterium as mentioned above. A carotenoid-producing ability can be imparted or enhanced by, for example, modifying a bacterium so that the bacterium has an increased activity of a carotenoid biosynthesis enzyme.

[0144]

<1-2> Enhancement of carotenoid biosynthesis enzyme activity

The bacterium of the present invention has been modified so that the activity of a carotenoid biosynthesis enzyme is increased. Specifically, the bacterium of the present invention has been modified so that the activity of a carotenoid biosynthesis enzyme is increased as compared with a non-modified strain. The bacterium of the present invention can be obtained by modifying a bacterium having an L-amino acid-producing ability so that the activity of a carotenoid biosynthesis enzyme is increased. The bacterium of the present invention can also be obtained by modifying a bacterium so that the activity of a carotenoid biosynthesis enzyme is increased, and then imparting or enhancing an L-amino acid-producing ability. The bacterium of the present invention may also be a bacterium that has acquired an L-amino acid-producing ability by being modified so that the activity of a carotenoid biosynthesis enzyme is increased. The bacterium of the present invention may have, as required, such a property possessed by an L-amino acid-producing bacterium as mentioned above. The modifications for

constructing the bacterium of the present invention can be performed in an arbitrary order.

[0145]

By modifying a bacterium so that the activity of a carotenoid biosynthesis enzyme is increased, an L-amino acid-producing ability of the bacterium can be improved, and that is, production of an L-amino acid by using the bacterium can be increased.

[0146]

Hereinafter, carotenoid biosynthesis enzymes and genes encoding them will be explained.

[0147]

The term "carotenoid biosynthesis enzyme" refers to a protein involved in the biosynthesis of a carotenoid. A gene encoding a carotenoid biosynthesis enzyme is also referred to as "carotenoid biosynthesis enzyme gene".

[0148]

Carotenoids are biosynthesized via farnesyl pyrophosphate as an intermediate. Hence, examples of the carotenoid biosynthesis enzyme include enzymes catalyzing the conversion from farnesyl pyrophosphate into carotenoids such as astaxanthine.

[0149]

Astaxanthine can be biosynthesized via, for example, the following reactions. That is, farnesyl pyrophosphate can be converted into geranylgeranyl pyrophosphate by the action of geranylgeranyl pyrophosphate synthase. Geranylgeranyl pyrophosphate can be converted into phytoene by the action of phytoene synthase. Phytoene can be converted into lycopene via 4 steps of desaturation by the action of phytoene desaturase. Lycopene can be converted into beta-carotene by the action of lycopene beta-cyclase. Beta-carotene can be converted into astaxanthine via 2 steps of ketolation and 2 steps of hydroxylation by the action of carotene ketolase and carotene hydroxylase. Hence, specific examples of the carotenoid biosynthesis enzyme include geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, lycopene beta-cyclase, carotene ketolase, and carotene hydroxylase.

[0150]

The term "geranylgeranyl pyrophosphate synthase" refers to a protein that has the activity of catalyzing the reaction of converting farnesyl pyrophosphate and

isopentenyl pyrophosphate into geranylgeranyl pyrophosphate and pyrophosphate (EC 2.5.1.29). This activity is also referred to as "geranylgeranyl pyrophosphate synthase activity". Examples of geranylgeranyl pyrophosphate synthase include CrtE protein, which is encoded by *crtE* gene.

[0151]

The term "phytoene synthase" refers to a protein that has the activity of catalyzing the reaction of converting two molecules of geranylgeranyl pyrophosphate into one molecule of phytoene and two molecules of pyrophosphate (EC 2.5.1.32 or EC 2.5.1.99). This activity is also referred to as "phytoene synthase activity". Examples of phytoene synthase include CrtB protein, which is encoded by *crtB* gene.

[0152]

The term "phytoene desaturase" refers to a protein that has the activity of catalyzing the reaction of desaturating a linear carotene such as phytoene, phytofluene, zeta-carotene, or neurosporene. This activity is also referred to as "phytoene desaturase activity". Phytoene can be converted into lycopene via 4 steps of desaturation. Specifically, phytoene can be successively desaturated into phytofluene, zeta-carotene, neurosporene, and lycopene. Specific examples of phytoene desaturase include phytoene desaturase (lycopene-forming), which catalyzes 4 steps of desaturation from phytoene into lycopene (EC 1.3.99.31 etc.); phytoene desaturase (zeta-carotene-forming), which catalyzes 2 steps of desaturation from phytoene into zeta-carotene (EC 1.3.5.6, EC 1.3.99.29, etc.); and zeta-carotene desaturase, which catalyzes 2 steps of desaturation from zeta-carotene into lycopene (EC 1.3.5.6, EC 1.3.99.26, etc.). Examples of phytoene desaturase (lycopene-forming) include CrtI protein, which is encoded by *crtI* gene. Examples of phytoene desaturase (zeta-carotene-forming) include CrtP protein (synonym: Pds protein), which is encoded by *crtP* gene (synonym: *pds* gene). Examples of zeta-carotene desaturase include CrtQ protein, which is encoded by *crtQ* gene. As phytoene desaturase, for example, phytoene desaturase (lycopene-forming) may be used, or a combination of phytoene desaturase (zeta-carotene-forming) and zeta-carotene desaturase may be used.

[0153]

The term "lycopene beta-cyclase" refers to a protein that has the activity of catalyzing the reaction of cyclizing lycopene to generate beta-carotene (EC 5.5.1.19). This activity is also referred to as "lycopene beta-cyclase activity". Examples of

lycopene beta-cyclase include CrtY and CrtL proteins, which are encoded by *crtY* and *crtL* genes, respectively.

[0154]

The term "carotene ketolase" refers to a protein that has the activity of catalyzing the reaction of introducing a keto group into the ionone ring of a cyclic carotene (EC 1.3.5.B4, EC 1.14.11.B16, EC 1.17.5.B2, etc.). This activity is also referred to as "carotene ketolase activity". Examples of carotene ketolase include CrtW and CrtO proteins, which are encoded by *crtW* and *crtO* genes, respectively.

[0155]

The term "carotene hydroxylase" refers to a protein that has the activity of catalyzing the reaction of introducing a hydroxyl group into the ionone ring of a cyclic carotene (EC 1.14.13.129). This activity is also referred to as "carotene hydroxylase activity". Examples of carotene hydroxylase include CrtZ and CrtR proteins, which are encoded by *crtZ* and *crtR* genes, respectively.

[0156]

In the present invention, the activity of one kind of carotenoid biosynthesis enzyme may be increased, or the activities of two or more kinds of carotenoid biosynthesis enzymes may be increased. The number and kind(s) of carotenoid biosynthesis enzyme(s) of which the activity or activities is/are increased are not particularly limited. The carotenoid biosynthesis enzyme(s) of which the activity or activities is/are increased may be selected so that, for example, a carotenoid-producing ability is imparted or enhanced. For example, at least the activity or activities of one or more kinds of enzymes selected from geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and lycopene beta-cyclase may be increased. Also, for example, at least the activities of geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and lycopene beta-cyclase may be increased. Also, for example, in addition to the activity or activities of this/these enzyme(s), the activity or activities of carotene ketolase and/or carotene hydroxylase may further be increased.

[0157]

Examples of the carotenoid biosynthesis enzyme gene include genes of various organisms such as plants, algae, and bacteria. Examples of bacteria having the carotenoid biosynthesis enzyme gene include, for example, bacteria belonging to the

family *Enterobacteriaceae*, such as *Pantoea* bacteria; bacteria belonging to the family *Caulobacteraceae*, such as *Brevundimonas* bacteria; bacteria belonging to the family *Rhodobacteraceae*, such as *Paracoccus* bacteria; bacteria belonging to the family *Alcaligenaceae*, such as *Alcaligenes* bacteria; coryneform bacteria such as *Corynebacterium* bacteria; cyanobacteria such as *Nostoc* bacteria and *Synechococcus* bacteria. The nucleotide sequences of the carotenoid biosynthesis enzyme genes derived from these various organisms and the amino acid sequences of the carotenoid biosynthesis enzymes encoded by these genes can be obtained from, for example, public databases such as NCBI. Specific examples of *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* genes include, for example, *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* genes of *Pantoea* bacteria such as *Pantoea ananatis* and *Pantoea agglomerans*. The nucleotide sequences of the *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* genes of *Pantoea ananatis* AJ13355 are shown in SEQ ID NOS: 11, 13, 15, 17, and 21, respectively. The amino acid sequences of the CrtE, CrtB, CrtI, CrtY, and CrtZ proteins encoded by these genes are shown in SEQ ID NOS: 12, 14, 16, 18, and 22, respectively. Furthermore, specific examples of *crtE*, *crtB*, and *crtI* genes include, for example, *crtE*, *crtB*, and *crtI* genes of *Corynebacterium* bacteria such as *Corynebacterium glutamicum*. The nucleotide sequences of the *crtE* (cg0723), *crtB* (cg0721), and *crtI* (cg0720) genes of *Corynebacterium glutamicum* ATCC 13032 are shown in SEQ ID NOS: 23, 25, and 27, respectively. The amino acid sequences of the CrtE, CrtB, and CrtI proteins encoded by these genes are shown in SEQ ID NOS: 24, 26, and 28, respectively. Furthermore, specific examples of *crtW* gene include, for example, *crtW* genes of *Nostoc* bacteria such as *Nostoc* sp. and *Nostoc punctiforme* and *Brevundimonas* bacteria such as *Brevundimonas aurantiaca*. The nucleotide sequence of the *crtW* gene of *Nostoc* sp. PCC 7120 and the amino acid sequence of the protein encoded by the gene are shown in SEQ ID NOS: 19 and 20, respectively. The nucleotide sequence of the *crtW* gene of *Nostoc punctiforme* (codon-optimized for expression in *E. coli*) and the amino acid sequence of the protein encoded by the gene are shown in SEQ ID NOS: 50 and 61, respectively. The nucleotide sequence of the *crtW* gene of *Brevundimonas aurantiaca* and the amino acid sequence of the protein encoded by the gene are shown in SEQ ID NOS: 29 and 30, respectively. Furthermore, specific examples of *crtZ* gene include, for example, *crtZ* genes of *Brevundimonas* bacteria such as *Brevundimonas* sp., *Paracoccus* bacteria such as *Paracoccus* sp., and *Alcaligenes* bacteria such as *Alcaligenes* sp. The nucleotide sequence of the *crtZ* gene

of *Brevundimonas* sp. SD-212 and the amino acid sequence of the protein encoded by the gene are shown in SEQ ID NOS: 37 and 58, respectively. The nucleotide sequence of the *crtZ* gene of *Paracoccus* sp. N81106 and the amino acid sequence of the protein encoded by the gene are shown in SEQ ID NOS: 42 and 59, respectively. The nucleotide sequence of the *crtZ* gene of *Alcaligenes* sp. and the amino acid sequence of the protein encoded by the gene are shown in SEQ ID NOS: 46 and 60, respectively. Furthermore, specific examples of *crtP*, *crtQ*, *crtO*, and *crtR* genes include, for example, *crtP* (*pds*), *crtQ*, *crtO*, and *crtR* genes of cyanobacteria such as *Synechococcus* bacteria. That is, the carotenoid biosynthesis enzyme gene may be, for example, a gene having the nucleotide sequence of any of the carotenoid biosynthesis enzyme genes exemplified above, such as the nucleotide sequence shown as SEQ ID NO: 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 37, 42, 46, or 50. Also, the carotenoid biosynthesis enzyme may be, for example, a protein having the amino acid sequence of any of the carotenoid biosynthesis enzymes exemplified above, such as the amino acid sequence shown as SEQ ID NO: 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 58, 59, 60, or 61. The expression "a gene or protein has a nucleotide or amino acid sequence" means that a gene or protein comprises the nucleotide or amino acid sequence unless otherwise stated, and also includes cases where a gene or protein consists of the nucleotide or amino acid sequence. [0158]

The carotenoid biosynthesis enzyme gene may be a variant of any of the carotenoid biosynthesis enzyme genes exemplified above (e.g. a gene having the nucleotide sequence shown as SEQ ID NO: 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 37, 42, 46, or 50), so long as the original function thereof is maintained. Similarly, the carotenoid biosynthesis enzyme may be a variant of any of the carotenoid biosynthesis enzymes exemplified above (e.g. a protein having the amino acid sequence shown as SEQ ID NO: 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 58, 59, 60, or 61), so long as the original function thereof is maintained. Such a variant that maintains the original function thereof is also referred to as "conservative variant". A gene defined with the above-mentioned gene name and a protein defined with the above-mentioned protein name include not only the genes and proteins exemplified above, respectively, but also include conservative variants thereof. Namely, for example, the term "*crtE* gene" includes not only the *crtE* genes exemplified above, but also includes conservative variants thereof. Similarly, the term "CrtE protein" includes not only the CrtE proteins

exemplified above, but also includes conservative variants thereof. Examples of the conservative variants include, for example, homologues and artificially modified versions of the carotenoid biosynthesis enzyme genes and carotenoid biosynthesis enzymes exemplified above.

[0159]

The expression "the original function is maintained" means that a variant of gene or protein has a function (such as activity or property) corresponding to the function (such as activity or property) of the original gene or protein. The expression "the original function is maintained" used for a gene means that a variant of the gene encodes a protein that maintains the original function. That is, the expression "the original function is maintained" used for the carotenoid biosynthesis enzyme gene means that a variant of the gene encodes a protein having the corresponding carotenoid biosynthesis enzyme activity, e.g. geranylgeranyl pyrophosphate synthase activity for a geranylgeranyl pyrophosphate synthase gene. Furthermore, the expression "the original function is maintained" used for the carotenoid biosynthesis enzyme means that a variant of the protein has the corresponding carotenoid biosynthesis enzyme activity, e.g. geranylgeranyl pyrophosphate synthase activity for geranylgeranyl pyrophosphate synthase.

[0160]

Each carotenoid biosynthesis enzyme activity can be measured by, for example, incubating the enzyme with a substrate corresponding to the activity to be measured, and measuring the enzyme- and substrate-dependent generation of a product corresponding to the activity to be measured. For example, geranylgeranyl pyrophosphate synthase activity can be measured by incubating the enzyme with a substrate (i.e. farnesyl pyrophosphate and isopentenyl pyrophosphate), and measuring the enzyme- and substrate-dependent generation of a product (i.e. geranylgeranyl pyrophosphate).

[0161]

Hereinafter, examples of the conservative variants will be explained.

[0162]

Homologues of the carotenoid biosynthesis enzyme genes or homologues of the carotenoid biosynthesis enzymes can be easily obtained from public databases by, for example, BLAST search or FASTA search using any of the nucleotide sequences of

the carotenoid biosynthesis enzyme genes exemplified above or any of the amino acid sequences of the carotenoid biosynthesis enzymes exemplified above as a query sequence. Furthermore, homologues of the carotenoid biosynthesis enzyme genes can be obtained by, for example, PCR using a chromosome of various organisms as the template, and oligonucleotides prepared on the basis of any of the nucleotide sequences of these known carotenoid biosynthesis enzyme genes as primers.

[0163]

The carotenoid biosynthesis enzyme gene may be a gene encoding a protein having any of the aforementioned amino acid sequences (e.g. the amino acid sequence shown as SEQ ID NO: 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 58, 59, 60, or 61), but which includes substitution, deletion, insertion, and/or addition of one or several amino acid residues at one or several positions, so long as the original function is maintained. For example, the N-terminus and/or the C-terminus of the encoded protein may be elongated or shortened. Although the number meant by the term "one or several" mentioned above may differ depending on the positions of amino acid residues in the three-dimensional structure of the protein or the types of amino acid residues, specifically, it is, for example, 1 to 50, 1 to 40, or 1 to 30, preferably 1 to 20, more preferably 1 to 10, still more preferably 1 to 5, particularly preferably 1 to 3.

[0164]

The aforementioned substitution, deletion, insertion, and/or addition of one or several amino acid residues are/is a conservative mutation that maintains the normal function of the protein. Typical examples of the conservative mutation are conservative substitutions. The conservative substitution is a mutation wherein substitution takes place mutually among Phe, Trp, and Tyr, if the substitution site is an aromatic amino acid; among Leu, Ile, and Val, if it is a hydrophobic amino acid; between Gln and Asn, if it is a polar amino acid; among Lys, Arg, and His, if it is a basic amino acid; between Asp and Glu, if it is an acidic amino acid; and between Ser and Thr, if it is an amino acid having a hydroxyl group. Examples of substitutions considered as conservative substitutions include, specifically, substitution of Ser or Thr for Ala, substitution of Gln, His, or Lys for Arg, substitution of Glu, Gln, Lys, His, or Asp for Asn, substitution of Asn, Glu, or Gln for Asp, substitution of Ser or Ala for Cys, substitution of Asn, Glu, Lys, His, Asp, or Arg for Gln, substitution of Gly, Asn, Gln, Lys, or Asp for Glu, substitution of Pro for Gly, substitution of Asn, Lys, Gln, Arg, or

Tyr for His, substitution of Leu, Met, Val, or Phe for Ile, substitution of Ile, Met, Val, or Phe for Leu, substitution of Asn, Glu, Gln, His, or Arg for Lys, substitution of Ile, Leu, Val, or Phe for Met, substitution of Trp, Tyr, Met, Ile, or Leu for Phe, substitution of Thr or Ala for Ser, substitution of Ser or Ala for Thr, substitution of Phe or Tyr for Trp, substitution of His, Phe, or Trp for Tyr, and substitution of Met, Ile, or Leu for Val. Furthermore, such substitution, deletion, insertion, addition, inversion, or the like of amino acid residues as mentioned above includes a naturally occurring mutation due to an individual difference, or a difference of species of the organism from which the gene is derived (mutant or variant).

[0165]

The carotenoid biosynthesis enzyme gene may be a gene encoding a protein having an amino acid sequence showing a homology of, for example, 50% or more, 65% or more, or 80% or more, preferably 90% or more, more preferably 95% or more, still more preferably 97% or more, particularly preferably 99% or more, to the total amino acid sequence of any of the aforementioned amino acid sequences, so long as the original function is maintained. In this description, "homology" means "identity".

[0166]

The carotenoid biosynthesis enzyme gene may also be a DNA that is able to hybridize under stringent conditions with a probe that can be prepared from any of the aforementioned nucleotide sequences (e.g. the nucleotide sequence shown as SEQ ID NO: 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 37, 42, 46, or 50), such as a sequence complementary to a partial or entire sequence of any of the aforementioned nucleotide sequences, so long as the original function is maintained. The term "stringent conditions" refers to conditions under which a so-called specific hybrid is formed, and a non-specific hybrid is not formed. Examples of the stringent conditions include those under which highly homologous DNAs hybridize to each other, for example, DNAs not less than 50%, 65%, or 80% homologous, preferably not less than 90% homologous, more preferably not less than 95% homologous, still more preferably not less than 97% homologous, particularly preferably not less than 99% homologous, hybridize to each other, and DNAs less homologous than the above do not hybridize to each other, or conditions of washing of typical Southern hybridization, i.e., conditions of washing once, preferably 2 or 3 times, at a salt concentration and temperature corresponding to 1 x SSC, 0.1% SDS at 60°C, preferably 0.1 x SSC, 0.1% SDS at 60°C, more preferably

0.1 x SSC, 0.1% SDS at 68°C.

[0167]

The probe used for the aforementioned hybridization may be a part of a sequence that is complementary to the gene as described above. Such a probe can be prepared by PCR using oligonucleotides prepared on the basis of a known gene sequence as primers and a DNA fragment containing any of the aforementioned genes as a template. As the probe, for example, a DNA fragment having a length of about 300 bp can be used. When a DNA fragment having a length of about 300 bp is used as the probe, the washing conditions of the hybridization may be, for example, 50°C, 2 x SSC and 0.1% SDS.

[0168]

Furthermore, since the degeneracy of codons differs depending on the host, arbitrary codons in the carotenoid biosynthesis enzyme gene may be replaced with respective equivalent codons. That is, the carotenoid biosynthesis enzyme gene may be a variant of any of the carotenoid biosynthesis enzyme genes exemplified above due to the degeneracy of the genetic code. For example, the carotenoid biosynthesis enzyme gene may be a gene modified so that it has optimal codons according to codon frequencies in a host to be used.

[0169]

The percentage of the sequence identity between two sequences can be determined by, for example, using a mathematical algorithm. Non-limiting examples of such a mathematical algorithm include the algorithm of Myers and Miller (1988) CABIOS 4:11-17, the local homology algorithm of Smith et al (1981) Adv. Appl. Math. 2:482, the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48:443-453, the method for searching homology of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. 85:2444-2448, and an modified version of the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87:2264, such as that described in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5877.

[0170]

By using a program based on such a mathematical algorithm, sequence comparison (i.e. alignment) for determining the sequence identity can be performed. The program can be appropriately executed by a computer. Examples of such a program include, but not limited to, CLUSTAL or PC/Gene program (available from

Intelligenetics, Mountain View, Calif.), ALIGN program (Version 2.0), and GAP, BESTFIT, BLAST, FASTA, and TFASTA of Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wis., USA). Alignment using these programs can be performed by using, for example, initial parameters. The CLUSTAL program is well described in Higgins et al. (1988) *Gene* 73:237-244, Higgins et al. (1989) *CABIOS* 5:151-153, Corpet et al. (1988) *Nucleic Acids Res.* 16:10881-90, Huang et al. (1992) *CABIOS* 8:155-65, and Pearson et al. (1994) *Meth. Mol. Biol.* 24:307-331.

[0171]

In order to obtain a nucleotide sequence homologous to a target nucleotide sequence, in particular, for example, BLAST nucleotide search can be performed by using BLASTN program with score of 100 and word length of 12. In order to obtain an amino acid sequence homologous to a target protein, in particular, for example, BLAST protein search can be performed by using BLASTX program with score of 50 and word length of 3. See <http://www.ncbi.nlm.nih.gov> for BLAST nucleotide search and BLAST protein search. In addition, Gapped BLAST (BLAST 2.0) can be used in order to obtain an alignment including gap(s) for the purpose of comparison. In addition, PSI-BLAST (BLAST 2.0) can be used in order to perform repetitive search for detecting distant relationships between sequences. See Altschul et al. (1997) *Nucleic Acids Res.* 25:3389 for Gapped BLAST and PSI-BLAST. When using BLAST, Gapped BLAST, or PSI-BLAST, initial parameters of each program (e.g. BLASTN for nucleotide sequences, and BLASTX for amino acid sequences) can be used. Alignment can also be manually performed.

[0172]

The sequence identity between two sequences is calculated as the ratio of residues matching in the two sequences when aligning the two sequences so as to fit maximally with each other.

[0173]

The aforementioned descriptions concerning conservative variants of the genes and proteins can be applied *mutatis mutandis* to variants of arbitrary proteins such as L-amino acid biosynthesis system enzymes and genes encoding them.

[0174]

<1-3> Methods for increasing activity of protein

Hereinafter, methods for increasing the activity of a protein such as the carotenoid biosynthesis enzyme will be explained.

[0175]

The expression "the activity of a protein is increased" means that the activity of the protein is increased as compared with a non-modified strain. Specifically, the expression "the activity of a protein is increased" may mean that the activity of the protein per cell is increased as compared with that of a non-modified strain. The term "non-modified strain" used herein refers to a control strain that has not been modified so that the activity of an objective protein is increased. Examples of the non-modified strain include a wild-type strain and parent strain. Specific examples of the non-modified strain include the respective type strains of the species of microorganisms. Specific examples of the non-modified strain also include strains exemplified above in relation to the description of microorganisms. That is, in an embodiment, the activity of a protein may be increased as compared with a type strain, i.e. the type strain of the species to which the microorganism of the present invention belongs. In another embodiment, the activity of a protein may also be increased as compared with the *E. coli* MG1655 strain. In another embodiment, the activity of a protein may also be increased as compared with the *P. ananatis* AJ13355 strain. In another embodiment, the activity of a protein may also be increased as compared with the *C. glutamicum* ATCC 13869 strain. The state that "the activity of a protein is increased" may also be expressed as "the activity of a protein is enhanced". More specifically, the expression "the activity of a protein is increased" may mean that the number of molecules of the protein per cell is increased, and/or the function of each molecule of the protein is increased as compared with those of a non-modified strain. That is, the term "activity" in the expression "the activity of a protein is increased" is not limited to the catalytic activity of the protein, but may also mean the transcription amount of a gene (i.e. the amount of mRNA) encoding the protein, or the translation amount of the gene (i.e. the amount of the protein). Furthermore, the state that "the activity of a protein is increased" includes not only a state that the activity of an objective protein is increased in a strain inherently having the activity of the objective protein, but also a state that the activity of an objective protein is imparted to a strain not inherently having the activity of the objective protein. Furthermore, so long as the activity of the protein is eventually increased, the activity of an objective protein inherently contained in a host

may be attenuated and/or eliminated, and then an appropriate type of the objective protein may be imparted to the host.

[0176]

The degree of the increase in the activity of a protein is not particularly limited, so long as the activity of the protein is increased as compared with a non-modified strain. The activity of the protein may be increased to, for example, 1.5 times or more, 2 times or more, or 3 times or more of that of a non-modified strain. Furthermore, when the non-modified strain does not have the activity of the objective protein, it is sufficient that the protein is produced as a result of introduction of the gene encoding the protein, and for example, the protein may be produced to such an extent that the activity thereof can be measured.

[0177]

The modification for increasing the activity of a protein can be attained by, for example, increasing the expression of a gene encoding the protein. The expression "the expression of a gene is increased" means that the expression of the gene is increased as compared with a non-modified strain such as a wild-type strain and parent strain. Specifically, the expression "the expression of a gene is increased" may mean that the expression amount of the gene per cell is increased as compared with that of a non-modified strain such as a wild-type strain and parent strain. More specifically, the expression "the expression of a gene is increased" may mean that the transcription amount of the gene (i.e. the amount of mRNA) is increased, and/or the translation amount of the gene (i.e. the amount of the protein expressed from the gene) is increased. The state that "the expression of a gene is increased" may also be referred to as "the expression of a gene is enhanced". The expression of a gene may be increased to, for example, 1.5 times or more, 2 times or more, or 3 times or more of that of a non-modified strain. Furthermore, the state that "the expression of a gene is increased" includes not only a state that the expression amount of an objective gene is increased in a strain that inherently expresses the objective gene, but also a state that the gene is introduced into a strain that does not inherently express the objective gene, and expressed therein. That is, the phrase "the expression of a gene is increased" may also mean, for example, that an objective gene is introduced into a strain that does not possess the gene, and is expressed therein.

[0178]

The expression of a gene can be increased by, for example, increasing the copy number of the gene.

[0179]

The copy number of a gene can be increased by introducing the gene into the chromosome of a host. A gene can be introduced into a chromosome by, for example, using homologous recombination (Miller, J.H., Experiments in Molecular Genetics, 1972, Cold Spring Harbor Laboratory). Examples of the gene transfer method utilizing homologous recombination include, for example, a method of using a linear DNA such as Red-driven integration (Datsenko, K.A., and Wanner, B.L., Proc. Natl. Acad. Sci. USA, 97:6640-6645 (2000)), a method of using a plasmid containing a temperature sensitive replication origin, a method of using a plasmid capable of conjugative transfer, a method of using a suicide vector not having a replication origin that functions in a host, and a transduction method using a phage. Only one copy, or two or more copies of a gene may be introduced. For example, by performing homologous recombination using a sequence which is present in multiple copies on a chromosome as a target, multiple copies of a gene can be introduced into the chromosome. Examples of such a sequence which is present in multiple copies on a chromosome include repetitive DNAs, and inverted repeats located at the both ends of a transposon. Alternatively, homologous recombination may be performed by using an appropriate sequence on a chromosome such as a gene unnecessary for production of an objective substance as a target. Furthermore, a gene can also be randomly introduced into a chromosome by using a transposon or Mini-Mu (Japanese Patent Laid-open (Kokai) No. 2-109985, U.S. Patent No. 5,882,888, EP805867B1).

[0180]

Introduction of a target gene into a chromosome can be confirmed by Southern hybridization using a probe having a sequence complementary to the whole gene or a part thereof, PCR using primers prepared on the basis of the sequence of the gene, or the like.

[0181]

Furthermore, the copy number of a gene can also be increased by introducing a vector containing the gene into a host. For example, the copy number of a target gene can be increased by ligating a DNA fragment containing the target gene with a vector that functions in a host to construct an expression vector of the gene, and transforming

the host with the expression vector. The DNA fragment containing the target gene can be obtained by, for example, PCR using the genomic DNA of a microorganism having the target gene as the template. As the vector, a vector autonomously replicable in the cell of the host can be used. The vector is preferably a multi-copy vector.

Furthermore, the vector preferably has a marker such as an antibiotic resistance gene for selection of transformant. Furthermore, the vector may have a promoter and/or terminator for expressing the introduced gene. The vector may be, for example, a vector derived from a bacterial plasmid, a vector derived from a yeast plasmid, a vector derived from a bacteriophage, cosmid, phagemid, or the like. Specific examples of vector autonomously replicable in *Enterobacteriaceae* bacteria such as *Escherichia coli* include, for example, pUC19, pUC18, pHSG299, pHSG399, pHSG398, pBR322, pSTV29 (all of these are available from Takara Bio), pACYC184, pMW219 (NIPPON GENE), pTrc99A (Pharmacia), pPROK series vectors (Clontech), pKK233-2 (Clontech), pET series vectors (Novagen), pQE series vectors (QIAGEN), pCold TF DNA (Takara Bio), pACYC series vectors, and the broad host spectrum vector RSF1010. Specific examples of vector autonomously replicable in coryneform bacteria include, for example, pHM1519 (Agric. Biol. Chem., 48, 2901-2903 (1984)); pAM330 (Agric. Biol. Chem., 48, 2901-2903 (1984)); plasmids obtained by improving these and having a drug resistance gene; plasmid pCRY30 (Japanese Patent Laid-open (Kokai) No. 3-210184); plasmids pCRY21, pCRY2KE, pCRY2KX, pCRY31, pCRY3KE, and pCRY3KX (Japanese Patent Laid-open (Kokai) No. 2-72876 and U.S. Patent No. 5,185,262); plasmids pCRY2 and pCRY3 (Japanese Patent Laid-open (Kokai) No. 1-191686); pAJ655, pAJ611, and pAJ1844 (Japanese Patent Laid-open (Kokai) No. 58-192900); pCG1 (Japanese Patent Laid-open (Kokai) No. 57-134500); pCG2 (Japanese Patent Laid-open (Kokai) No. 58-35197); pCG4 and pCG11 (Japanese Patent Laid-open (Kokai) No. 57-183799); pVK7 (Japanese Patent Laid-open (Kokai) No. 10-215883); pVK9 (US2006-0141588); pVC7 (Japanese Patent Laid-open (Kokai) No. 9-070291); pVS7 (WO2013/069634).

[0182]

When a gene is introduced, it is sufficient that the gene is expressibly harbored by a host. Specifically, it is sufficient that the gene is harbored by a host so that it is expressed under control by a promoter sequence that functions in the host. The promoter is not particularly limited so long as it functions in the host. The term

"promoter that functions in a host" refers to a promoter that shows a promoter activity in the host. The promoter may be a promoter derived from the host, or a heterogenous promoter. The promoter may be the native promoter of the gene to be introduced, or a promoter of another gene. As the promoter, for example, such a stronger promoter as mentioned later may also be used.

[0183]

A terminator for termination of gene transcription may be located downstream of the gene. The terminator is not particularly limited so long as it functions in the host. The terminator may be a terminator derived from the host, or a heterogenous terminator. The terminator may be the native terminator of the gene to be introduced, or a terminator of another gene. Specific examples of the terminator include, for example, T7 terminator, T4 terminator, fd phage terminator, *tet* terminator, and *trpA* terminator.

[0184]

Vectors, promoters, and terminators available in various microorganisms are disclosed in detail in "Fundamental Microbiology Vol. 8, Genetic Engineering, KYORITSU SHUPPAN CO., LTD, 1987", and those can be used.

[0185]

Furthermore, when two or more of genes are introduced, it is sufficient that the genes each are expressibly harbored by the host. For example, all the genes may be carried by a single expression vector or a chromosome. Furthermore, the genes may be separately carried by two or more expression vectors, or separately carried by a single or two or more expression vectors and a chromosome. An operon constituted by two or more genes may also be introduced. The case of "introducing two or more genes" include, for example, cases of introducing respective genes encoding two or more kinds of proteins (such as enzymes), introducing respective genes encoding two or more subunits constituting a single protein complex (such as enzyme complex), and a combination of the foregoing cases.

[0186]

The gene to be introduced is not particularly limited so long as it encodes a protein that functions in the host. The gene to be introduced may be a gene derived from the host, or may be a heterogenous gene. The gene to be introduced can be obtained by, for example, PCR using primers designed on the basis of the nucleotide

sequence of the gene, and using the genomic DNA of an organism having the gene, a plasmid carrying the gene, or the like as a template. The gene to be introduced may also be totally synthesized, for example, on the basis of the nucleotide sequence of the gene (Gene, 60(1), 115-127 (1987)). The obtained gene can be used as it is, or after being modified as required. That is, a variant of a gene may be obtained by modifying the gene. A gene can be modified by a known technique. For example, an objective mutation can be introduced into an objective site of DNA by the site-specific mutation method. That is, the coding region of a gene can be modified by the site-specific mutation method so that a specific site of the encoded protein include substitution, deletion, insertion, and/or addition of amino acid residues. Examples of the site-specific mutation method include the method utilizing PCR (Higuchi, R., 61, in PCR Technology, Erlich, H.A. Eds., Stockton Press (1989); Carter, P., Meth. in Enzymol., 154, 382 (1987)), and the method utilizing phage (Kramer, W. and Frits, H.J., Meth. in Enzymol., 154, 350 (1987); Kunkel, T.A. et al., Meth. in Enzymol., 154, 367 (1987)). Alternatively, a variant of a gene may be totally synthesized.

[0187]

Incidentally, when a protein functions as a complex consisting of a plurality of subunits, a part or all of the plurality of subunits may be modified, so long as the activity of the protein is eventually increased. That is, for example, when the activity of a protein is increased by increasing the expression of a gene, the expression of a part or all of the plurality of genes that encode the subunits may be enhanced. It is usually preferable to enhance the expression of all of the plurality of genes encoding the subunits. Furthermore, the subunits constituting the complex may be derived from a single kind of organism or two or more kinds of organisms, so long as the complex has a function of the objective protein. That is, for example, genes of the same organism encoding a plurality of subunits may be introduced into a host, or genes of different organisms encoding a plurality of subunits may be introduced into a host.

[0188]

Furthermore, the expression of a gene can be increased by improving the transcription efficiency of the gene. In addition, the expression of a gene can also be increased by improving the translation efficiency of the gene. The transcription efficiency of the gene and the translation efficiency of the gene can be improved by, for example, modifying an expression control sequence of the gene. The term "expression

control sequence" collectively refers to sites that affect the expression of a gene. Examples of the expression control sequence include, for example, promoter, Shine-Dalgarno (SD) sequence (also referred to as ribosome binding site (RBS)), and spacer region between RBS and the start codon. Expression control sequences can be identified by using a promoter search vector or gene analysis software such as GENETYX. These expression control sequences can be modified by, for example, a method of using a temperature sensitive vector, or the Red driven integration method (WO2005/010175).

[0189]

The transcription efficiency of a gene can be improved by, for example, replacing the promoter of the gene on a chromosome with a stronger promoter. The term "stronger promoter" refers to a promoter providing an improved transcription of a gene compared with an inherently existing wild-type promoter of the gene. Examples of stronger promoters include, for example, the known high expression promoters such as T7 promoter, *trp* promoter, *lac* promoter, *thr* promoter, *tac* promoter, *trc* promoter, *tet* promoter, *araBAD* promoter, *rpoH* promoter, *msrA* promoter, Pml promoter (derived from the genus *Bifidobacterium*), PR promoter, and PL promoter. Examples of stronger promoters usable in coryneform bacteria include, for example, the artificially modified P54-6 promoter (Appl. Microbiol. Biotechnol., 53, 674-679 (2000)), *pta*, *aceA*, *aceB*, *adh*, and *amyE* promoters inducible in coryneform bacteria with acetic acid, ethanol, pyruvic acid, or the like, *cspB*, *SOD*, and *tuf* (EF-Tu) promoters, which are potent promoters capable of providing a large expression amount in coryneform bacteria (Journal of Biotechnology, 104 (2003) 311-323; Appl. Environ. Microbiol., 2005 Dec; 71 (12):8587-96), as well as *lac* promoter, *tac* promoter, and *trc* promoter. Furthermore, as the stronger promoter, a highly-active type of an existing promoter may also be obtained by using various reporter genes. For example, by making the -35 and -10 regions in a promoter region closer to the consensus sequence, the activity of the promoter can be enhanced (WO00/18935). Examples of highly active-type promoter include various *tac*-like promoters (Katashkina JI et al., Russian Federation Patent Application No. 2006134574) and *pnlp8* promoter (WO2010/027045). Methods for evaluating the strength of promoters and examples of strong promoters are described in the paper of Goldstein et al. (Prokaryotic Promoters in Biotechnology, Biotechnol. Annu. Rev., 1, 105-128 (1995)), and so forth.

[0190]

The translation efficiency of a gene can be improved by, for example, replacing the Shine-Dalgarno (SD) sequence (also referred to as ribosome binding site (RBS)) for the gene on a chromosome with a stronger SD sequence. The "stronger SD sequence" means a SD sequence that provides an improved translation of mRNA compared with the inherently existing wild-type SD sequence of the gene. Examples of stronger SD sequences include, for example, RBS of the gene *10* derived from phage T7 (Olins P.O. et al, Gene, 1988, 73, 227-235). Furthermore, it is known that substitution, insertion, or deletion of several nucleotides in a spacer region between RBS and the start codon, especially in a sequence immediately upstream of the start codon (5'-UTR), significantly affects the stability and translation efficiency of mRNA, and hence, the translation efficiency of a gene can also be improved by modifying them.

[0191]

The translation efficiency of a gene can also be improved by, for example, modifying codons. For example, the translation efficiency of the gene can be improved by replacing a rare codon present in the gene with a synonymous codon more frequently used. That is, the gene to be introduced may be modified, for example, so as to contain optimal codons according to the frequencies of codons observed in a host to be used. Codons can be replaced by, for example, the site-specific mutation method. Alternatively, a gene fragment in which objective codons are replaced may be totally synthesized. Frequencies of codons in various organisms are disclosed in the "Codon Usage Database" (<http://www.kazusa.or.jp/codon>; Nakamura, Y. et al, Nucl. Acids Res., 28, 292 (2000)).

[0192]

Furthermore, the expression of a gene can also be increased by amplifying a regulator that increases the expression of the gene, or deleting or attenuating a regulator that reduces the expression of the gene.

[0193]

Such methods for increasing the gene expression as mentioned above may be used independently or in an arbitrary combination.

[0194]

Furthermore, the modification that increases the activity of a protein can also be attained by, for example, enhancing the specific activity of the protein.

Enhancement of the specific activity also includes desensitization to feedback inhibition. That is, when a protein is subject to feedback inhibition by a metabolite, the activity of the protein can be increased by making the bacterium harbor a gene encoding a mutant protein that has been desensitized to the feedback inhibition. In the present invention, the term "desensitization to feedback inhibition" includes complete elimination of the feedback inhibition, and attenuation of the feedback inhibition, unless otherwise stated. Also, a state of "being desensitized to feedback inhibition", i.e. a state that feedback inhibition is eliminated or attenuated, may also be referred to as "tolerant to feedback inhibition". A protein showing an enhanced specific activity can be obtained by, for example, searching various organisms. Furthermore, a highly-active type of an existing protein may also be obtained by introducing a mutation into the existing protein. The mutation to be introduced may be, for example, substitution, deletion, insertion, or addition of one or several amino acid residues at one or several position of the protein. The mutation can be introduced by, for example, such a site-specific mutation method as mentioned above. The mutation may also be introduced by, for example, a mutagenesis treatment. Examples of the mutagenesis treatment include irradiation of X-ray, irradiation of ultraviolet, and a treatment with a mutation agent such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), and methyl methanesulfonate (MMS). Furthermore, a random mutation may be induced by directly treating DNA in vitro with hydroxylamine. Enhancement of the specific activity may be independently used, or may be used in an arbitrary combination with such methods for enhancing gene expression as mentioned above.

[0195]

The method for the transformation is not particularly limited, and conventionally known methods can be used. There can be used, for example, a method of treating recipient cells with calcium chloride so as to increase the permeability thereof for DNA, which has been reported for the *Escherichia coli* K-12 strain (Mandel, M. and Higa, A., J. Mol. Biol., 1970, 53, 159-162), and a method of preparing competent cells from cells which are in the growth phase, followed by transformation with DNA, which has been reported for *Bacillus subtilis* (Duncan, C.H., Wilson, G.A. and Young, F.E., Gene, 1997, 1:153-167). Alternatively, there can also be used a method of making DNA-recipient cells into protoplasts or spheroplasts, which can easily take up recombinant DNA, followed by introducing a recombinant DNA into

the DNA-recipient cells, which is known to be applicable to *Bacillus subtilis*, actinomycetes, and yeasts (Chang, S. and Choen, S.N., 1979, Mol. Gen. Genet., 168:111-115; Bibb, M.J., Ward, J.M. and Hopwood, O.A., 1978, Nature, 274:398-400; Hinnen, A., Hicks, J.B. and Fink, G.R., 1978, Proc. Natl. Acad. Sci. USA, 75:1929-1933). Furthermore, the electric pulse method reported for coryneform bacteria (Japanese Patent Laid-open (Kokai) No. 2-207791) can also be used.

[0196]

An increase in the activity of a protein can be confirmed by measuring the activity of the protein.

[0197]

An increase in the activity of a protein can also be confirmed by confirming an increase in the expression of a gene encoding the protein. An increase in the expression of a gene can be confirmed by confirming an increase in the transcription amount of the gene, or by confirming an increase in the amount of a protein expressed from the gene.

[0198]

An increase of the transcription amount of a gene can be confirmed by comparing the amount of mRNA transcribed from the gene with that of a non-modified strain such as a wild-type strain or parent strain. Examples of the method for evaluating the amount of mRNA include Northern hybridization, RT-PCR, and so forth (Sambrook, J., et al., Molecular Cloning A Laboratory Manual/Third Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001). The amount of mRNA may be increased to, for example, 1.5 times or more, 2 times or more, or 3 times or more of that of a non-modified strain.

[0199]

An increase in the amount of a protein can be confirmed by Western blotting using antibodies (Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001). The amount of the protein may be increased to, for example, 1.5 times or more, 2 times or more, or 3 times or more of that of a non-modified strain.

[0200]

The aforementioned methods for increasing the activity of a protein can be used for enhancement of the activities of arbitrary proteins such as L-amino acid

biosynthesis enzymes, and enhancement of the expression of arbitrary genes such as genes encoding those arbitrary proteins, besides enhancement of carotenoid biosynthesis enzyme activity.

[0201]

<1-4> Method for reducing activity of protein

Hereinafter, the methods for reducing the activity of a protein will be explained.

[0202]

The expression "the activity of a protein is reduced" means that the activity of the protein is reduced as compared with a non-modified strain. Specifically, the expression "the activity of a protein is reduced" may mean that the activity of the protein per cell is reduced as compared with that of a non-modified strain. The term "non-modified strain" used herein refers to a control strain that has not been modified so that the activity of an objective protein is reduced. Examples of the non-modified strain include a wild-type strain and parent strain. Specific examples of the non-modified strain include the respective type strains of the species of microorganisms. Specific examples of the non-modified strain also include strains exemplified above in relation to the description of microorganisms. That is, in an embodiment, the activity of a protein may be reduced as compared with a type strain, i.e. the type strain of the species to which the microorganism of the present invention belongs. In another embodiment, the activity of a protein may also be reduced as compared with the *E. coli* MG1655 strain. In another embodiment, the activity of a protein may also be reduced as compared with the *P. ananatis* AJ13355 strain. In another embodiment, the activity of a protein may also be reduced as compared with the *C. glutamicum* ATCC 13869 strain. The state that "the activity of a protein is reduced" also includes a state that the activity of the protein has completely disappeared. More specifically, the expression "the activity of a protein is reduced" may mean that the number of molecules of the protein per cell is reduced, and/or the function of each molecule of the protein is reduced as compared with those of a non-modified strain. That is, the term "activity" in the expression "the activity of a protein is reduced" is not limited to the catalytic activity of the protein, but may also mean the transcription amount of a gene (i.e. the amount of mRNA) encoding the protein or the translation amount of the protein (i.e. the amount of the protein). The state that "the number of molecules of the protein per cell is reduced" also includes a state that the protein does not exist at all. The state that

"the function of each molecule of the protein is reduced" also includes a state that the function of each protein molecule has completely disappeared. The degree of the reduction in the activity of a protein is not particularly limited, so long as the activity is reduced as compared with that of a non-modified strain. The activity of a protein may be reduced to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0% of that of a non-modified strain.

[0203]

The modification for reducing the activity of a protein can be attained by, for example, reducing the expression of a gene encoding the protein. The expression "the expression of a gene is reduced" means that the expression of the gene is reduced as compared with a non-modified strain such as a wild-type strain and parent strain. Specifically, the expression "the expression of a gene is reduced" may mean that the expression of the gene per cell is reduced as compared with that of a non-modified strain such as a wild-type strain and parent strain. More specifically, the expression "the expression of a gene is reduced" may mean that the transcription amount of the gene (i.e. the amount of mRNA) is reduced, and/or the translation amount of the gene (i.e. the amount of the protein expressed from the gene) is reduced. The state that "the expression of a gene is reduced" also includes a state that the gene is not expressed at all. The state that "the expression of a gene is reduced" is also referred to as "the expression of a gene is attenuated". The expression of a gene may be reduced to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0% of that of a non-modified strain.

[0204]

The reduction in gene expression may be due to, for example, a reduction in the transcription efficiency, a reduction in the translation efficiency, or a combination of them. The expression of a gene can be reduced by modifying an expression control sequence of the gene such as promoter, Shine-Dalgarno (SD) sequence (also referred to as ribosome-binding site (RBS)), and spacer region between RBS and the start codon of the gene. When an expression control sequence is modified, preferably one or more nucleotides, more preferably two or more nucleotides, particularly preferably three or more nucleotides, of the expression control sequence are modified. The transcription efficiency of a gene can be reduced by, for example, replacing the promoter of the gene on a chromosome with a weaker promoter. The term "weaker promoter" means a promoter providing an attenuated transcription of a gene compared with an inherently

existing wild-type promoter of the gene. Examples of weaker promoters include, for example, inducible promoters. That is, an inducible promoter may function as a weaker promoter under a non-induced condition, such as in the absence of the corresponding inducer. Furthermore, a part or the whole of an expression control sequence may be deleted. The expression of a gene can also be reduced by, for example, manipulating a factor responsible for expression control. Examples of the factor responsible for expression control include low molecules responsible for transcription or translation control (inducers, inhibitors, etc.), proteins responsible for transcription or translation control (transcription factors etc.), nucleic acids responsible for transcription or translation control (siRNA etc.), and so forth. Furthermore, the expression of a gene can also be reduced by, for example, introducing a mutation that reduces the expression of the gene into the coding region of the gene. For example, the expression of a gene can be reduced by replacing a codon in the coding region of the gene with a synonymous codon used less frequently in a host. Furthermore, for example, the gene expression may be reduced due to disruption of a gene as described later.

[0205]

The modification for reducing the activity of a protein can also be attained by, for example, disrupting a gene encoding the protein. The expression "a gene is disrupted" means that a gene is modified so that a protein that can normally function is not produced. The state that "a protein that normally functions is not produced" includes a state that the protein is not produced at all from the gene, and a state that the protein of which the function (such as activity or property) per molecule is reduced or eliminated is produced from the gene.

[0206]

Disruption of a gene can be attained by, for example, deleting the gene on a chromosome. The term "deletion of a gene" refers to deletion of a partial or entire region of the coding region of the gene. Furthermore, the whole of a gene including sequences upstream and downstream from the gene on a chromosome may be deleted. The region to be deleted may be any region such as an N-terminus region, an internal region, or a C-terminus region, so long as the activity of the protein can be reduced. Deletion of a longer region can usually more surely inactivate the gene. The region to be deleted may be, for example, a region having a length of 10% or more, 20% or more,

30% or more, 40% or more, 50% or more, 60% or more, 70% or more, 80% or more, 90% or more, or 95% or more of the total length of the coding region of the gene.

Furthermore, it is preferred that reading frames of the sequences upstream and downstream from the region to be deleted are not the same.

[0207]

Disruption of a gene can also be attained by, for example, introducing a mutation for an amino acid substitution (missense mutation), a stop codon (nonsense mutation), a frame shift mutation which adds or deletes one or two nucleotide residues, or the like into the coding region of the gene on a chromosome (Journal of Biological Chemistry, 272:8611-8617 (1997); Proceedings of the National Academy of Sciences, USA, 95 5511-5515 (1998); Journal of Biological Chemistry, 26 116, 20833-20839 (1991)).

[0208]

Disruption of a gene can also be attained by, for example, inserting another sequence into a coding region of the gene on a chromosome. Site of the insertion may be in any region of the gene, and insertion of a longer region can usually more surely inactivate the gene. It is preferred that reading frames of the sequences upstream and downstream from the insertion site are not the same. The other sequence is not particularly limited so long as a sequence that reduces or eliminates the activity of the encoded protein is chosen, and examples thereof include, for example, a marker gene such as antibiotic resistance genes, and a gene useful for production of an objective substance.

[0209]

Such modification of a gene on a chromosome as described above can be attained by, for example, preparing a deficient type gene modified so that it is unable to produce a protein that normally functions, and transforming a host with a recombinant DNA containing the deficient type gene to cause homologous recombination between the deficient type gene and the wild-type gene on a chromosome and thereby substitute the deficient type gene for the wild-type gene on the chromosome. In this procedure, if a marker gene selected according to the characteristics of the host such as auxotrophy is included in the recombinant DNA, the operation becomes easier. Examples of the deficient type gene include a gene including deletion of all or a part of the gene, gene including a missense mutation, gene including a nonsense mutation, gene including a

frame shift mutation, and gene including insertion of a transposon or marker gene. The protein encoded by the deficient type gene has a conformation different from that of the wild-type protein, even if it is produced, and thus the function thereof is reduced or eliminated. Such gene disruption based on gene substitution utilizing homologous recombination has already been established, and there are methods of using a linear DNA such as a method called "Red driven integration" (Datsenko, K.A, and Wanner, B.L., Proc. Natl. Acad. Sci. USA, 97:6640-6645 (2000)), and a method utilizing the Red driven integration in combination with an excision system derived from λ phage (Cho, E.H., Gumport, R.I., Gardner, J.F., J. Bacteriol., 184:5200-5203 (2002)) (refer to WO2005/010175), a method of using a plasmid having a temperature sensitive replication origin, a method of using a plasmid capable of conjugative transfer, a method of utilizing a suicide vector not having a replication origin that functions in a host (U.S. Patent No. 6,303,383, Japanese Patent Laid-open (Kokai) No. 05-007491), and so forth.

[0210]

Modification for reducing activity of a protein can also be attained by, for example, a mutagenesis treatment. Examples of the mutagenesis treatment include irradiation of X-ray or ultraviolet and treatment with a mutation agent such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), and methyl methanesulfonate (MMS).

[0211]

When a protein functions as a complex consisting of a plurality of subunits, a part or all of the plurality of subunits may be modified, so long as the activity of the protein is eventually reduced. That is, for example, a part or all of a plurality of genes that encode the respective subunits may be disrupted or the like. Furthermore, when there is a plurality of isozymes of a protein, a part or all of the activities of the plurality of isozymes may be reduced, so long as the activity of the protein is eventually reduced. That is, for example, a part or all of a plurality of genes that encode the respective isozymes may be disrupted or the like.

[0212]

A reduction in the activity of a protein can be confirmed by measuring the activity of the protein.

[0213]

A reduction in the activity of a protein can also be confirmed by confirming a reduction in the expression of a gene encoding the protein. A reduction in the expression of a gene can be confirmed by confirming a reduction in the transcription amount of the gene or a reduction in the amount of the protein expressed from the gene.

[0214]

A reduction in the transcription amount of a gene can be confirmed by comparing the amount of mRNA transcribed from the gene with that of a non-modified strain. Examples of the method for evaluating the amount of mRNA include Northern hybridization, RT-PCR, and so forth (Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001). The amount of mRNA is preferably reduced to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0% of that of a non-modified strain.

[0215]

A reduction in the amount of a protein can be confirmed by Western blotting using antibodies (Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA) 2001). The amount of the protein is preferably reduced to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0% of that of a non-modified strain.

[0216]

Disruption of a gene can be confirmed by determining nucleotide sequence of a part or the whole of the gene, restriction enzyme map, full length, or the like of the gene depending on the means used for the disruption.

[0217]

The aforementioned methods for reducing the activity of a protein as mentioned above can be applied to reduction in the activities of arbitrary proteins such as an enzyme that catalyzes a reaction branching away from the biosynthesis pathway of an objective L-amino acid to generate a compound other than the objective L-amino acid, and reduction in the expression of arbitrary genes such as genes encoding those arbitrary proteins.

[0218]

<2> Method of the present invention

<2-1> Method for producing L-amino acid

An embodiment of the method of the present invention (also referred to as

"first embodiment of the method of the present invention") is a method for producing an L-amino acid comprising culturing the bacterium of the present invention in a medium (a culture medium) to accumulate an L-amino acid in the medium, and collecting the L-amino acid from the medium. In the first embodiment of the present invention, one kind of L-amino acid may be produced, or two or more kinds of L-amino acids may be produced.

[0219]

The medium to be used is not particularly limited, so long as the bacterium of the present invention can proliferate in it, and an objective L-amino acid can be produced. As the medium, for example, a usual medium used for culture of bacteria such as coryneform bacteria and *Enterobacteriaceae* bacteria can be used. As the medium, for example, a medium containing carbon source, nitrogen source, phosphorus source, and sulfur source, as well as components selected from other various organic components and inorganic components as required can be used. Types and concentrations of the medium components can be appropriately determined according to various conditions such as the type of bacterium to be used.

[0220]

Specific examples of the carbon source include, for example, saccharides such as glucose, fructose, sucrose, lactose, galactose, xylose, arabinose, blackstrap molasses, hydrolysates of starches, and hydrolysates of biomass, organic acids such as acetic acid, fumaric acid, citric acid, and succinic acid, alcohols such as glycerol, crude glycerol, and ethanol, and aliphatic acids. As the carbon source, plant-derived materials can be preferably used. Examples of the plant include, for example, corn, rice, wheat, soybean, sugarcane, beet, and cotton. Examples of the plant-derived materials include, for example, organs such as root, stem, trunk, branch, leaf, flower, and seed, plant bodies including them, and decomposition products of these plant organs. The forms of the plant-derived materials at the time of use thereof are not particularly limited, and they can be used in any form such as unprocessed product, juice, ground product, and purified product. Pentoses such as xylose, hexoses such as glucose, or mixtures of them can be obtained from, for example, plant biomass, and used. Specifically, these saccharides can be obtained by subjecting a plant biomass to such a treatment as steam treatment, hydrolysis with concentrated acid, hydrolysis with diluted acid, hydrolysis with an enzyme such as cellulase, and alkaline treatment. Since hemicellulose is

generally more easily hydrolyzed compared with cellulose, hemicellulose in a plant biomass may be hydrolyzed beforehand to liberate pentoses, and then cellulose may be hydrolyzed to generate hexoses. Furthermore, xylose may be supplied by conversion from hexoses by, for example, imparting a pathway for converting hexose such as glucose to xylose to the bacterium of the present invention. As the carbon source, a single kind of carbon source may be used, or two or more kinds of carbon sources may be used in combination.

[0221]

Specific examples of the nitrogen source include, for example, ammonium salts such as ammonium sulfate, ammonium chloride, and ammonium phosphate, organic nitrogen sources such as peptone, yeast extract, meat extract, and soybean protein decomposition products, ammonia, and urea. Ammonia gas or aqueous ammonia used for adjusting pH may also be used as the nitrogen source. As the nitrogen source, a single kind of nitrogen source may be used, or two or more kinds of nitrogen sources may be used in combination.

[0222]

Specific examples of the phosphate source include, for example, phosphoric acid salts such as potassium dihydrogenphosphate and dipotassium hydrogenphosphate, and phosphoric acid polymers such as pyrophosphoric acid. As the phosphate source, a single kind of phosphate source may be used, or two or more kinds of phosphate sources may be used in combination.

[0223]

Specific examples of the sulfur source include, for example, inorganic sulfur compounds such as sulfates, thiosulfates, and sulfites, and sulfur-containing amino acids such as cysteine, cystine, and glutathione. As the sulfur source, a single kind of sulfur source may be used, or two or more kinds of sulfur sources may be used in combination.

[0224]

Specific examples of other various organic components and inorganic components include, for example, inorganic salts such as sodium chloride and potassium chloride; trace metals such as iron, manganese, magnesium, and calcium; vitamins such as vitamin B1, vitamin B2, vitamin B6, nicotinic acid, nicotinamide, and vitamin B12; amino acids; nucleic acids; and organic components containing those such as peptone, casamino acid, yeast extract, and soybean protein decomposition product.

As other various organic components and inorganic components, a single kind of component may be used, or two or more kinds of components may be used in combination.

[0225]

Furthermore, when an auxotrophic mutant that requires an amino acid or the like for growth thereof is used, it is preferable to supply a required nutrient to the medium. For example, in many of L-lysine-producing bacteria, the L-lysine biosynthetic pathway is enhanced and the L-lysine decomposition ability is attenuated. When such an L-lysine-producing bacterium is cultured, for example, one or more kinds of amino acids selected from L-threonine, L-homoserine, L-isoleucine, and L-methionine are preferably added to the medium.

[0226]

Furthermore, it is also preferable to, for example, restrict the amount of biotin in the medium, or add a surfactant or penicillin to the medium.

[0227]

The culture conditions are not particularly limited so long as the bacterium of the present invention can proliferate, and an objective L-amino acid can be produced. The culture can be performed, for example, under usual conditions used for culturing bacteria such as coryneform bacteria and *Enterobacteriaceae* bacteria. The culture conditions can be appropriately set according to various conditions such as the type of bacterium to be used.

[0228]

The culture can be performed by using a liquid medium. At the time of the culture, the bacterium of the present invention cultured on a solid medium such as agar medium may be directly inoculated into a liquid medium, or the bacterium of the present invention cultured in a liquid medium as seed culture may be inoculated into a liquid medium for main culture. That is, the culture may be performed separately as seed culture and main culture. In such a case, the culture conditions of the seed culture and the main culture may be or may not be the same. Amount of the bacterium of the present invention contained in the medium at the time of the start of the culture is not particularly limited. The main culture may be performed by, for example, inoculating a seed culture broth to a medium for main culture at an amount of 1 to 50%(v/v).

[0229]

The culture can be performed as batch culture, fed-batch culture, continuous culture, or a combination of these. The medium used at the time of the start of the culture is also referred to as "starting medium". The medium supplied to a culture system (fermentation tank) in fed-batch culture or continuous culture is also referred to as "feed medium". Furthermore, to supply a feed medium to a culture system in fed-batch culture or continuous culture is also referred to as to "feed". Furthermore, when the culture is performed separately as seed culture and main culture, for example, both the seed culture and the main culture may be performed as batch culture. Alternatively, for example, the seed culture may be performed as batch culture, and the main culture may be performed as fed-batch culture or continuous culture.

[0230]

In the present invention, the medium components each may be contained in the starting medium, feed medium, or the both. The types of the components contained in the starting medium may be or may not be the same as the types of the components contained in the feed medium. The concentration of each component contained in the starting medium may be or may not be the same as the concentration of the component contained in the feed medium. Furthermore, two or more kinds of feed media containing different types and/or different concentrations of components may be used. For example, when medium is intermittently fed a plurality of times, the types and/or concentrations of components contained in the feed media may be or may not be the same for each feeding.

[0231]

The concentration of the carbon source in the medium is not particularly limited, so long as the bacterium of the present invention can proliferate and produce an L-amino acid. The concentration of the carbon source in the medium may be as high as possible within such a range that production of the L-amino acid is not inhibited. The concentration of the carbon source in the medium may be, as the initial concentration (the concentration in the starting medium), for example, 1 to 30% (w/v), preferably 3 to 10% (w/v). Furthermore, the carbon source may be additionally supplied to the medium as required. For example, the carbon source may be additionally supplied to the medium in proportion to consumption of the carbon source accompanying progress of the fermentation.

[0232]

The culture can be performed, for example, under an aerobic condition. The term "aerobic condition" refers to a condition where the dissolved oxygen concentration in the liquid medium is not lower than 0.33 ppm, which is the detection limit for the detection with an oxygen membrane electrode, or may preferably to a condition where the dissolved oxygen concentration in the liquid medium is not lower than 1.5 ppm. The oxygen concentration can be controlled to, for example, 5 to 50%, preferably about 10%, of the saturated oxygen concentration. Specifically, the culture under an aerobic condition can be performed by aeration culture, shaking culture, stirring culture, or a combination thereof. The pH of the medium may be, for example, 3 to 10, preferably 4.0 to 9.5. During the culture, the pH of the medium can be adjusted as required. The pH of the medium can be adjusted by using various alkaline and acidic substances such as ammonia gas, aqueous ammonia, sodium carbonate, sodium bicarbonate, potassium carbonate, potassium bicarbonate, magnesium carbonate, sodium hydroxide, calcium hydroxide, and magnesium hydroxide. The culture temperature may be, for example, 20 to 40°C, preferably 25 to 37°C. The culture period may be, for example, 10 to 120 hours. The culture may be continued, for example, until the carbon source contained in the medium is consumed, or until the bacterium of the present invention loses the activity. By culturing the bacterium of the present invention under such conditions as described above, an L-amino acid is accumulated in the medium.

[0233]

For producing a basic amino acid such as L-lysine, the culture step (fermentation step) may be carried out so that bicarbonate ions and/or carbonate ions serve as counter ions for the basic amino acid. Such a fermentation mode is also referred to as "carbonate fermentation". By the carbonate fermentation, a basic amino acid can be produced by fermentation while reducing the amounts of sulfate ions and/or chloride ions to be used, which have been conventionally used as counter ions for a basic amino acid.

[0234]

The carbonate fermentation can be carried out, for example, as described in US2002-0025564A, EP1813677A, and Japanese Patent Laid-open (Kokai) No. 2002-65287.

[0235]

Specifically, the carbonate fermentation can be carried out so that, for example,

there is a culture period where 20 mM or more, preferably 30 mM or more, more preferably 40 mM or more, of bicarbonate ions and/or carbonate ions are present in the medium. The above-exemplified concentrations each are interpreted as the total concentration of bicarbonate ions and carbonate ions. The phrase "there is a culture period where bicarbonate ions and/or carbonate ions are present in a medium at a certain concentration" means that bicarbonate ions and/or carbonate ions are present in the medium at the certain concentration during at least a partial period of culture. That is, bicarbonate ions and/or carbonate ions may be present in the medium at the above-exemplified concentration over the whole period of culture or during a partial period of culture. The "partial period" is not particularly limited so long as a desired productivity of the basic amino acid is attained. The "partial period" may be, for example, a period of 10% or more, 20% or more, 30% or more, 50% or more, 70% or more, or 90% or more of that of the whole period of culture. When the culture is performed separately as seed culture and main culture, the term "whole period of culture" refers to the whole period of the main culture. It is preferable that bicarbonate ions and/or carbonate ions are present in the medium at the above-exemplified concentration during a period where the basic amino acid is produced. That is, for example, when the culture step (fermentation step) comprises a stage of proliferating the basic amino acid-producing bacterium (growth period) and a stage of producing the basic amino acid (production period), it is preferable that bicarbonate ions and/or carbonate ions are present in the medium at the above-exemplified concentration at least during the production period. The term "growth period" refers to a period where the carbon source is utilized mainly for cell growth, and may specifically refer to a period until the time point of 3 hr, 6 hr, or 10 hr after the start of culture. The term "production period" refers to a period where the carbon source is utilized mainly for production of a substance, and may specifically refer to a period from the time point of 3 hr, 6 hr, or 10 hr after the start of culture.

[0236]

Bicarbonate ions and/or carbonate ions can be made exist in the medium by controlling the internal pressure of the fermentation tank to be positive, supplying carbon dioxide gas into the medium, or a combination thereof. The internal pressure of the fermentation tank during fermentation can be controlled to be positive by, for example, making the gas supply pressure higher than the exhaust pressure. If the

internal pressure of the fermentation tank is controlled to be positive, the carbon dioxide gas generated by fermentation dissolves in the medium to generate bicarbonate ions and/or carbonate ions, and these can serve as counter ions for the basic amino acid. The internal pressure of the fermentation tank may specifically be, for example, 0.03 to 0.2 MPa, preferably 0.05 to 0.15 MPa, more preferably 0.1 to 0.3 MPa, in terms of the gage pressure (pressure difference with respect to the atmospheric pressure). Carbon dioxide gas can be supplied to the medium by, for example, bubbling pure carbon dioxide gas or a mixed gas containing carbon dioxide gas into the medium. Examples of the mixed gas containing carbon dioxide gas include a mixed gas containing 5 volume % or more of carbon dioxide gas. The internal pressure in the fermentation tank, supply volume of carbon dioxide gas, and limited aeration volume can be appropriately set according to various conditions such as pH of the medium, bicarbonate and/or carbonate ion concentration in the medium, and ammonia concentration in the medium.

[0237]

In the conventional methods for producing a basic amino acid, a sufficient amount of ammonium sulfate and/or ammonium chloride is usually added to the medium as a source of counter ions for the basic amino acid, or sulfuric acid decomposition products and/or hydrochloric acid decomposition products of proteins etc. as nutrient components are added to the medium. Therefore, large amounts of sulfate ions and/or chloride ions are present in the medium, and the concentration of the weakly acidic carbonate ions is extremely low, i.e., it is at a ppm order.

[0238]

On the other hand, the carbonate fermentation is characterized in that the amounts of sulfate ions and/or chloride ions to be used are reduced so that carbon dioxide gas released by a bacterium during fermentation and/or carbon dioxide gas externally supplied is dissolved in the medium, and used as counter ions for the basic amino acid. That is, in the carbonate fermentation, one of the objects is to reduce the amounts of sulfate ions and/or chloride ions to be used, and therefore, it is not necessary to add sulfate ions or chloride ions to the medium in an amount larger than that required for growth of the basic amino acid-producing bacterium. It is preferred that ammonium sulfate or the like is fed to the medium at an early stage of the culture at an amount required for growth, and the feeding is terminated in the middle of the culture.

Alternatively, ammonium sulfate or the like may be fed to the medium while maintaining the balance with respect to the amounts of carbonate ions and/or bicarbonate ions dissolved in the medium. By reducing the amounts of sulfate ions and/or chloride ions to be used, the concentrations of sulfate ions and/or chloride ions in the medium can be lowered. By lowering the concentrations of sulfate ions and/or chloride ions, bicarbonate ions and/or carbonate ions can be made more easily exist in the medium. That is, in the carbonate fermentation, the pH of the medium for making bicarbonate ions and/or carbonate ions exist in the medium in an amount required as the counter ions for the basic amino acid can be suppressed to be lower compared with the conventional methods. The total molar concentration of sulfate ions and chloride ions contained in the medium may specifically be, for example, 900 mM or lower, preferably 700 mM or lower, more preferably 500 mM or lower, further preferably 300 mM or lower, further preferably 200 mM or lower, particularly preferably 100 mM or lower. [0239]

In the carbonate fermentation, the pH of the medium is not particularly limited so long as bicarbonate ions and/or carbonate ions are made exist in the medium at a desired concentration, and a desired productivity of the basic amino acid is attained. The pH of the medium can be appropriately set according to various conditions such as the type of bacterium to be used, bicarbonate and/or carbonate ion concentration in the medium, and ammonia concentration in the medium. The pH of the medium may be controlled to, for example, 6.5 to 9.0, preferably 7.2 to 9.0, during the culture. The pH of the medium may be controlled to the above-exemplified value during at least a partial period of culture. That is, the pH of the medium may be controlled to the above-exemplified value over the whole period of culture or during a partial period of culture. The descriptions concerning the culture period where bicarbonate ions and/or carbonate ions are present in a medium can be applied *mutatis mutandis* to the "partial period" during which the pH of the medium is controlled. That is, for example, the pH of the medium may be controlled to the above-exemplified value during a period where the basic amino acid is produced. Furthermore, the pH of the medium may be, for example, 7.2 or higher, preferably 7.2 to 9.0, at the completion of culture. That is, the pH of the medium may be controlled so as to attain the above-exemplified value of pH at the completion of culture. The pH of the medium may be controlled, for example, directly by using the pH value *per se* as an indicator, or indirectly by controlling the

total ammonia concentration (WO2006/038695).

[0240]

Furthermore, the medium may contain anions other than bicarbonate ions and/or carbonate ions (also referred to as other anions). It is preferred that the concentrations of the other anions in the medium are low so long as they are present in amounts required for growth of the basic amino acid-producing bacterium. Examples of the other anions include chloride ions, sulfate ions, phosphate ions, organic acid ions, and hydroxide ions. The total molar concentration of these other anions may specifically be, for example, 900 mM or lower, preferably 700 mM or lower, more preferably 500 mM or lower, still more preferably 300 mM or lower, particularly preferably 200 mM or lower, 100 mM or lower, 50 mM or lower, or 20 mM or lower.

[0241]

Furthermore, the medium may contain cations other than the basic amino acid (also referred to as other cations). Examples of the other cations include K ions, Na ions, Mg ions, and Ca ions originating in medium components. The total molar concentration of the other cations may specifically be, for example, 50% or lower, preferably 10% or lower, more preferably 5% or lower, particularly preferably 2% or lower, of the molar concentration of the total cations.

[0242]

In the carbonate fermentation, it is also preferable to control the total ammonia concentration in the medium to a concentration at which production of the basic amino acid is not inhibited (WO2006/038695, WO2015/050234). The term "total ammonia concentration" refers to the total concentration of non-dissociated ammonia (NH_3) and ammonium ions (NH_4^+). Examples of the total ammonia concentration at which "production of the basic amino acid is not inhibited" include, for example, a total ammonia concentration providing yield and/or productivity corresponding to preferably 50% or more, more preferably 70% or more, particularly preferably 90% or more, of the yield and/or productivity obtainable in the production of the basic amino acid under optimal conditions. The total ammonia concentration in the medium may specifically be, for example, 300 mM or lower, 250 mM or lower, 200 mM or lower, 100 mM or lower, or 50 mM or lower. The dissociation degree of ammonia decreases as the pH becomes higher. Non-dissociated ammonia is more toxic to bacteria compared with ammonium ions. Therefore, the upper limit of the total ammonia concentration also

depends on the pH of the medium. That is, as the pH of the medium increases, the acceptable total ammonia concentration decreases. Therefore, the total ammonia concentration at which "production of the basic amino acid is not inhibited" is preferably determined for each specific pH value. However, the total ammonia concentration range that is acceptable at the highest pH level during the culture can be used as the total ammonia concentration range over the whole period of culture. The total ammonia concentration in the medium may be controlled to the above-exemplified concentration during at least a partial period of culture. That is, the total ammonia concentration in the medium may be controlled to the above-exemplified concentration over the whole period of culture or during a partial period of culture. The descriptions concerning the culture period where bicarbonate ions and/or carbonate ions are present in a medium can be applied *mutatis mutandis* to the "partial period" during which the total ammonia concentration in the medium is controlled. That is, for example, the total ammonia concentration in the medium may be controlled to the above-exemplified concentration during a period where the basic amino acid is produced. Furthermore, specific examples of the "partial period" during which the total ammonia concentration in the medium include a period during which the pH of the medium increases (e.g. the pH of the medium increases to 7.2 or higher) due to the shortage of counter ions such as sulfate ions and chloride ions with respect to accumulation of the basic amino acid.

[0243]

Meanwhile, the total concentration of ammonia as a source of nitrogen required for growth of the basic amino acid-producing bacterium and production of the basic amino acid is not particularly limited, and can be appropriately set, so long as depletion of ammonia does not continue during the culture, and decrease in the productivity of the basic amino acid due to the shortage of the nitrogen source does not occur. For example, the ammonia concentration can be measured over time during the culture, and if ammonia in the medium is depleted, a small amount of ammonia can be added to the medium. The ammonia concentration after the addition of ammonia may specifically be, for example, preferably 1 mM or higher, more preferably 10 mM or higher, particularly preferably 20 mM or higher, as the total ammonia concentration.

[0244]

The total ammonia concentration in the medium can be controlled by, for example, using an apparatus for controlling ammonia and a method for controlling

ammonia described in WO2015/050234.

[0245]

Moreover, when L-glutamic acid is produced, the culture can be performed by using a liquid medium adjusted to satisfy a condition under which L-glutamic acid is precipitated, while precipitating L-glutamic acid in the medium. Examples of the condition under which L-glutamic acid is precipitated include, for example, pH 5.0 to 4.0, preferably pH 4.5 to 4.0, more preferably pH 4.3 to 4.0, particularly preferably around pH 4.0 (EP1078989A).

[0246]

Production of an L-amino acid can be confirmed by known methods used for detection or identification of compounds. Examples of such methods include, for example, HPLC, LC/MS, GC/MS, and NMR. These methods can be independently used, or can be used in an appropriate combination.

[0247]

The produced L-amino acid can be collected (harvested) from the fermentation broth by known methods used for separation and purification of compounds. Examples of such methods include, for example, ion-exchange resin method (Nagai, H. et al., Separation Science and Technology, 39(16), 3691-3710), precipitation, membrane separation (Japanese Patent Laid-open (Kokai) No. 9-164323 and Japanese Patent Laid-open (Kokai) No. 9-173792), and crystallization (WO2008/078448 and WO2008/078646). These methods can be independently used, or can be used in an appropriate combination. The L-amino acid to be collected may be a free compound, a salt thereof, or a mixture thereof. Examples of the salt include, for example, sulfate, hydrochloride, carbonate, ammonium salt, sodium salt, and potassium salt. When L-lysine is produced, L-lysine to be collected may specifically be, for example, free L-lysine, L-lysine sulfate, L-lysine hydrochloride, L-lysine carbonate, or a mixture of these. When L-glutamic acid is produced, L-glutamic acid to be collected may specifically be, for example, free L-glutamic acid, sodium L-glutamate (monosodium L-glutamate, MSG), ammonium L-glutamate (monoammonium L-glutamate), or a mixture of these. For example, monosodium L-glutamate (MSG) can be obtained by adding an acid to the fermentation broth to crystallize ammonium L-glutamate contained therein, and then by adding an equimolar of sodium hydroxide to the crystals. In addition, decolorization can be performed by using activated carbon before and/or after the

crystallization (see, Tetsuya KAWAKITA, "Industrial Crystallization for Monosodium L-Glutamate.", Bulletin of the Society of Sea Water Science, Japan, Vol.56:5). The monosodium L-glutamate crystal can be used as, for example, an umami seasoning. The monosodium L-glutamate crystal may also be used as a seasoning in combination with a nucleic acid such as sodium guanylate and sodium inosinate, which also have umami taste.

[0248]

When the L-amino acid is precipitated in the medium, it can be collected by centrifugation, filtration, or the like. The L-amino acid precipitated in the medium may also be isolated together with the L-amino acid dissolving in the medium, after the L-amino acid dissolving in the medium is crystallized.

[0249]

The collected L-amino acid may contain such components as bacterial cells, medium components, moisture, and by-product metabolites of the bacterium in addition to the L-amino acid. The collected L-amino acid may also be purified at a desired extent. Purity of the collected L-amino acid may be, for example, 50% (w/w) or higher, preferably 85% (w/w) or higher, particularly preferably 95% (w/w) or higher (JP1214636B, USP5,431,933, USP4,956,471, USP4,777,051, USP4,946,654, USP5,840,358, USP6,238,714, and US2005/0025878). The L-amino acid may also be provided as a dried granulated product containing the L-amino acid. For example, examples of methods for producing a dried granulated product containing L-lysine include a method described in U.S. Patent No. 7,416,740.

[0250]

In addition, by culturing the bacterium of the present invention under such conditions as described above, a carotenoid can be accumulated in cells of the bacterium. Specifically, when the bacterium of the present invention has a carotenoid-producing ability, a carotenoid can be accumulated in cells of the bacterium by culturing the bacterium. In the present invention, one kind of carotenoid may be accumulated in cells of the bacterium, or two or more kinds of carotenoids may be accumulated in cells of the bacterium.

[0251]

When a carotenoid is accumulated in cells of the bacterium, the cells (i.e. bacterial cells containing a carotenoid) can be collected. That is, the first embodiment

of the method of the present invention may further comprise collecting the cells (i.e. bacterial cells containing a carotenoid). That is, the first embodiment of the method of the present invention may include a method for producing bacterial cells containing a carotenoid. Methods for collecting the cells from the fermentation broth are not particularly limited. The cells can be collected from the fermentation broth by known methods. Examples of methods for collecting the cells from the fermentation broth include, for example, spontaneous precipitation, centrifugation, and filtration. These methods can be independently used, or can be used in an appropriate combination.

[0252]

<2-2> Method for producing bacterial cells containing carotenoid

As described above, when a carotenoid is accumulated in cells of the bacterium, bacterial cells containing the carotenoid can be obtained. That is, another embodiment of the method of the present invention (also referred to as "second embodiment of the method of the present invention") is a method for producing bacterial cells containing a carotenoid comprising culturing the bacterium of the present invention having a carotenoid-producing ability in a medium to accumulate a carotenoid in cells of the bacterium, and collecting the cells of the bacterium. In the present invention, one kind of carotenoid may be accumulated in the cells, or two or more kinds of carotenoids may be accumulated in the cells.

[0253]

Furthermore, the bacterium of the present invention has an L-amino acid-producing ability. Hence, in the second embodiment of the method of the present invention, by culturing the bacterium of the present invention, an L-amino acid can be accumulated in a medium. When an L-amino acid is accumulated in the medium, the L-amino acid can be collected (harvested). That is, the second embodiment of the method of the present invention may further comprise collecting the L-amino acid from the medium. That is, the second embodiment of the method of the present invention may include a method for producing an L-amino acid. In the second embodiment of the present invention, one kind of L-amino acid may be produced, or two or more kinds of L-amino acids may be produced.

[0254]

Conditions for carrying out the second embodiment of the method of the present invention, such as medium composition, culture conditions, and methods for

collecting the cells, are not particularly limited so long as bacterial cells containing a carotenoid are obtained. The descriptions concerning the conditions for carrying out the first embodiment of the method of the present invention can be applied *mutatis mutandis* to the conditions for carrying out the second embodiment of the method of the present invention.

[0255]

In an embodiment, the first embodiment of the method of the present invention and the second embodiment of the method of the present invention may be identical to each other. Specifically, when both an L-amino acid and bacterial cells containing a carotenoid are produced in the first and second embodiments of the method of the present invention, both the embodiments may be identical to each other.

[0256]

<3> Bacterial cells of the present invention and use of the same

The bacterial cells of the present invention are bacterial cells containing a carotenoid. The bacterial cells of the present invention may be cells of the bacterium of the present invention containing a carotenoid. Specifically, when the bacterium of the present invention has a carotenoid-producing ability, cells of the bacterium can contain a carotenoid. That is, more specifically, the bacterial cells of the present invention may be cells of a bacterium, wherein the cells contain a carotenoid, the bacterium has an L-amino acid-producing ability and a carotenoid-producing ability, and the bacterium has been modified so that the activity of a carotenoid biosynthesis enzyme is increased. The bacterial cells of the present invention may contain one kind of carotenoid, or two or more kinds of carotenoids. The bacterial cells of the present invention can be produced by, for example, culturing the bacterium of the present invention and collecting (harvesting) the cells of the bacterium. That is, the bacterial cells of the present invention can be produced by, for example, the method of the present invention. The contained amount of the carotenoid in the cells can be measured according to the usual manner. The contained amount of the carotenoid in the cells can be measured by, for example, the method described in the Examples section. The bacterial cells of the present invention can be used as any form such as wet cells or dry cells.

[0257]

The use purpose of the bacterial cells of the present invention is not particularly

limited. The bacterial cells of the present invention can be used for, for example, culturing (breeding) of organisms such as aquatic organisms. Examples of aquatic organisms include, for example, fishes and crustaceans. Specific examples of aquatic organisms include, for example, salmon, shrimp (prawn), and seabream. The term "salmon" collectively refers to fishes belonging to the family *Salmonidae*. Specific examples of salmon include, for example, chum salmon (*Oncorhynchus keta*), sockeye salmon (*Oncorhynchus nerka*), coho salmon (*Oncorhynchus kisutch*), Atlantic salmon (*Salmo salar*), king salmon (*Oncorhynchus tshawytscha*), and rainbow trout (*Oncorhynchus mykiss*).

[0258]

That is, the present invention provides a feed, such as a feed for an aquatic organism, containing the bacterial cells of the present invention. This feed is also referred to as "feed of the present invention".

[0259]

The composition of the feed of the present invention is not particularly limited, except that it contains the bacterial cells of the present invention. The types and contained amounts of ingredients contained in the feed of the present invention can be appropriately chosen according to various conditions such as the type and growth stage of the objective organism, and the usage scheme of the feed of the present invention.

[0260]

The feed of the present invention may contain, for example, an ingredient similar to those contained in feeds such as usual feeds for aquatic organisms, in addition to the bacterial cells of the present invention. Examples of such an ingredient include animal feed ingredients, vegetable feed ingredients, and other various feed ingredients. Specific examples of the animal feed ingredients include, for example, fish meal, fish soluble, powdered bone, and chicken meal. Specific examples of the vegetable feed ingredients include, for example, cereal powder (wheat flour etc.), vegetable protein (wheat gluten, corn gluten, etc.), starch (cornstarch etc.), and vegetable oil meal (soybean meal etc.). Specific examples of the other ingredients include, for example, amino acid fermentation meal (lysine fermentation meal, etc.), thickening agent (carboxymethylcellulose, alginic acid, etc.), yeast, eicosapentaenoic acid, and docosahexaenoic acid. Specific examples of the other ingredients also include, for example, additives for fishery such as drugs for fishery, vitamins, and amino acids.

[0261]

The contained amount of the bacterial cells of the present invention in the feed of the present invention, for example, may be 1%(w/w) or more, 2%(w/w) or more, 3%(w/w) or more, 4%(w/w) or more, 5%(w/w) or more, 7%(w/w) or more, or 10%(w/w) or more, may be 50%(w/w) or less, 30%(w/w) or less, 20%(w/w) or less, 15%(w/w) or less, 10%(w/w) or less, 7%(w/w) or less, or 5%(w/w) or less, or may be within a range defined as a non-contradictory combination thereof, in terms of dry cell weight. Specifically, the contained amount of the bacterial cells of the present invention in the feed of the present invention may be, for example, 1%(w/w) to 10%(w/w).

[0262]

The contained amount of the bacterial cells of the present invention in the feed of the present invention may also be, for example, such an amount that the contained amount of total carotenoids in the feed of the present invention comes to be within a predetermined range. The contained amount of total carotenoids in the feed of the present invention, for example, may be 2 mg/100 g or more, 3 mg/100 g or more, 4 mg/100 g or more, 5 mg/100 g or more, or 10 mg/100 g or more, may be 30 mg/100 g or less, 20 mg/100 g or less, 15 mg/100 g or less, 10 mg/100 g or less, or 5 mg/100 g or less, or may be within a range defined as a non-contradictory combination thereof. Specifically, the contained amount of total carotenoids in the feed of the present invention may be, for example, 2 mg/100 g to 10 mg/100 g.

[0263]

The contained amount of the bacterial cells of the present invention in the feed of the present invention may also be, for example, such an amount that the contained amount of astaxanthine in the feed of the present invention comes to be within a predetermined range. The contained amount of astaxanthine in the feed of the present invention, for example, may be 0.3 mg/100 g or more, 0.5 mg/100 g or more, 1 mg/100 g or more, 1.5 mg/100 g or more, 2 mg/100 g or more, or 3 mg/100 g or more, may be 20 mg/100 g or less, 15 mg/100 g or less, 10 mg/100 g or less, 5 mg/100 g or less, or 2 mg/100 g or less, or may be within a range defined as a non-contradictory combination thereof. Specifically, the contained amount of astaxanthine in the feed of the present invention may be, for example, 0.3 mg/100 g to 5 mg/100 g.

[0264]

The feed of the present invention can be produced by combining the bacterial cells of the present invention and the other ingredient(s) so that the bacterial cells of the present invention is contained at a desired concentration in the feed. Methods for producing the feed of the present invention are not particularly limited. For example, the feed of the present invention can be produced by the same method as that for usual feeds for organisms using the same raw materials as those for usual feeds for organisms except that the bacterial cells of the present invention is added (blended).

[0265]

The form of the feed of the present invention is not particularly limited, so long as it can be ingested by an objective organism. That is, the feed of the present invention may be in any form such as powder, granule, crumble, pellet, cube, paste, and liquid. The feed of the present invention may be produced in the form of, for example, pellet such as dry pellet and moist pellet. The form of the feed of the present invention can be appropriately chosen depending on various conditions such as the type and growth stage of the objective organism. For example, for fry, the feed in the form of powder or crumble can usually be preferably used. For adult fish, dry pellets can usually be preferably used.

[0266]

The present invention also provides a composition for culturing (breeding) of an organism such as an aquatic organism, containing the bacterial cells of the present invention. This composition is also referred to as "composition of the present invention".

[0267]

The composition of the composition of the present invention is not particularly limited, except that it contains the bacterial cells of the present invention. The types and contained amounts of ingredients contained in the composition of the present invention can be appropriately chosen according to various conditions such as the type and growth stage of the objective organism, and the usage scheme of the composition of the present invention.

[0268]

The composition of the present invention may consist of the bacterial cells of the present invention, or may contain an ingredient other than the bacterial cells of the present invention. Examples of the ingredient other than the bacterial cells of the

present invention include, for example, ingredients usually used for use in foods, feeds, drugs, and so forth. Specific examples of such ingredients include, for example, additives such as excipients, binders, disintegrants, lubricants, stabilizers, flavoring agent, odor improving agents, perfumes, diluents, and surfactants. Specific examples of the ingredient other than the bacterial cells of the present invention also include, for example, such ingredients in the feed of the present invention as described above other than the bacterial cells of the present invention. That is, the composition of the present invention may be constituted as a feed for an aquatic organism. That is, an embodiment of the composition of the present invention may be such a composition of the present invention as described above. The composition of the present invention may be in any form such as powder, granule, crumble, pellet, cube, paste, and liquid.

[0269]

The contained amount of the bacterial cells of the present invention in the composition of the present invention, for example, may be 1%(w/w) or more, 2%(w/w) or more, 3%(w/w) or more, 4%(w/w) or more, 5%(w/w) or more, 7%(w/w) or more, or 10%(w/w) or more, may be 100%(w/w) or less, 99.9%(w/w) or less, 70%(w/w) or less, 50%(w/w) or less, 30%(w/w) or less, 20%(w/w) or less, 15%(w/w) or less, 10%(w/w) or less, 7%(w/w) or less, or 5%(w/w) or less, or may be within a range defined as a non-contradictory combination thereof, in terms of dry cell weight.

[0270]

The composition of the present invention may be used, for example, in combination with a feed such as a feed for an aquatic organism. The composition of the present invention may be used in combination with a feed so that, for example, the contained amount of the bacterial cells of the present invention, total carotenoids, or astaxanthine comes to be such a contained amount of the bacterial cells of the present invention, total carotenoids, or astaxanthine as described above in the feed of the present invention. To such a feed, the descriptions concerning the feed of the present invention mentioned above can be applied *mutatis mutandis*. When the composition of the present invention is constituted as a feed, the composition of the present invention itself may be used as the feed of the present invention.

[0271]

The present invention also provides a method for culturing an organism such as an aquatic organism, comprising feeding the bacterial cells of the present invention to

the organism. By culturing an organism, the organism can be produced. That is, the term "method for culturing an organism" may be synonymous to "method for producing an organism". By culturing an organism with feeding of the bacterial cells of the present invention, color tone of the organism can be improved. By culturing an organism with feeding of the bacterial cells of the present invention, particularly, reddish color of the organism can be enhanced. That is, the organism to be produced may be an organism having an improved color tone, or may particularly be an organism having an enhanced reddish color. The color tone and reddish color may be, for example, color tone and reddish color inside of the organism, such as those of fresh, or color tone and reddish color outside of the organism, such as those of scale or exoskeleton. Specifically, the color tone and reddish color may be improved or enhanced as compared with cases of feeding of a usual feed (i.e. a feed not added with the bacterial cells of the present invention or a carotenoid).

[0272]

The bacterial cells of the present invention can be fed to an organism by, for example, utilizing the feed of the present invention (namely, by feeding the feed of the present invention). The expression "to feed the feed of the present invention" includes not only to feed the feed of the present invention prepared beforehand, but also to feed the bacterial cells of the present invention and other ingredient(s) in combination so that they satisfy the requirement(s) of the feed of the present invention described above. For example, the bacterial cells of the present invention for addition and a feed before addition of the bacterial cells of the present invention may be separately provided, and fed as a mixture of them prepared before or at the time of feeding, or fed separately. The feeding amount of the bacterial cells of the present invention can be chosen so that, for example, the contained amount of the bacterial cells of the present invention, total carotenoids, or astaxanthine comes to be such a contained amount of the bacterial cells of the present invention, total carotenoids, or astaxanthine as described above in the feed of the present invention.

[0273]

The feeding amount of the feed of the present invention can be appropriately chosen according to various conditions such as the type of the objective organism. The feeding amount of the feed of the present invention may be, for example, the satiation amount. The feed of the present invention may be fed once a day, or two or

more times a day as divided portions. The feed of the present invention may also be fed once in several days. The feeding amount of the feed of the present invention at the time of each feeding may or may not be constant in terms of the amount of the bacterial cells of the present invention, total carotenoids, or astaxanthine. The contained amount of the bacterial cells of the present invention, total carotenoids, or astaxanthine in the feed of the present invention at the time of each feeding may or may not be constant.

[0274]

The period of feeding the feed of the present invention can be appropriately chosen according to various conditions such as the type of the objective organism. The feed of the present invention may be continuously fed over the entire period of culturing (breeding), or may be fed only in a partial period of culturing (breeding). The term "partial period" may refer to, for example, a period consisting of 10% or more, 20% or more, 30% or more, 50% or more, 70% or more, or 90% or more of the entire period of culturing (breeding). The period of feeding of the feed of the present invention, for example, may be 3 days or longer, one week or longer, 2 weeks or longer, 3 weeks or longer, 4 weeks or longer, 2 months or longer, or 3 months or longer, may be one year or shorter, 6 months or shorter, 4 months or shorter, 3 months or shorter, 2 months or shorter, 4 weeks or shorter, or 3 weeks or shorter, or may be within a range defined by any uncontradictory combination of these ranges. Specifically, the period of feeding of the feed of the present invention may be, for example, 2 weeks to 6 months. Feeding of the feed of the present invention may be repeatedly continued and interrupted for arbitrary periods and with arbitrary intervals.

[0275]

Cultivation (breeding) of an organism can be performed by the same method as usual methods for culturing (breeding) organisms except that the feed of the present invention is fed. The culturing (breeding) of aquatic organisms can be performed in, for example, a fish pen in the sea or fish tank on land. The term "culturing" includes propagation culturing in which only production of seed organism (fry production etc.) is performed, and culturing for production of adult organism (parent fish etc.).

[0276]

Similarly, the bacterial cells of the present invention can be fed to an organism by, for example, using the composition of the present invention (namely, by feeding the

composition of the present invention). The composition of the present invention can be fed to an organism, for example, independently or together with a feed. The descriptions concerning the feeding scheme of the feed of the present invention mentioned above can be applied *mutatis mutandis* to feeding scheme of the composition of the present invention. That is, the composition of the present invention can be fed, for example, together with a feed so that the contained amount of the bacterial cells of the present invention, total carotenoids, or astaxanthine comes to be such a contained amount of the bacterial cells of the present invention, total carotenoids, or astaxanthine as described above in the feed of the present invention.

[0277]

Such descriptions concerning the feeding scheme of the feed of the present invention or the composition of the present invention as mentioned above may be applied *mutatis mutandis* to any other case of feeding the bacterial cells of the present invention.

Examples

[0278]

Hereinafter, the present invention will be more specifically explained with reference to examples. However, the present invention is not limited by these examples.

[0279]

Example 1: L-Lysine production and carotenoid accumulation using *E. coli* strain having enhanced expression of carotenoid biosynthesis enzyme genes (1)

In this Example, L-lysine production was performed by using an L-lysine-producing strain of *E. coli* introduced with carotenoid biosynthesis enzyme genes (*crtEYIB*), and the effect of an enhanced expression of carotenoid biosynthesis enzyme genes on L-lysine production and carotenoid accumulation was evaluated.

[0280]

(1) Materials

Materials used in this Example are as follows.

[0281]

Table 1 <Strains used>

Strain	Description
<i>Escherichia coli</i>	
MG1655	ATCC 47076, a wild-type strain
WC196LC	FERM BP-11027, also referred to as WC196 Δ cadA Δ ldc, a model strain for L-lysine production
WC196LC_crtEYIB	WC196LC Δ argT::P _{tac} _crtEYIB_Km ^r (present application)
<i>Pantoea ananatis</i>	
SC17(0)	VKPM B-9246
SC17(0)::Ptac-kdp	A strain corresponding to <i>P. ananatis</i> SC17(0) except that λ attL_Km ^r _ λ attR_P _{tac} sequence has been inserted into an upstream portion of <i>kdp</i> operon (WO2008/090770)
SC17(0) Δ crtEX:: P _{tac} _crtE_Km ^r	A strain corresponding to <i>P. ananatis</i> SC17(0) except that <i>crtX</i> gene has been deleted and the promoter of <i>crtE</i> gene has been replaced with <i>tac</i> promoter (present application)

[0282]

Table 2 <Primers used>

Primer	SEQ ID NO	Nucleotide Sequence (5'→3')
crtE-attL-F	1	ccgcatctttcgcggtgccgtaaagtatccgttataag gacagcccgatgaagcctgctttttataactaagttggca
crtE-Ptac407X-R	2	gtgttttttgcgcagaccgtcatggcagtccttgtgt gaaattgttatccg
Ptac407X-crtE-F	3	cggataacaatttcacacaaggagacttgccatgacggtc tgcgcaaaaaaacac
crtY-SD-crtE-R	4	cgcgagtcagccccacgagaatcagatcataatgcggt tgcatagccgtctcctgtcgattaactgacggcagcgagt
argT_attL_fw	5	cacaacacaatacacacataaaaaagccattttcacttg agggttatgttgaagcctgctttttataactaagttggca
argT_crtB_rv	6	ggcgtgcacatgatgatcattccatcaggtacagettcc cagcgacgtactagagcgggcgctgccagagatgcgcagg

[0283]

<Medium composition>

LB medium: 10 g/L Bacto tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0 (adjusted with NaOH). Agar (15 g/L) was further contained in the case of an agar medium.

[0284]

LBGM9 medium: the composition of LB medium added with a minimal medium components (5 g/L glucose, 2 mM magnesium sulfate, 3 g/L potassium dihydrogen phosphate, 0.5 g/L sodium chloride, 1 g/L ammonium chloride, 6 g/L disodium hydrogen phosphate), pH 7.0 (adjusted with KOH). Agar (15 g/L) was further contained in the case of an agar medium.

[0285]

MS medium: 40 g/L glucose, 1 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 24 g/L $(\text{NH}_4)_2\text{SO}_4$, 1 g/L KH_2PO_4 , 2 g/L yeast extract, 10 mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 8 mg/L $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, pH 7.0 (adjusted with KOH).

[0286]

(2) Construction of strains

(2-1) Construction of *P. ananatis* SC17(0) Δ crtEX::P_{tac}_crtE_Km^r

PCR was performed using the chromosomal DNA of the *P. ananatis* SC17(0)::Ptac-kdp strain (WO2008/090770) as the template, and primers crtE-attL-F and crtE-Ptac407X-R, to amplify a DNA fragment λ attL_Km^r_ λ attR_P_{tac} (WO2008/090770), which contains a kanamycin resistance gene added with λ attL and λ attR sequences at the respective ends and the *tac* promoter sequence. Separately, PCR was performed using the chromosomal DNA of the *P. ananatis* SC17(0) strain (VKPM B-9246) as the template, and primers Ptac407X-crtE-F and crtY-SD-crtE-R, to amplify a DNA fragment containing a *crtE* gene. The SC17(0) strain was deposited in Russian National Collection of Industrial Microorganisms (VKPM, FGUP GosNII Genetika, Russian Federation, 117545 Moscow, 1st Dorozhny proezd, 1) on September 21, 2005 under the accession number VKPM B-9246. The PCRs were performed by using PrimeSTAR GXL polymerase (Takara Bio) according to the protocol attached to the enzyme. Cross-over PCR was performed using both the obtained DNA fragments as the template, and primers crtE-attL-F and crtY-SD-crtE-R, to obtain a DNA fragment λ attL_Km^r_ λ attR_P_{tac}_crtE, which contains a nucleotide sequence consisting of the *crtE* gene sequence and the λ attL_Km^r_ λ attR_P_{tac} sequence ligated to the 5' end side of the *crtE* gene.

[0287]

According to a previous report (WO2010/027022A1), the *P. ananatis* SC17(0) strain was transformed with λ attL_Km^r_ λ attR_P_{tac}_crtE by the λ -red method (US2006-0160191A; Datsenko, K. A, and Wanner, B. L. Proc. Natl. Acad. Sci. U S A. 97:6640-6645 (2000)), and transformants were selected on LBGM9 agar medium containing 40 μ g/mL kanamycin. Among the obtained transformants, a strain in which the *crtX* gene on the *crtEXYIB* operon has been deleted and the promoter of *crtE* gene has been replaced with the *tac* promoter was designated as *P. ananatis* SC17(0) Δ crtEX::P_{tac}_crtE_Km^r strain.

[0288]

(2-2) Construction of *E. coli* WC196LC_crtEYIB

PCR was performed using the DNA extracted from the *P. ananatis* SC17(0) Δ crtEX::P_{tac}_crtE_Km^r strain as the template, and primers argT_attL_fw and argT_crtB_rv, to amplify a DNA fragment λ attL_Km^r_ λ attR_P_{tac}_crtEYIB, which

contains a nucleotide sequence consisting of the λ attL_Km^r_ λ attR_P_{tac} sequence and the *crtEYIB* genes ligated to the downstream of the λ attL_Km^r_ λ attR_P_{tac} sequence.

[0289]

According to a previous report, the *E. coli* MG1655 strain was transformed with λ attL_Km^r_ λ attR_P_{tac}_crtEYIB by the λ -red method (US2006-0160191A; Datsenko, K. A, and Wanner, B. L. Proc. Natl. Acad. Sci. U S A. 97:6640-6645 (2000)), and transformants were selected on LB agar medium containing 50 μ g/mL kanamycin. Among the obtained transformants, a strain in which insertion of λ attL_Km^r_ λ attR_P_{tac}_crtEYIB in the *argT* gene on the chromosome was confirmed was designated as *E. coli* MG1655 Δ argT::P_{tac}_crtEYIB_Km^r strain.

[0290]

P1 transduction was performed using the *E. coli* MG1655 Δ argT::P_{tac}_crtEYIB_Km^r strain as the donor and an *E. coli* L-lysine-producing strain WC196LC (FERM BP-11027) as the recipient according to the usual manner, to obtain the *E. coli* WC196LC Δ argT::P_{tac}_crtEYIB_Km^r strain (hereinafter, referred to as WC196LC_crtEYIB strain), which has been introduced with carotenoid biosynthesis enzyme genes (*crtEYIB*).

[0291]

(3) Extraction and quantification of carotenoids

The strains WC196LC and WC196LC_crtEYIB each were cultured on LB agar medium for 48 hr at 37°C. Cells were scraped off in an amount corresponding to about 8-cm diameter of the cultured plate, and suspended in 1 mL of sterilized water. A part of the obtained cell suspension was separated, and used for measuring dry cell weight. The remaining part of the cell suspension was centrifuged, and the obtained cells were re-suspended in an equal volume of ethanol. The cell suspension in ethanol was added with an equal volume of ϕ 0.5 mm glass beads (YGBLA05, YASUI KIKAI), and then cells were disrupted by repeating a step consisting of treating for 30 sec at 2,700 rpm and thereafter leaving at rest for 30 sec by using Multi Beads Shocker^R (YASUI KIKAI) for 20 cycles. The cell disruption product was centrifuged for 2 min at 20,000 $\times$ g, to obtain a supernatant. The obtained supernatant was diluted twice with ethanol, and A450 was measured. The amount of carotenoids was calculated from the measured value of A450 according to the following formula (I) (Britton G., Geteral carotenoid methods., Methods. Enzymol., 111: 113-149 (1985)).

Amount of total carotenoids ($\mu\text{g/L}$) = $A_{450} \times 2 / (2620 \times 100) \times 1000 \times 1000000 \dots(I)$

[0292]

Results are shown in Table 3. While accumulation of carotenoids was not observed for the strain WC196LC, accumulation of carotenoids was observed for the strain WC196LC_crtEYIB.

[0293]

Table 3: Accumulated amount of carotenoids in *E. coli* L-lysine-producing bacterium

Strain	Carotenoids ($\mu\text{g/g-DCW}$)
WC196LC	N.D.
WC196LC_crtEYIB	21.6 ± 0.8 (n = 3)

[0294]

(4) L-lysine production culture

The strains WC196LC and WC196LC_crtEYIB each were uniformly applied to LB agar medium, and cultured for 24 hr at 37°C. Cells were scraped off in an amount corresponding to about 1-cm square of the cultured plate, and suspended in 0.5 mL of physiological saline. The turbidity at a wavelength of 620 nm (OD620) was measured for the obtained cell suspension by using a spectrophotometer U-2800 (Hitachi High-Technologies). The cell suspension was inoculated into 20 mL of MS medium contained in a 500-mL-volume Sakaguchi flask so as to obtain a final OD620 of 0.15, and 0.6 g of Japanese Pharmacopeia CaCO_3 preliminarily sterilized by dry heating was added thereto. Culture was performed for about 48 hr at 37°C with stirring at 120 rpm by using a reciprocal shaking culture apparatus. After completion of the culture, the amount of L-lysine accumulated in the medium and the amount of residual glucose in the medium were quantified by using a Biotech-analyzer AS-210 (SAKURA SI).

[0295]

Results are shown in Fig. 1. The strain WC196LC_crtEYIB showed an improved L-lysine production as compared to the strain WC196LC.

[0296]

Example 2: L-Lysine production and carotenoid accumulation using *E. coli* strain having enhanced expression of carotenoid biosynthesis enzyme genes (2)

In this Example, L-lysine production was performed by using an L-lysine-producing strain of *E. coli* introduced with carotenoid biosynthesis enzyme genes (*crtZ* and/or *crtW* as well as *crtEYIB*), and the effect of an enhanced expression of carotenoid biosynthesis enzyme genes on L-lysine production and carotenoid accumulation was evaluated.

[0297]

(1) Construction of strains

(1-1) Introduction of *crtZ* gene into *E. coli*

The *crtZ* genes derived from the *P. ananatis* SC17 strain, *Brevundimonas* sp. SD-212 strain, *Paracoccus* sp. N81106 strain, and *Alcaligenes* sp. were each introduced into the chromosome of the *E. coli* MG1655 strain as described below.

[0298]

(1-1-1) Introduction of *crtZ* gene of *P. ananatis* SC17 strain

PCR was performed using the chromosomal DNA of the *P. ananatis* SC17 strain (FERM BP-11091) as the template, and primers CrtZ-FwP-tac40 and CrtZ-RvP (SEQ ID NOS: 31 and 32), to amplify a DNA fragment containing the *crtZ* gene (SEQ ID NO: 21). The SC17 strain was deposited at the independent administrative agency, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depository (currently independent administrative agency, National Institute of Technology and Evaluation, International Patent Organism Depository, #120, 2-5-8 Kazusakamatar, Kisarazu-shi, Chiba-ken, 292-0818, Japan) on February 4, 2009, and assigned an accession number of FERM BP-11091. Then, PCR was performed using the chromosomal DNA of the *P. ananatis* SC17(0)::Ptac-kdp strain as the template, and primers yhfK-crtZ-Fw1 and 71_Pa_rv (SEQ ID NOS: 34 and 35), to amplify a DNA fragment containing λ attL_Km^r λ attR_P_{tac} sequence. These two kinds of PCR products obtained above were purified, and then, PCR was performed using a mixture thereof as the template, and primers yhfK-crtZ-Fw1 and Pa_crtZ_yhfK_rv (SEQ ID NOS: 34 and 36), to obtain a fragment λ attL_Km^r λ attR_P₄₀₇₁_crtZ(Pa). The *E. coli* MG1655 strain was transformed with the obtained DNA fragment by the λ -red method, to obtain a strain MG1655 Δ yhfK::P4071_crtZ(Pa), in which the *crtZ* gene derived from the *P. ananatis* SC17 strain was inserted into a *yhfK* gene region on the chromosome.

[0299]

(1-1-2) Introduction of *crtZ* gene of *Brevundimonas* sp. SD-212 strain

The *crtZ* gene derived from the *Brevundimonas* sp. SD-212 strain (SEQ ID NO: 37) was totally-synthesized (Eurofins Genomics). PCR was performed using the obtained DNA fragment as the template, and primers 71_Bre212_crtZ_fw and Bre212_crtZ_yhfK_rv (SEQ ID NOS: 38 and 39), to amplify a DNA fragment containing the *crtZ* gene. Then, PCR was performed using the chromosomal DNA of the *P. ananatis* SC17(0)::Ptac-kdp strain as the template, and primers yhfK_P4071_fw and 71_Bre212_rv (SEQ ID NOS: 40 and 41), to amplify a DNA fragment containing λ attL_Km^r_ λ attR_P_{tac} sequence. These two kinds of PCR products obtained above were purified, and then, PCR was performed using a mixture thereof as the template, and primers yhfK_P4071_fw and Bre212_crtZ_yhfK_rv (SEQ ID NOS: 40 and 39), to obtain a fragment λ attL_Km^r_ λ attR_P₄₀₇₁_crtZ(Br). The *E. coli* MG1655 strain was transformed with the obtained DNA fragment by the λ -red method, to obtain a strain MG1655 Δ yhfK::P4071_crtZ(Br), in which the *crtZ* gene derived from the *Brevundimonas* sp. SD-212 strain was inserted into a *yhfK* gene region on the chromosome.

[0300]

(1-1-3) Introduction of *crtZ* gene of *Paracoccus* sp. N81106 strain

The *crtZ* gene derived from the *Paracoccus* sp. N81106 strain (SEQ ID NO: 42) was totally-synthesized (Eurofins Genomics). PCR was performed using the obtained DNA fragment as the template, and primers 71_Pc_crtZ_fw and Pc_crtZ_yhfK_rv (SEQ ID NOS: 43 and 44), to amplify a DNA fragment containing the *crtZ* gene. Then, PCR was performed using the chromosomal DNA of the *P. ananatis* SC17(0)::Ptac-kdp strain as the template, and primers yhfK_P4071_fw and 71_Pc_rv (SEQ ID NOS: 40 and 45), to amplify a DNA fragment containing λ attL_Km^r_ λ attR_P_{tac} sequence. These two kinds of PCR products obtained above were purified, and then, PCR was performed using a mixture thereof as the template, and primers yhfK_P4071_fw and Pc_crtZ_yhfK_rv (SEQ ID NOS: 40 and 44), to obtain a fragment λ attL_Km^r_ λ attR_P₄₀₇₁_crtZ(Pc). The *E. coli* MG1655 strain was transformed with the obtained DNA fragment by the λ -red method, to obtain a strain MG1655 Δ yhfK::P4071_crtZ(Pc), in which the *crtZ* gene derived from the *Paracoccus* sp. N81106 strain was inserted into a *yhfK* gene region on the chromosome.

[0301]

(1-1-4) Introduction of *crtZ* gene of *Alcaligenes* sp.

The *crtZ* gene derived from the *Alcaligenes* sp. (SEQ ID NO: 46) was totally-synthesized (Eurofins Genomics). PCR was performed using the obtained DNA fragment as the template, and primers 71_Al_crtZ_fw and Al_crtZ_yhfK_rv (SEQ ID NOS: 47 and 48), to amplify a DNA fragment containing the *crtZ* gene. Then, PCR was performed using the chromosomal DNA of the *P. ananatis* SC17(0)::Ptac-kdp strain as the template, and primers yhfK_P4071_fw and 71_Al_rv (SEQ ID NOS: 40 and 49), to amplify a DNA fragment containing λ attL_Km^r λ attR_P_{tac} sequence. These two kinds of PCR products obtained above were purified, and then, PCR was performed using a mixture thereof as the template, and primers yhfK_P4071_fw and Al_crtZ_yhfK_rv (SEQ ID NOS: 40 and 48), to obtain a fragment λ attL_Km^r λ attR_P₄₀₇₁_crtZ(Al). The *E. coli* MG1655 strain was transformed with the obtained DNA fragment by the λ -red method, to obtain a strain MG1655 Δ yhfK::P4071_crtZ(Al), in which the *crtZ* gene derived from the *Alcaligenes* sp. was inserted into a *yhfK* gene region on the chromosome.

[0302]

(1-2) Construction of plasmid pMIV-Pnlp8-crtW

The *crtW* gene derived from *Nostoc punctiforme* (codon-optimized for expression in *E. coli*; SEQ ID NO: 50) was totally-synthesized (Takara BIO). PCR was performed using the obtained DNA fragment as the template, and primers nlp-crtW_fw and nlp-crtW-Rv (SEQ ID NO: 51 and 52), to amplify a DNA fragment containing the *crtW* gene. Separately, PCR was performed using pMIV-Pnlp8 (Japanese Patent Laid-open (Kokai) No. 2010-187552) as the template, and primers pMIV-Pnlp8-Fw and pMIV-Rv (SEQ ID NO: 53 and 54), to amplify a vector fragment. These two kinds of obtained DNA fragments were mutually ligated by using In-Fusion(R) HD Cloning Kit (Takara BIO), to obtain pMIV-Pnlp8-crtW, an expression plasmid for the *crtW* gene derived from *Nostoc punctiforme*.

[0303]

(1-3) Construction of plasmid pMW_Ptac_crtW

A DNA (SEQ ID NO: 56) consisting of tac promoter sequence, the *crtW* gene derived from *Brevundimonas aurantiaca*, and rrnB terminator sequence ligated in this order was totally-synthesized (Eurofins Genomics). The obtained DNA was cloned between BamHI (5'-terminal side) and XbaI (3'-terminal side) of pMW219, to obtain pMW_Ptac_crtW, an expression plasmid for the *crtW* gene derived from *B. aurantiaca*.

[0304]

(1-4) Introduction of *crtZ* gene and *crtW* gene into *E. coli* L-lysine-producing bacterium having beta-carotene-producing ability

In order to remove the kanamycin resistance marker gene from the *E. coli* WC196LC_crtEYIB strain, which is an L-lysine-producing bacterium having beta-carotene-producing ability, the WC196LC_crtEYIB strain was transformed with pMW-int-xis (WO2007/037460 and Japanese Patent Laid-open (Kokai) No. 2005-058827) by the electroporation method. Transformants were selected by culturing on LB agar medium containing 50 mg/L ampicillin for 24 hr at 30°C. Obtained colonies of the transformants were subcultured on LB agar medium and cultured for 24 hr at 42°C. A strain sensitive to kanamycin and resistant to ampicillin was selected from obtained colonies, and designated as strain WC196LC_crtEYIB(ML).

[0305]

Then, a P1 lysate was obtained from each of the strains MG1655ΔyhfK::P4071_crtZ(Pa), MG1655ΔyhfK::P4071_crtZ(Br), MG1655ΔyhfK::P4071_crtZ(Pc), and MG1655ΔyhfK::P4071_crtZ(Al), and P1 transduction was performed using the WC196LC_crtEYIB(ML) strain as the host. Transformants were selected by culturing on LB agar medium containing 40 mg/L kanamycin for 24 hr at 37°C. From the obtained transformants, there were obtained strains WC196LC_crtEYIB(ML)_ΔyhfK::P4071_crtZ(Pa), WC196LC_crtEYIB(ML)_ΔyhfK::P4071_crtZ(Br), WC196LC_crtEYIB(ML)_ΔyhfK::P4071_crtZ(Pc), and WC196LC_crtEYIB(ML)_ΔyhfK::P4071_crtZ(Al), in which the *crtZ* genes derived from the *P. ananatis* SC17 strain, *Brevundimonas* sp. SD-212 strain, *Paracoccus* sp. N81106 strain, and *Alcaligenes* sp., respectively, were inserted into the chromosome.

[0306]

Then, the WC196LC_crtEYIB(ML)_ΔyhfK::P4071_crtZ(Pa) strain was transformed with pMIV-Pnlp8-crtW and pMW_Ptac_crtW by the electroporation method, to obtain strains WC196LC_crtEYIB(ML)_ΔyhfK::P4071_crtZ(Pa)/pMIV-Pnlp8-crtW and WC196LC_crtEYIB(ML)_ΔyhfK::P4071_crtZ(Pa)/pMW_Ptac_crtW, respectively, into which the *crtZ* genes were introduced.

[0307]

Similarly, the WC196LC strain was transformed with pMIV5JS (Japanese

Patent Laid-open (Kokai) No. 2008-99668) and pMW219 by the electroporation method, to obtain vector control strains WC196LC/pMIV5JS and WC196LC/pMW219, respectively.

[0308]

Hereinafter, unless otherwise stated, strains having plasmids were cultured using a medium added with the following antibiotic: 25 mg/L chloramphenicol for strains having pMIV5JS and pMIV-Pnlp8-crtW, and 50 mg/L kanamycin for strains having pMW219 and pMW_Ptac_crtW.

[0309]

(2) Extraction and quantification of carotenoids

Carotenoids were extracted from bacterial cells with ethanol, and quantified by using HPLC. The procedure is described below.

[0310]

Each strain was cultured on LB agar medium for 48 hr at 37°C. Cells were scraped off in an amount corresponding to about 8-cm diameter of the cultured plate, and suspended in 1 mL of sterilized water. A part of the obtained cell suspension was separated, and used for measuring dry cell weight. The remaining part of the cell suspension was also centrifuged, and the obtained cells were re-suspended in an equal volume of ethanol. The cell suspension in ethanol was added with an equal volume of ϕ 0.5 mm glass beads (YGBLA05, YASUI KIKAI), and then cells were disrupted by repeating a step consisting of treating for 30 sec at 2,700 rpm and thereafter leaving at rest for 30 sec by using Multi Beads Shocker^R (YASUI KIKAI) for 20 cycles. The cell disruption product was centrifuged for 2 min at 20,000 x g, to obtain a supernatant. The obtained supernatant was subjected to HPLC, to quantify the amount of carotenoids. The conditions of HPLC were as follows:

UHPLC system: solvent delivery unit LC-30AD, autosampler SIL-30AC, degasser DGU-20A5R, column oven CTO-20AC, and detector SPD-20A UV-VIS (Shimadzu Corporation);

Column: phenomenex kinetex 2.6 μ m C18 100A 75 x 4.6 mm (Shimadzu Corporation);

Analysis conditions:

Mobile phase, (A) Acetonitrile / H₂O / Formate (43/5/2), , (B) Ethyl acetate / Formate (24/1);

Gradient, 0.0 min, 0% B, 0.0 - 6.0 min, to 100% B, 6.0 min - 7.4 min, 100% B, 7.4 - 9.2 min, to 0% B, 9.2 - 10 min, 0% B;

Flow rate, 2.0 mL/min;

Column temperature, 30°C.

[0311]

(3) L-lysine production culture

Each strain was uniformly applied to LB agar medium, and cultured for 24 hr at 37°C. Cells were scraped off in an amount corresponding to about 1-cm square of the cultured plate, and suspended in 1 mL of physiological saline. The turbidity at a wavelength of 620 nm (OD₆₂₀) was measured for the obtained cell suspension by using a spectrophotometer U-2800 (Hitachi High-Technologies). The cell suspension was inoculated into 20 mL of MS medium contained in a 500-mL-volume Sakaguchi flask so as to obtain a final OD₆₂₀ of 0.126, and 0.6 g of Japanese Pharmacopeia CaCO₃ preliminarily sterilized by dry heating was added thereto. Culture was performed for about 48 hr at 37°C with stirring at 120 rpm by using a reciprocal shaking culture apparatus. After completion of the culture, the amount of L-lysine accumulated in the medium and the amount of residual glucose in the medium were quantified by using a Biotech-analyzer AS-210 (SAKURA SI).

[0312]

(4) Results

(4-1) Effect of introduction of *crtZ* gene

As a result of extraction and quantification of carotenoids, accumulation of zeaxanthin was confirmed in the strains introduced with *crtEYIB* genes and *crtZ* gene (Table 4). In addition, as a result of L-lysine production culture, the strains introduced with *crtEYIB* genes and *crtZ* gene each showed an improved L-lysine accumulation as compared to the control strain WC196LC (Fig. 2).

[0313]

Table 4: Accumulated amount of carotenoids in *E. coli* L-lysine-producing bacterium

Strain	Zeaxanthin ($\mu\text{g/g-DCW}$)	Astaxanthin ($\mu\text{g/g-DCW}$)
WC196LC	N.D.	N.D.
WC196LC_crtEYIB(ML) Δ yhfk::P4071_crtZ(Pa)	34.3 ± 4.7	N.D.
WC196LC_crtEYIB(ML) Δ yhfk::P4071_crtZ(Br)	16.2 ± 0.6	N.D.
WC196LC_crtEYIB(ML) Δ yhfk::P4071_crtZ(Pc)	17.1 ± 1.6	N.D.
WC196LC_crtEYIB(ML) Δ yhfk::P4071_crtZ(Al)	20.9 ± 1.4	N.D.

N.D., Not Detected.

[0314]

(4-2) Effect of introduction of *crtW* gene

As a result of extraction and quantification of carotenoids, accumulation of astaxanthin was confirmed in the strains introduced with *crtW* gene as well as *crtEYIB* genes and *crtZ* gene (Table 5). In addition, as a result of L-lysine production culture, the strains introduced with *crtW* gene as well as *crtEYIB* genes and *crtZ* gene each showed an improved L-lysine accumulation as compared to the corresponding vector control strain (Fig. 3).

[0315]

Table 5: Accumulated amount of carotenoids in *E. coli* L-lysine-producing bacterium (n = 3)

Strain	Zeaxanthin ($\mu\text{g/g-DCW}$)	Astaxanthin ($\mu\text{g/g-DCW}$)
WC196LC/pMIV5JS	N.D.	N.D.
WC196LC_crtEYIB(ML) Δ yhfk::P4071_crtZ(Pa) /pMIV-Pnlp8-crtW	13.2 ± 1.1	25.4 ± 4.9
WC196LC/pMW219	N.D.	N.D.
WC196LC_crtEYIB(ML) Δ yhfk::P4071_crtZ(Pa) /pMW_Ptac_crtW	N.D.	28.6 ± 3.0

N.D., Not Detected.

[0316]

Example 3: L-threonine production and carotenoid accumulation using *E. coli* strain having enhanced expression of carotenoid biosynthesis enzyme genes

In this Example, L-threonine production was performed by using an L-threonine-producing strain of *E. coli* introduced with carotenoid biosynthesis enzyme genes (*crtEYIB*), and the effect of an enhanced expression of carotenoid biosynthesis enzyme genes on L-threonine production and carotenoid accumulation was evaluated.

[0317]

(1) Construction of strains

(1-1) Construction of pMW119-*crtEYIB*

PCR was performed using the chromosomal DNA of the WC196LC-*crtEYIB*(ML) strain as the template, and primers infu-1F and infu-2R (SEQ ID NOS: 56 and 57), to amplify a DNA fragment Ptac-*crtEYIB*, which contains a nucleotide sequence consisting of Ptac promoter sequence and the *crtEYIB* genes ligated to the downstream of the Ptac promoter sequence. PCR was performed by using PrimeSTAR(R) MAX DNA polymerase (Takara BIO) according to the protocol attached to the enzyme. Separately, pMW119 was treated with XbaI and KpnI, and then mixed with Ptac-*crtEYIB*. The mixture was subjected to a reaction using In-Fusion(R) HD Cloning Kit, to obtain pMW119-*crtEYIB*, an expression plasmid for the *crtEYIB* genes in which Ptac-*crtEYIB* was inserted into the pMW119 vector.

[0318]

(1-2) Introduction of carotenoid biosynthesis genes into *E. coli* L-threonine-producing bacterium

The *E. coli* VKPM B-3996 strain (US patent No. 5,175,107) was transformed with pMW119-*crtEYIB* by the electroporation method, to obtain a strain VKPM B-3996/pMW119-*crtEYIB*, which is an L-threonine-producing bacterium introduced with carotenoid biosynthesis genes.

[0319]

Similarly, the VKPM B-3996 strain was transformed with pMW119, to obtain a vector control strain VKPM B-3996/pMW119.

[0320]

Hereinafter, unless otherwise stated, these transformants were cultured using a

medium added with 50 mg/L ampicillin.

[0321]

(2) Extraction and quantification of carotenoids

Carotenoids were extracted from bacterial cells with ethanol, and quantified by using HPLC. The procedure was the same as that described in Examples 2(2).

[0322]

(3) L-threonine production culture

Each strain was uniformly applied to LB agar medium, and cultured for 24 hr at 37°C. Cells were scraped off in an amount corresponding to about 1-cm square of the cultured plate, and suspended in 1 mL of physiological saline. The turbidity at a wavelength of 620 nm (OD₆₂₀) was measured for the obtained cell suspension by using a spectrophotometer U-2800 (Hitachi High-Technologies). The cell suspension was inoculated into 20 mL of MS medium contained in a 500-mL-volume Sakaguchi flask so as to obtain a final OD₆₂₀ of 0.126, and 0.6 g of Japanese Pharmacopeia CaCO₃ preliminarily sterilized by dry heating was added thereto. Culture was performed for about 48 hr at 37°C with stirring at 120 rpm by using a reciprocal shaking culture apparatus. After completion of the culture, the amount of L-threonine accumulated in the medium was quantified by using a biosensor BF-7 (Oji Scientific Instruments).

[0323]

(4) Results

As a result of extraction and quantification of carotenoids, accumulation of beta-carotene was confirmed in the strain introduced with pMW119-crtEYIB (Table 6). In addition, as a result of L-threonine production culture, the strain introduced with pMW119-crtEYIB showed an improved L-threonine accumulation as compared to the vector control strain (Fig. 4).

[0324]

Table 6: Accumulated amount of carotenoids in *E. coli* L-threonine-producing bacterium

Strain	beta-carotene (μg/g-DCW)
VKPM B-3996/pMW119	N.D.
VKPM B-3996/pMW119-crtEYIB	37.5 ± 1.6

N.D., Not Detected.

[0325]

Example 4: L-lysine production and carotenoid accumulation using *C. glutamicum* strain having enhanced expression of carotenoid biosynthesis enzyme genes

In this Example, L-lysine production was performed by using an L-lysine-producing strain of *C. glutamicum* introduced with carotenoid biosynthesis enzyme genes (*crtYZ*), and the effect of an enhanced expression of carotenoid biosynthesis enzyme genes on L-lysine production and carotenoid accumulation was evaluated.

[0326]

(1) Media used

CM-Dex medium: 10 g/L polypeptone, 10 g/L yeast extract, 5 g/L glucose, 1 g/L KH_2PO_4 , 3 g/L urea, 0.4 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, 1.2 g/L (T-N) filtrated soybean hydrolysate, pH 7.5 (adjusted with KOH). Agar (15 g/L) was further contained in the case of an agar medium.

[0327]

Coryne.-Lys production medium: 100 g/L glucose, 55 g/L $(\text{NH}_4)_2\text{SO}_4$, 1.05 g/L (T-N) filtrated soybean hydrolysate, 1 g/L KH_2PO_4 , 1 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, 2 mg/L vitamin B1-HCl, 0.5 mg/L biotin, 5 mg/L nicotinamide, pH 7.0 (adjusted with KOH).

[0328]

(2) Construction of strains

(2-1) Construction of pVS7_PmsrA_crtYZ

A DNA (PmsrA_crtYZ; SEQ ID NO: 33) comprising an operon structure consisting of a promoter sequence of *msrA* gene (PmsrA) and *crtY* and *crtZ* genes derived from *P. ananatis* ligated to the downstream of PmsrA was totally-synthesized (Eurofins Genomics). PmsrA_crtYZ was inserted into pVS7 (WO2013/069634), to construct pVS7_PmsrA_crtYZ, an expression plasmid for the *crtY* gene derived from *P. ananatis*. Upon insertion into pVS7, the 5'-terminal and 3'-terminal sides of PmsrA_crtYZ were ligated to PstI and BamHI sites of pVS7, respectively.

[0329]

(2-2) Introduction of pVS7_PmsrA_crtYZ into *C. glutamicum*

The *C. glutamicum* AJ3990 strain (ATCC 31269) was transformed with pVS7_PmsrA_crtYZ by the electroporation method, and cultured on CM-Dex agar medium containing 25 mg/L spectinomycin at 31.5°C, to obtain a strain

AJ3990/pVS7_PmsrA_crtYZ, which is a transformant introduced with *crtY* and *crtZ* genes derived from *P. ananatis*.

[0330]

Similarly, the AJ3990 strain was transformed with pVS7, to obtain a vector control strain AJ3990/pVS7.

[0331]

(3) Extraction and quantification of carotenoids

Carotenoids were extracted from bacterial cells with an ethanol/acetone mixture (ethanol:acetone = 7:3), and quantified by using HPLC. The procedure is described below.

[0332]

Each strain was cultured on CM-Dex agar medium for 48 hr at 31.5°C. The subsequent procedure was the same as that described in Examples 2(2), except that the ethanol/acetone mixture was used instead of ethanol.

[0333]

(4) L-lysine production culture

Each strain was uniformly applied to CM-Dex agar medium containing 25 mg/L spectinomycin, and cultured for 24 hr at 31.5°C. Cells were scraped off in an amount corresponding to about 1-cm square of the cultured plate, and suspended in 1 mL of physiological saline. The turbidity at a wavelength of 620 nm (OD₆₂₀) was measured for the obtained cell suspension by using a spectrophotometer U-2800 (Hitachi High-Technologies). The cell suspension was inoculated into 20 mL of Coryne.-Lys production medium contained in a 500-mL-volume Sakaguchi flask so as to obtain a final OD₆₂₀ of 0.126, and 1.0 g of Japanese Pharmacopeia CaCO₃ preliminarily sterilized by dry heating was added thereto. Culture was performed for about 48 hr at 37°C with stirring at 120 rpm by using a reciprocal shaking culture apparatus. After completion of the culture, the amount of L-lysine accumulated in the medium was quantified by using a Biotech-analyzer AS-210 (SAKURA SI).

[0334]

(5) Results

As a result of extraction and quantification of carotenoids, accumulation of beta-carotene was confirmed in the strain introduced with pVS7_PmsrA_crtYZ, and this strain also showed an increased amount of total carotenoids as compared to the vector

control strain (Fig. 5). In addition, as a result of L-lysine production culture, the strain introduced with pVS7_PmsrA_crtYZ showed an improved L-lysine accumulation as compared to the vector control strain (Fig. 6).

[0335]

Example 5: L-glutamic acid production and carotenoid accumulation using *P. ananatis* strain having enhanced expression of carotenoid biosynthesis enzyme gene(s)

In this Example, L-glutamic acid production was performed by using an L-glutamic acid-producing strain of *P. ananatis* having an enhanced expression of carotenoid biosynthesis enzyme gene(s) (*crtE* and/or *crtW*), and the effect of an enhanced expression of carotenoid biosynthesis enzyme gene(s) on L-glutamic acid production and carotenoid accumulation was evaluated.

[0336]

(1) Medium used

MSII medium: 40 g/L glucose, 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 g/L $(\text{NH}_4)_2\text{SO}_4$, 2 g/L KH_2PO_4 , 0.5 g/L NaCl, 2 g/L yeast extract, 0.25 g/L $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$, 20 mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg/L $\text{MnSO}_4 \cdot n\text{H}_2\text{O}$, 4 mL/L Trace element (0.66 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.18 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.16 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.15 g/L $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.18 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.10 g/L H_3BO_3 , 0.30 g/L Na_2MoO_4), 200 mg/L L-Lys, 200 mg/L DL-Met, 200 mg/L DAP, pH 7.0 (adjusted with KOH). Japanese Pharmacopeia CaCO_3 preliminarily sterilized by dry heating was added at a concentration of 50 g/L immediately before culture.

[0337]

(2) Construction of strains

(2-1) Enhanced expression of *crtE* gene

The chromosomal DNA of the *P. ananatis* SC17(0) Δ crtEX::P_{tac}_crtE_Km^r strain was extracted, and the *P. ananatis* NA1 Δ sdhA strain (Japanese Patent Laid-open (Kokai) No. 2010-041920) was transformed with this chromosomal DNA by the electroporation method. The NA1 Δ sdhA strain is an L-glutamic acid-producing strain derived from the *P. ananatis* AJ13601 strain (FERM BP-7207). Transformants were selected by culturing on LBGM9 agar medium containing 40 mg/L kanamycin for 24 hr at 34°C. Among the obtained strains, a strain in which insertion of the λ attL_Km^r_ λ attR_P_{tac}_crtE sequence was confirmed was designated as NA1 Δ sdhA Δ crtEX::P_{tac}_crtE_Km^r strain.

[0338]

(2-2) Introduction of *crtW* gene

The NA1ΔsdhAΔcrtEX::P_{tac}_crtE_Km^r strain was transformed with pMIV_Pnlp8_crtW by the electroporation method. Transformants were selected by culturing on LBGM9 agar medium containing 25 mg/L chloramphenicol for 24 hr at 34°C, and one of them was designated as NA1ΔsdhAΔcrtEX::P_{tac}_crtE_Km^r/pMIV_Pnlp8_crtW strain.

[0339]

Similarly, the NA1ΔsdhA strain was transformed with pMIV5JS, to obtain a vector control strain NA1ΔsdhA/pMIV5JS.

[0340]

Hereinafter, unless otherwise stated, these transformants were cultured using a medium added with 25 mg/L chloramphenicol.

[0341]

(3) Extraction and quantification of carotenoids

Carotenoids were extracted from bacterial cells with ethanol, and quantified by using HPLC. The procedure is described below.

[0342]

Each strain was cultured on LBGM9 agar medium for 48 hr at 34°C. The subsequent procedure was the same as that described in Examples 2(2).

[0343]

(4) L-glutamic acid production culture

Each strain was uniformly applied to LBGM9 agar medium, and cultured for 24 hr at 34°C. Cells were scraped off in an amount corresponding to about 1-cm square of the cultured plate, inoculated into 5 mL of MSII medium contained in a large test tube, and cultured for 24 hr at 34°C at 120 rpm. After completion of the culture, the amount of L-glutamic acid accumulated in the medium was quantified by using a Biotech-analyzer AS-210 (SAKURA SI).

[0344]

(5) Results

(5-1) Effect of enhanced expression of *crtE* gene

As a result of extraction and quantification of carotenoids, accumulation of zeaxanthin was confirmed in the strain having an enhanced expression of *crtE* gene

(NA1ΔsdhAΔcrtEX::P_{tac}_crtE_Km^r strain), and this strain also showed an increased amount of total carotenoids as compared to the control strain NA1ΔsdhA (Fig. 7). No accumulation of canthaxanthin or astaxanthin was confirmed for any strain. In addition, as a result of L-glutamic acid production culture, the strain having an enhanced expression of *crtE* gene (NA1ΔsdhAΔcrtEX::P_{tac}_crtE_Km^r strain) showed an improved L-glutamic acid accumulation as compared to the control strain NA1ΔsdhA (Fig. 8).

[0345]

(5-2) Effect of introduction of *crtW* gene

As a result of extraction and quantification of carotenoids, accumulation of canthaxanthin and astaxanthin was confirmed in the strain introduced with the *crtW* gene derived from *N. punctiforme* (NA1ΔsdhAΔcrtEX::P_{tac}_crtE_Km^r/pMIV_Pnlp8_crtW strain), and this strain also showed an increased amount of total carotenoids as compared to the vector control strain NA1ΔsdhA/pMIV5JS (Fig. 9). No accumulation of zeaxanthin was confirmed for any strain. In addition, as a result of L-glutamic acid production culture, the strain introduced with the *crtW* gene (NA1ΔsdhAΔcrtEX::P_{tac}_crtE_Km^r/pMIV_Pnlp8_crtW strain) showed an improved L-glutamic acid accumulation as compared to the vector control strain NA1ΔsdhA/pMIV5JS (Fig. 10).

[0346]

Example 6: Culturing of rainbow trout using astaxanthin-containing bacterial cells (1)

In this Example, astaxanthin was quantified by using a high performance chromatography (HPLC, LC-20AT, Shimadzu Corporation). The column used was Luna 3μ Silica Φ4.6 mm x 150 mm. A mixture of hexane and acetone (82:18) as the mobile phase was passed through at a flow rate of 1.2 ml/min at a constant temperature, and detection was carried out at a wavelength of 470 nm. The amount of total carotenoids was quantified by extraction of the carotenoids with acetone and detection at wavelength of 478 nm using a spectrophotometer.

[0347]

For test groups, a common feed for trout mainly containing fishmeal was ground, and then amino acid cells (dry cells of an L-lysine-producing strain of *E. coli* having an enhanced expression of astaxanthin-biosynthesis enzyme genes) in an amount of 3.3% or 5.5% were added to the feed. They were mixed, and the mixture was re-

pelletized, to obtain feeds for rainbow trout of test groups (amino acid cell groups). As a positive control, the same common feed for trout after grinding was added and mixed with commercially-available synthesized astaxanthin (CAROPHYLL Pink; DSM) in such an amount that the addition amount of astaxanthin to the feed should be equal to that of the group added with 5.5% amino acid cells. The mixture was re-pelletized, to obtain a feed for rainbow trout of control group (synthesized product group). As a negative control, a feed not added with astaxanthin (control group) was prepared. This feed for control group was prepared by re-pelletizing without addition of any additive after grinding.

[0348]

Results of analysis of the amounts of total carotenoids and astaxanthin in the feeds used in the experiment are shown in Table 7. The synthesized product group and the 5.5% and 3.3% amino acid cell groups showed higher amounts of total carotenoids and astaxanthin than the control group. Meanwhile, the 5.5% amino acid cell group contained almost the same amount of astaxanthin with that in the synthesized product group, and showed a higher amount of total carotenoids than the synthesized product group.

[0349]

Table 7: Contained amounts of carotenoids in feeds used in the experiment

	Control group	Synthesized product group	5.5% amino acid cell group	3.3% amino acid cell group
Total carotenoids (mg/100 g)	1.49	2.91	4.93	3.8
Astaxanthin (mg/100 g)	0.26	1.84	1.79	1.39

[0350]

Two hundred and fifty (250) rainbow trout having a body weight of about 100 g were introduced into 10 water tanks (200 L) of closed recirculating system so that each tank contained 25 rainbow trout, and cultured for 3 months. Due to the limited number of the tanks, there were used 2 tanks for the control group, 2 tanks for the synthesized product group, 3 tanks for the 3.3% amino acid cell group, and 3 tanks for the 5.5% amino acid cell group. As an evaluation item, the body weight of all

individuals was measured after 1 month, 2 months, and 3 months. The mean body weight of each group during the experimental period is shown in Table 8. Four (4) individuals per tank having a body weight close to the mean value were sampled, and the color tone of fillet was measured at 2 positions, i.e. at a head-side position and a tail-side position, by using a spectrophotometer (CM-700d, Konica Minolta). The mean values thereof are shown in Table 9. The spectrophotometer enables measurement of L, brightness; a, redness; and b, yellowness, and a higher value of "a" indicates a higher degree of coloring of an object toward reddish color. In addition, the amounts of carotenoids and astaxanthin in fillet of the same individuals were analyzed. The mean values thereof are shown in Table 10.

[0351]

No difference in the mean body weight during the experimental period was observed among the test groups (Table 8).

[0352]

The "a" value of fillet of individuals of the 5.5% amino acid cell group was higher than that of the synthesized product group containing an equal amount of astaxanthin.

[0353]

The contained amount of astaxanthin in fillet of individuals of the 5.5% amino acid cell group was equal to that of the synthesized product group containing an equal amount of astaxanthin, and the amounts of total carotenoids in fillet of individuals of the 5.5% amino acid cell group was higher than that of the synthesized product group.

[0354]

Table 8: Mean body weight (g) during experimental period

	At start	After 1 month	After 2 months	After 3 months
Control group	106.4	142.6	182.8	241.7
Synthesized product group	106.6	146.8	184.7	233.9
5.5% amino acid cell group	105.9	143.0	195.7	254.1
3.3% amino acid cell group	105.5	147.5	202.5	257.3

[0355]

Table 9: Color tone of fillet

After 1 month		Control group	Synthesized product group	5.5% amino acid cell group	3.3% amino acid cell group
Head side	L	51.2	48.2	47.1	49.2
	a	-0.6	2.3	3.8	1.9
	b	4.8	8.5	7.8	6.4
Tail side	L	51.3	48.8	45.5	49.0
	a	1.0	4.7	7.0	4.7
	b	6.5	9.4	10.5	8.8

After 2 months		Control group	Synthesized product group	5.5% amino acid cell group	3.3% amino acid cell group
Head side	L	48.8	47.6	47.9	47.7
	a	0.1	3.5	4.1	3.1
	b	6.3	6.9	7.3	7.1
Tail side	L	48.0	46.0	46.3	46.4
	a	1.6	6.8	7.1	5.9
	b	7.0	9.0	9.7	8.5

After 3 months		Control group	Synthesized product group	5.5% amino acid cell group	3.3% amino acid cell group
Head side	L	51.1	47.6	43.6	44.4
	a	0.4	4.0	5.5	4.6
	b	6.7	7.7	7.4	7.3
Tail side	L	48.4	44.9	42.8	54.4
	a	2.2	7.5	8.4	7.7
	b	8.0	10.8	10.3	10.1

[0356]

Table 10: Contained amount of carotenoids in fillet

After 1 month	Control group	Synthesized product group	5.5% amino acid cell group	3.3% amino acid cell group
Astaxanthin mg/100 g	0.04	0.16	0.13	0.11
Total carotenoids mg/100 g	0.07	0.17	0.22	0.17

After 2 months	Control group	Synthesized product group	5.5% amino acid cell group	3.3% amino acid cell group
Astaxanthin mg/100 g	0.06	0.31	0.20	0.16
Total carotenoids mg/100 g	0.07	0.30	0.30	0.24

After 3 months	Control group	Synthesized product group	5.5% amino acid cell group	3.3% amino acid cell group
Astaxanthin mg/100 g	0.08	0.33	0.31	0.27
Total carotenoids mg/100 g	0.10	0.34	0.46	0.40

[0357]

Example 7: Culturing of rainbow trout using astaxanthin-containing bacterial cells (2)

For tests, a common feed for trout mainly containing fishmeal is ground, and then amino acid cells (dry cells of an L-lysine-producing strain of *E. coli* having an enhanced expression of astaxanthin-biosynthesis enzyme genes) are added to the feed. They are mixed, and the mixture is re-pelletized, to obtain a feed for rainbow trout of test group (amino acid cell group). A feed blended with astaxanthin derived from *Phaffia* yeast (*Phaffia* yeast group), a feed blended with astaxanthin derived from *Paracoccus* (*Paracoccus* group), and a feed blended with astaxanthin derived from

Haematococcus (*Haematococcus* group) are prepared by a similar manner so that the contained amount of astaxanthin should be equal to that in the feed of the amino acid cell group, to obtain test feeds. A feed for control group is prepared by re-pelletizing without addition of any additive after grinding. There are used 5 test groups: control group, amino acid cell group, *Phaffia* yeast group, *Paracoccus* group, and *Haematococcus* (*Haematococcus* group).

[0358]

Two hundred and fifty (250) rainbow trout having a body weight of about 100 g are introduced into 10 water tanks (200 L) of closed recirculating system so that each tank contained 25 rainbow trout, and cultured for 3 months. There are used 2 tanks for each group. As an evaluation item, the body weight of all individuals is measured after 1 month, 2 months, and 3 months, and the mean body weights during the experimental period are compared among the groups. Four (4) individuals per tank having a body weight close to the mean value are sampled, the color tone of fillet is measured at 2 positions, i.e. at a head-side position and a tail-side position, by using a spectrophotometer (CM-700d, Konica Minolta), and the mean values thereof are compared.

[0359]

Example 8: Culturing of Atlantic salmon using astaxanthin-containing bacterial cells (1)

[0360]

There are prepared feeds for 5 test groups: a feed for salmon not added with astaxanthin (control group), a feed for salmon added with 50 ppm of synthesized astaxanthin (synthesized product group), feed for salmon added with amino acid cells (dry cells of an L-lysine-producing strain of *E. coli* having an enhanced expression of astaxanthin-biosynthesis enzyme genes) in terms of 25, 50, and 75 ppm of astaxanthin (amino acid cell groups; 25 ppm, 50 ppm, and 75 ppm).

[0361]

Atlantic salmon having a body weight of about 200 g at the start of test are introduced into 10 water tanks containing 850 L of seawater so that each tank contained 30 Atlantic salmon, and cultured for 84 days. There are used 2 tanks for each group. As evaluation items, the body weight and the feeding amount of the feed are measured once every 4 weeks, and the feed efficiency is calculated. In addition, Atlantic salmon are sampled once every 4 weeks, the color tone of fillet is measured and compared by

using a spectrophotometer and SalmoFanTM, and the contained amount of astaxanthin in fillet is measured and compared.

[0362]

Example 9: Culturing of Atlantic salmon using astaxanthin-containing bacterial cells (2)

[0363]

There are prepared feeds for 6 test groups: a feed for salmon not added with astaxanthin (control group), a feed for salmon added with amino acid cells (dry cells of an L-lysine-producing strain of *E. coli* having an enhanced expression of astaxanthin-biosynthesis enzyme genes) in an optimal amount determined in Example 8 (amino acid cell group), a feed for salmon added with astaxanthin derived from *Phaffia* yeast (*Phaffia* yeast group), a feed for salmon added with astaxanthin derived from *Paracoccus* (*Paracoccus* group), and a feed for salmon added with astaxanthin derived from *Haematococcus* (*Haematococcus* group).

[0364]

Atlantic salmon having a body weight of about 200 g at the start of test are introduced into 12 water tanks containing 850 L of seawater so that each tank contained 30 Atlantic salmon, and cultured for 84 days. There are used 2 tanks for each group. As evaluation items, the body weight and the feeding amount of the feed are measured once every 4 weeks, and the feed efficiency is calculated. In addition, Atlantic salmon are sampled once every 4 weeks, the color tone of fillet is measured and compared by using a spectrophotometer and SalmoFanTM, and the contained amount of astaxanthin in fillet is measured and compared.

Industrial applicability

[0365]

According to the present invention, an L-amino acid-producing ability of a bacterium can be improved, and an L-amino acid can be efficiently produced. In addition, in an embodiment of the present invention, by allowing an L-amino acid-producing bacterium to produce a carotenoid, bacterial cells containing the carotenoid can be obtained and used as a by-product of L-amino acid production.

[0366]

Explanation of Sequence Listing

SEQ ID NOS:

1-6: Primers

7: Nucleotide sequence of *yggB* gene of *Corynebacterium glutamicum* 2256 (ATCC 13869)

8: Amino acid sequence of YggB protein of *Corynebacterium glutamicum* 2256 (ATCC 13869)

9: Nucleotide sequence of mutant *yggB* gene (V419::IS) of *Corynebacterium glutamicum* 2256 (ATCC 13869)

10: Amino acid sequence of protein encoded by mutant *yggB* gene (V419::IS) of *Corynebacterium glutamicum* 2256 (ATCC 13869)

11: Nucleotide sequence of *crtE* gene of *Pantoea ananatis* AJ13355

12: Amino acid sequence of CrtE protein of *Pantoea ananatis* AJ13355

13: Nucleotide sequence of *crtB* gene of *Pantoea ananatis* AJ13355

14: Amino acid sequence of CrtB protein of *Pantoea ananatis* AJ13355

15: Nucleotide sequence of *crtI* gene of *Pantoea ananatis* AJ13355

16: Amino acid sequence of CrtI protein of *Pantoea ananatis* AJ13355

17: Nucleotide sequence of *crtY* gene of *Pantoea ananatis* AJ13355

18: Amino acid sequence of CrtY protein of *Pantoea ananatis* AJ13355

19: Nucleotide sequence of *crtW* gene of *Nostoc* sp. PCC 7120

20: Amino acid sequence of CrtW protein of *Nostoc* sp. PCC 7120

21: Nucleotide sequence of *crtZ* gene of *Pantoea ananatis* AJ13355

22: Amino acid sequence of CrtZ protein of *Pantoea ananatis* AJ13355

23: Nucleotide sequence of *crtE* gene of *Corynebacterium glutamicum* ATCC 13032

24: Amino acid sequence of CrtE protein of *Corynebacterium glutamicum* ATCC 13032

25: Nucleotide sequence of *crtB* gene of *Corynebacterium glutamicum* ATCC 13032

26: Amino acid sequence of CrtB protein of *Corynebacterium glutamicum* ATCC 13032

27: Nucleotide sequence of *crtI* gene of *Corynebacterium glutamicum* ATCC 13032

28: Amino acid sequence of CrtI protein of *Corynebacterium glutamicum* ATCC 13032

29: Nucleotide sequence of *crtW* gene of *Brevundimonas aurantiaca*

30: Amino acid sequence of CrtW protein of *Brevundimonas aurantiaca*

31-32: Primers

33: Nucleotide sequence of PmsrA_crtYZ

34-36: Primers

37: Nucleotide sequence of *crtZ* gene of *Brevundimonas* sp. SD-212

38-41: Primers

42: Nucleotide sequence of *crtZ* gene of *Paracoccus* sp. N81106

43-45: Primers

46: Nucleotide sequence of *crtZ* gene of *Alcaligenes* sp.

47-49: Primers

50: Nucleotide sequence of *crtW* gene of *Nostoc punctiforme* (codon-optimized for expression in *E. coli*)

51-54: Primers

55: Nucleotide sequence of DNA containing *crtW* gene of *Brevundimonas aurantiaca*

56-57: Primers

58: Amino acid sequence of CrtZ protein of *Brevundimonas* sp. SD-212

59: Amino acid sequence of CrtZ protein of *Paracoccus* sp. N81106

60: Amino acid sequence of CrtZ protein of *Alcaligenes* sp.

61: Amino acid sequence of CrtW protein of *Nostoc punctiforme*

CLAIMS

1. A method for producing an L-amino acid, the method comprising:
culturing a bacterium having an L-amino acid-producing ability in a medium to accumulate an L-amino acid in the medium; and
collecting the L-amino acid from the medium,
wherein the bacterium has been modified so that the activity of a carotenoid biosynthesis enzyme is increased as compared with a non-modified strain.
2. The method according to claim 1, wherein the carotenoid biosynthesis enzyme consists of one or more kinds of enzymes selected from geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, lycopene beta-cyclase, carotene ketolase, and carotene hydroxylase.
3. The method according to claim 2, wherein at least the activity or activities of one or more kinds of enzymes selected from geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and lycopene beta-cyclase is/are increased.
4. The method according to claim 2, wherein at least the activities of geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and lycopene beta-cyclase are increased.
5. The method according to claim 3 or 4, wherein the activity or activities of carotene ketolase and/or carotene hydroxylase is/are further increased.
6. The method according to any one of claims 2 to 5, wherein the geranylgeranyl pyrophosphate synthase is a protein defined in (a), (b), or (c) mentioned below:
(a) a protein comprising the amino acid sequence of SEQ ID NO: 12 or 24;
(b) a protein comprising the amino acid sequence of SEQ ID NO: 12 or 24, but which includes substitution, deletion, insertion, and/or addition of 1 to 10 amino acid residues, and having geranylgeranyl pyrophosphate synthase activity;
(c) a protein comprising an amino acid sequence showing an identity of 90% or higher to the amino acid sequence of SEQ ID NO: 12 or 24, and having geranylgeranyl

pyrophosphate synthase activity.

7. The method according to any one of claims 2 to 6, wherein the phytoene synthase is a protein defined in (a), (b), or (c) mentioned below:

- (a) a protein comprising the amino acid sequence of SEQ ID NO: 14 or 26;
- (b) a protein comprising the amino acid sequence of SEQ ID NO: 14 or 26, but which includes substitution, deletion, insertion, and/or addition of 1 to 10 amino acid residues, and having phytoene synthase activity;
- (c) a protein comprising an amino acid sequence showing an identity of 90% or higher to the amino acid sequence of SEQ ID NO: 14 or 26, and having phytoene synthase activity.

8. The method according to any one of claims 2 to 7, wherein the phytoene desaturase is a protein defined in (a), (b), or (c) mentioned below:

- (a) a protein comprising the amino acid sequence of SEQ ID NO: 16 or 28;
- (b) a protein comprising the amino acid sequence of SEQ ID NO: 16 or 28, but which includes substitution, deletion, insertion, and/or addition of 1 to 10 amino acid residues, and having phytoene desaturase activity;
- (c) a protein comprising an amino acid sequence showing an identity of 90% or higher to the amino acid sequence of SEQ ID NO: 16 or 28, and having phytoene desaturase activity.

9. The method according to any one of claims 2 to 8, wherein the lycopene beta-cyclase is a protein defined in (a), (b), or (c) mentioned below:

- (a) a protein comprising the amino acid sequence of SEQ ID NO: 18;
- (b) a protein comprising the amino acid sequence of SEQ ID NO: 18, but which includes substitution, deletion, insertion, and/or addition of 1 to 10 amino acid residues, and having lycopene beta-cyclase activity;
- (c) a protein comprising an amino acid sequence showing an identity of 90% or higher to the amino acid sequence of SEQ ID NO: 18, and having lycopene beta-cyclase activity.

10. The method according to any one of claims 2 to 9, wherein the carotene

ketolase is a protein defined in (a), (b), or (c) mentioned below:

- (a) a protein comprising the amino acid sequence of SEQ ID NO: 20, 30, or 61;
- (b) a protein comprising the amino acid sequence of SEQ ID NO: 20, 30, or 61, but which includes substitution, deletion, insertion, and/or addition of 1 to 10 amino acid residues, and having carotene ketolase activity;
- (c) a protein comprising an amino acid sequence showing an identity of 90% or higher to the amino acid sequence of SEQ ID NO: 20, 30, or 61, and having carotene ketolase activity.

11. The method according to any one of claims 2 to 10, wherein the carotene hydroxylase is a protein defined in (a), (b), or (c) mentioned below:

- (a) a protein comprising the amino acid sequence of SEQ ID NO: 22, 58, 59, or 60;
- (b) a protein comprising the amino acid sequence of SEQ ID NO: 22, 58, 59, or 60, but which includes substitution, deletion, insertion, and/or addition of 1 to 10 amino acid residues, and having carotene hydroxylase activity;
- (c) a protein comprising an amino acid sequence showing an identity of 90% or higher to the amino acid sequence of SEQ ID NO: 22, 58, 59, or 60, and having carotene hydroxylase activity.

12. The method according to any one of claims 1 to 11, wherein the activity of the carotenoid biosynthesis enzyme is increased by increasing the expression of a gene encoding the carotenoid biosynthesis enzyme.

13. The method according to claim 12, wherein the expression of the gene is increased by increasing the copy number of the gene and/or modifying an expression control sequence of the gene.

14. The method according to any one of claims 1 to 13, wherein the bacterium is a bacterium belonging to the family *Enterobacteriaceae* or a coryneform bacterium.

15. The method according to any one of claims 1 to 14, wherein the bacterium is a bacterium belonging to the genus *Pantoea* or *Escherichia*.

16. The method according to claim 15, wherein the bacterium is *Pantoea ananatis* or *Escherichia coli*.
17. The method according to any one of claims 1 to 14, wherein the bacterium is a bacterium belonging to the genus *Corynebacterium*.
18. The method according to claim 17, wherein the bacterium is *Corynebacterium glutamicum*.
19. The method according to any one of claims 1 to 18, wherein the L-amino acid consists of an L-amino acid of aspartate family and/or an L-amino acid of glutamate family.
20. The method according to any one of claims 1 to 19, wherein the L-amino acid consists of one or more kinds of L-amino acids selected from L-lysine, L-threonine, and L-glutamic acid.
21. The method according to any one of claims 1 to 20, wherein the bacterium has a carotenoid-producing ability, and a carotenoid is accumulated in cells of the bacterium by the culturing.
22. The method according to claim 21, wherein the cells contain the carotenoid in an amount of 10 µg/g-DCW or more.
23. The method according to claim 21 or 22, wherein the carotenoid consists of one or more kinds of carotenoids selected from beta-carotene, zeaxanthin, canthaxanthin, and astaxanthine.
24. The method according to any one of claims 21 to 23, which further comprises collecting the cells.
25. A method for producing bacterial cells containing a carotenoid, the method comprising:

culturing a bacterium having an L-amino acid-producing ability and a carotenoid-producing ability in a medium to accumulate a carotenoid in cells of the bacterium; and

collecting the cells,

wherein the bacterium has been modified so that the activity of a carotenoid biosynthesis enzyme is increased as compared with a non-modified strain.

26. The method according to claim 25, wherein the cells contain the carotenoid in an amount of 10 µg/g-DCW or more.

27. The method according to claim 25 or 26, wherein the carotenoid consists of one or more kinds of carotenoids selected from beta-carotene, zeaxanthin, canthaxanthin, and astaxanthine.

28. Cells of a bacterium,
wherein the cells contain a carotenoid,
wherein the bacterium having an L-amino acid-producing ability and a carotenoid-producing ability, and
wherein the bacterium has been modified so that the activity of a carotenoid biosynthesis enzyme is increased as compared with a non-modified strain.

29. The cells according to claim 28, wherein the cells contain the carotenoid in an amount of 10 µg/g-DCW or more.

30. The cells according to claim 28 or 29, wherein the carotenoid consists of one or more kinds of carotenoids selected from beta-carotene, zeaxanthin, canthaxanthin, and astaxanthine.

31. A composition for culturing an aquatic organism, the composition containing the cells according to any one of claims 28 to 30,
wherein the aquatic organism is salmon, shrimp, or seabream.

32. A feed for an aquatic organism, the feed containing the cells according to any

one of claims 28 to 30,

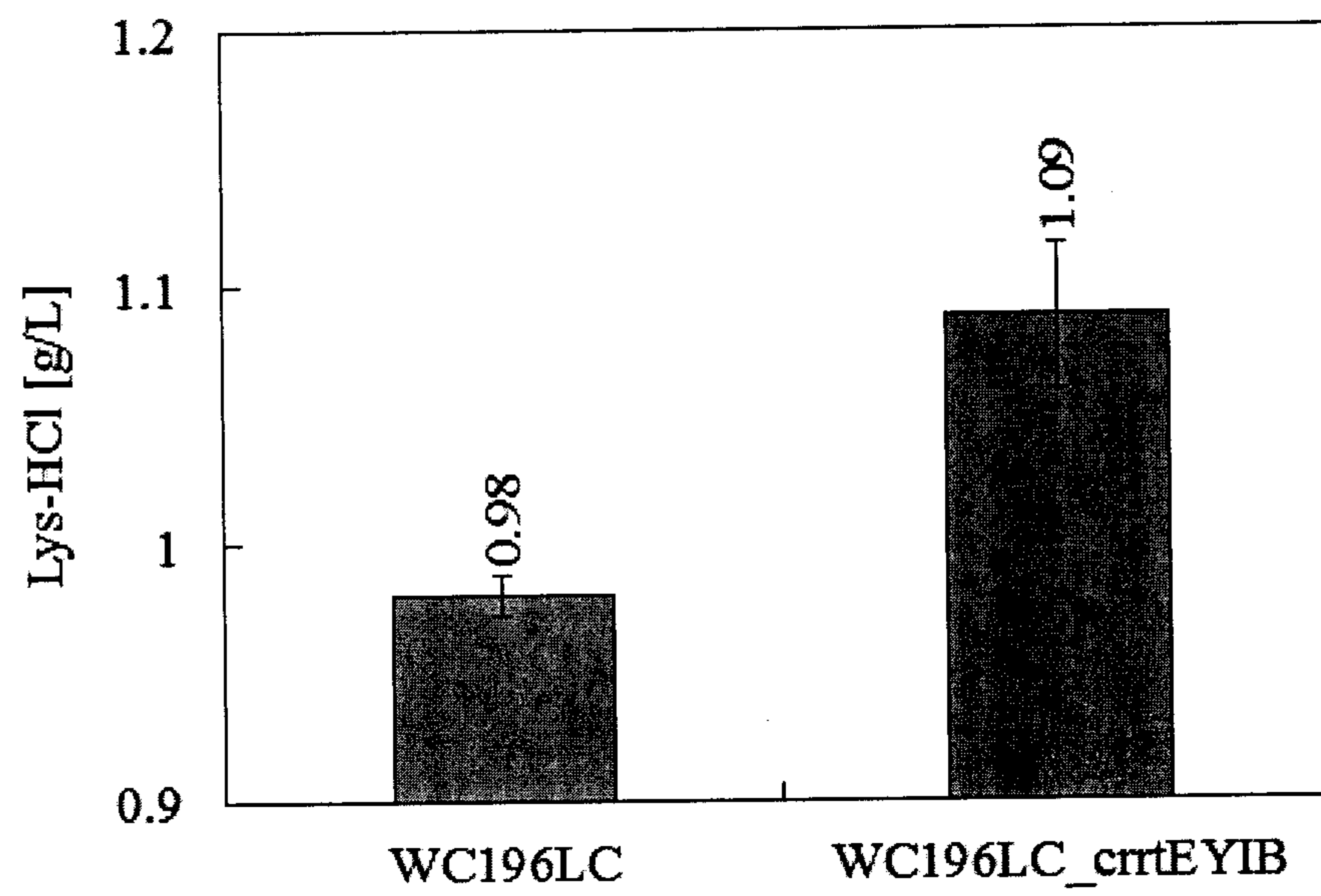
wherein the aquatic organism is salmon, shrimp, or seabream.

33. A method for culturing an aquatic organism, the method comprising:
feeding cells produced by the method according to any one of claims 25 to 27,
the cells according to any one of claims 28 to 30, the composition according to claim 31,
or the feed according to claim 32 to an aquatic organism,
wherein the aquatic organism is salmon, shrimp, or seabream.

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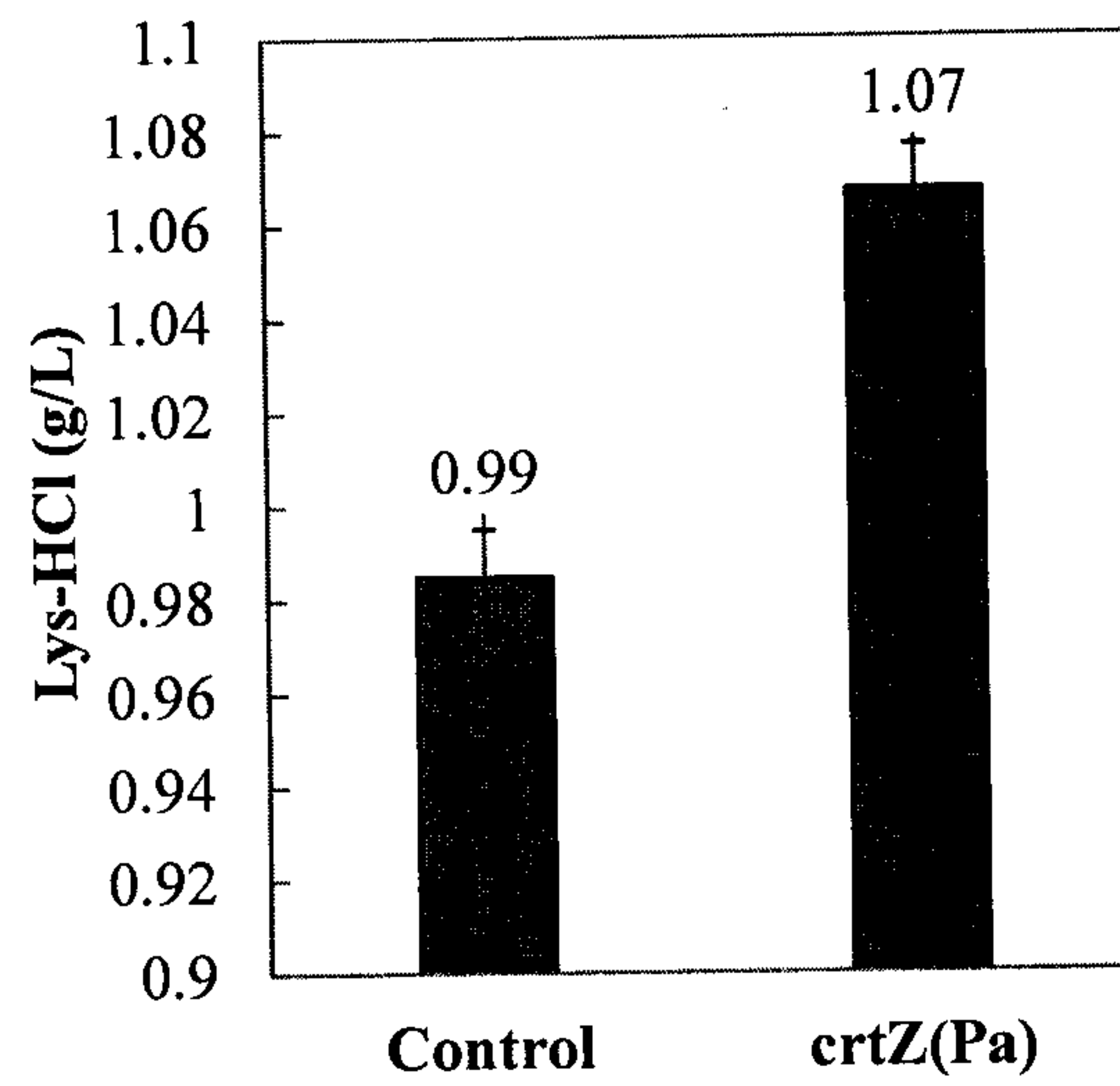
DRAWINGS

[Fig. 1]

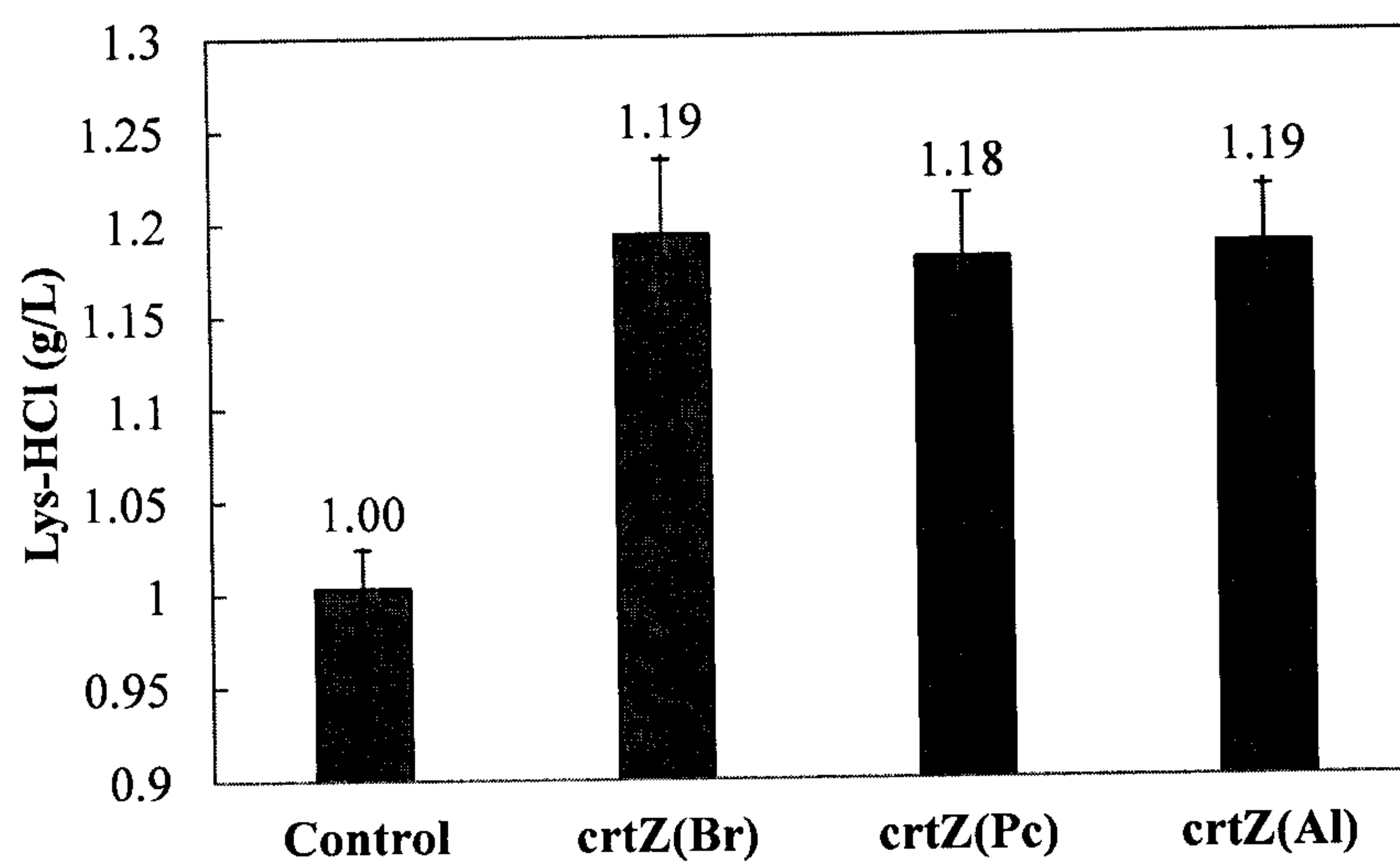


[Fig. 2]

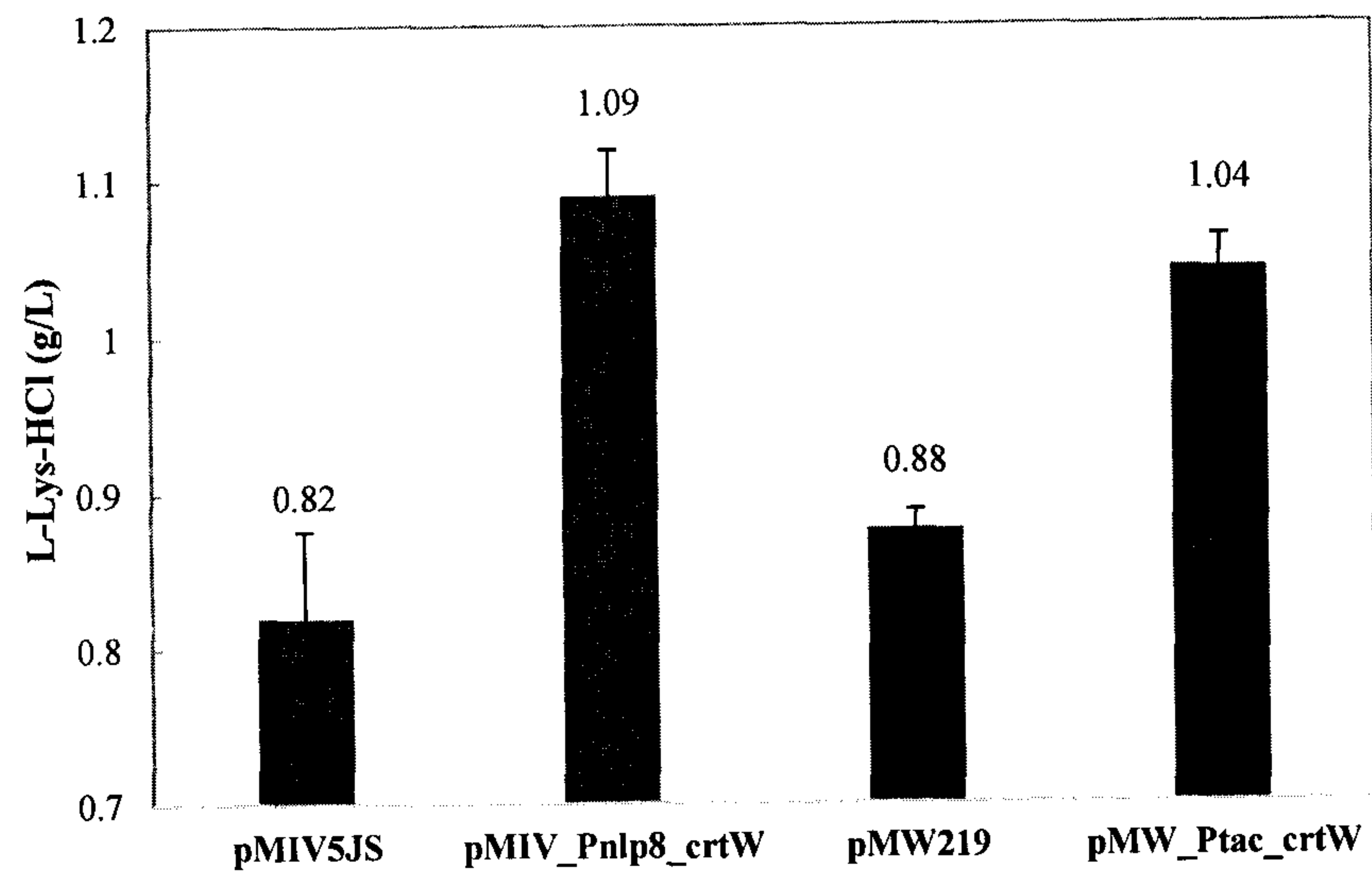
(A)



(B)

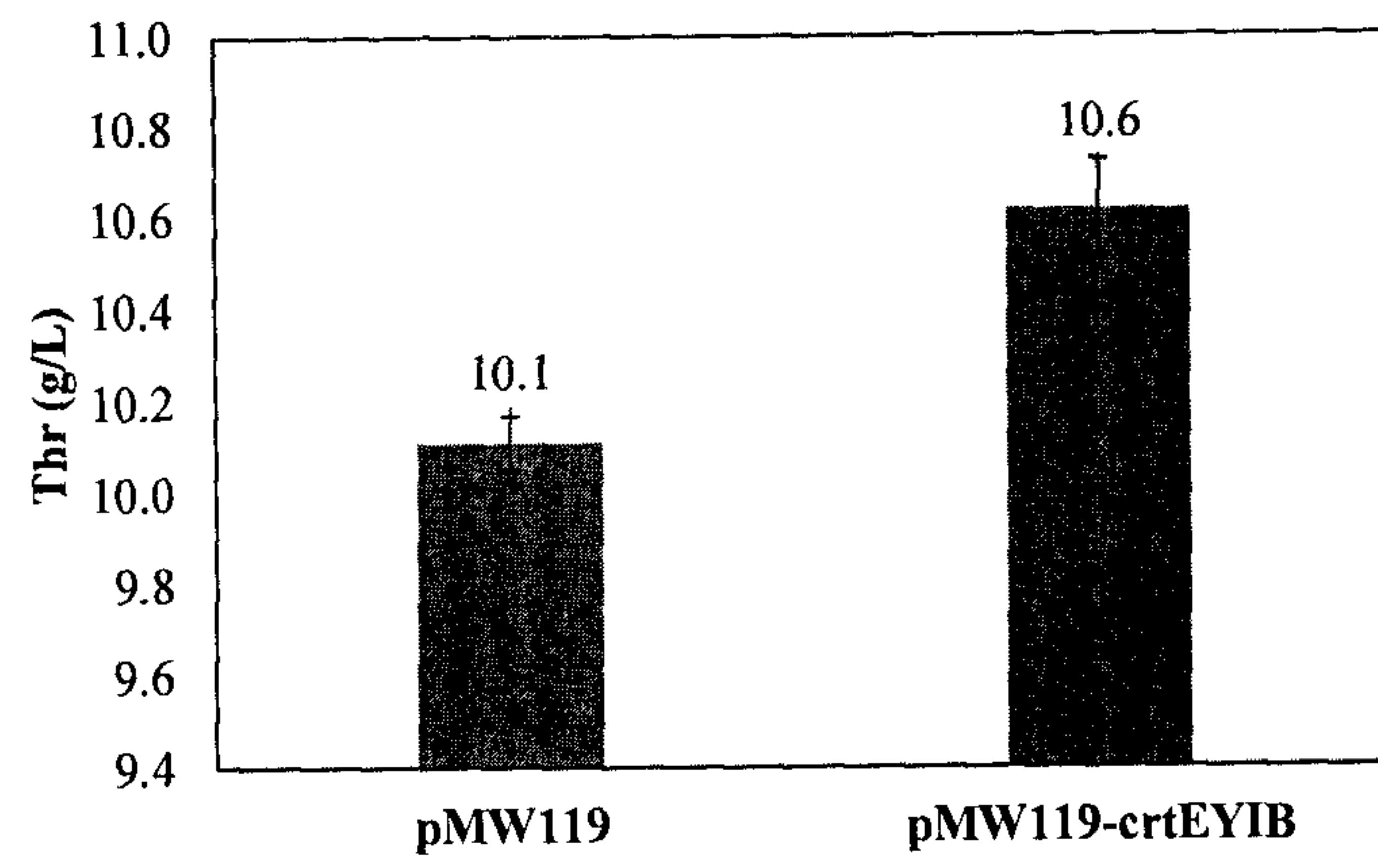


[Fig. 3]

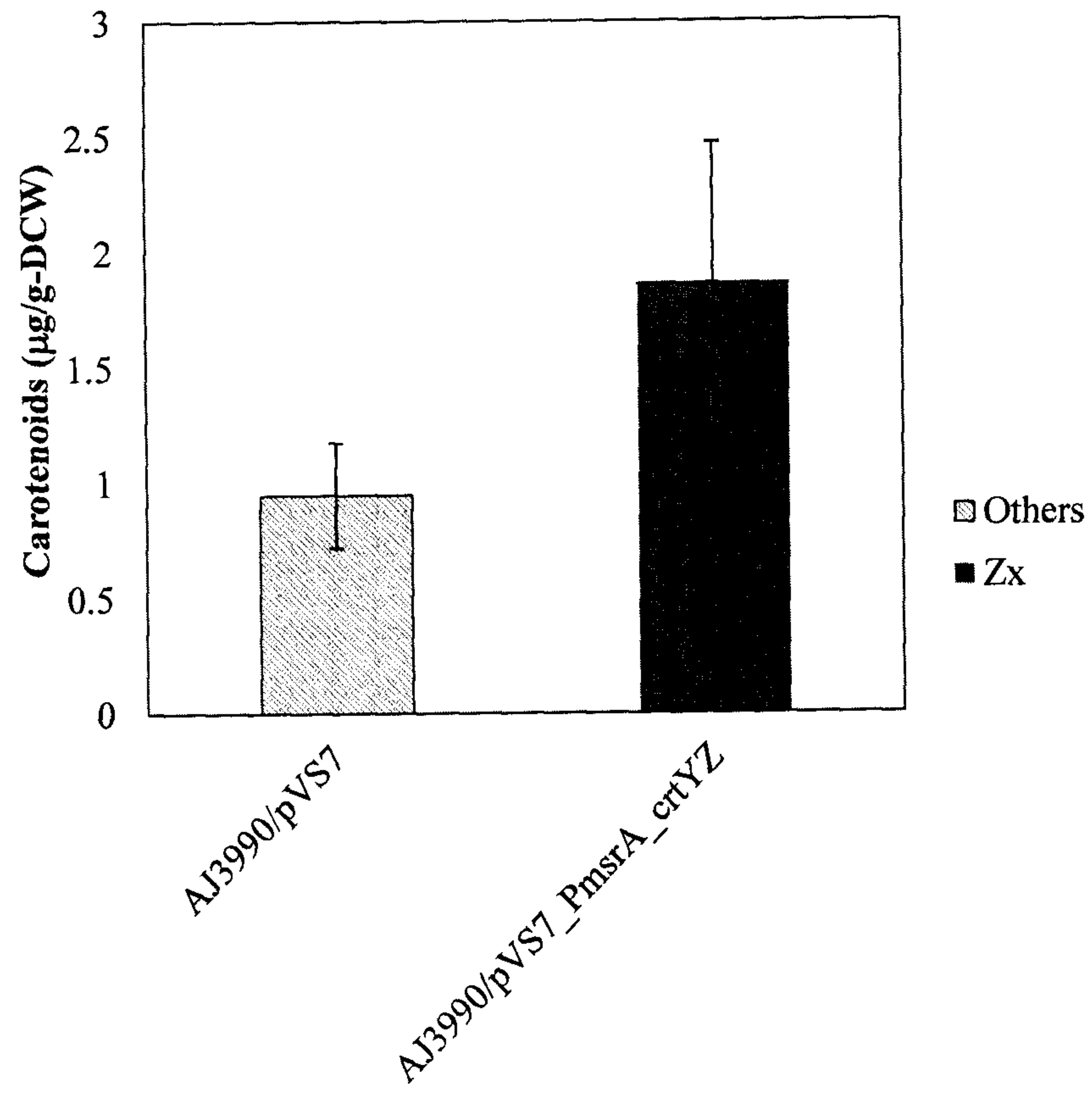


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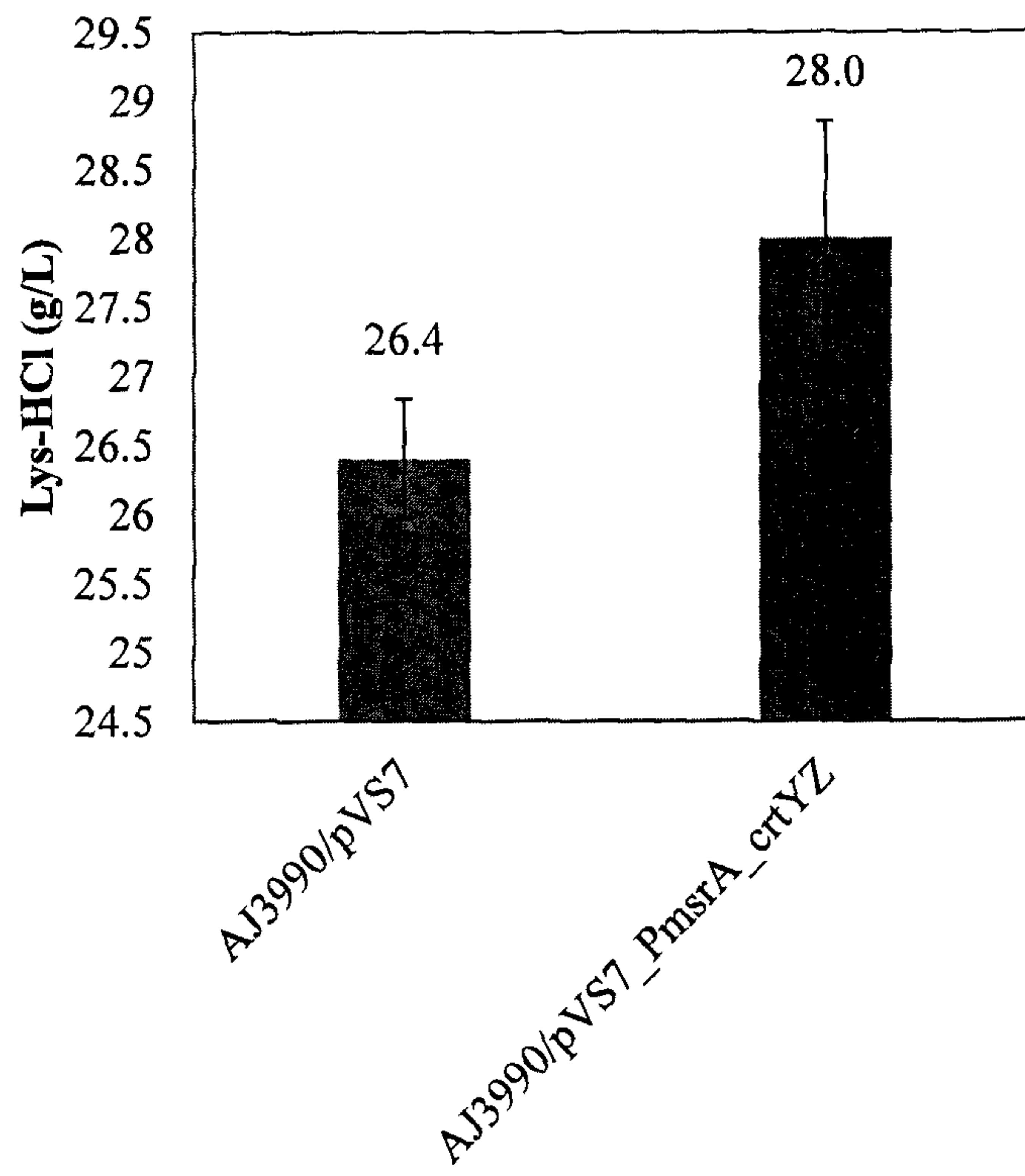
[Fig. 4]



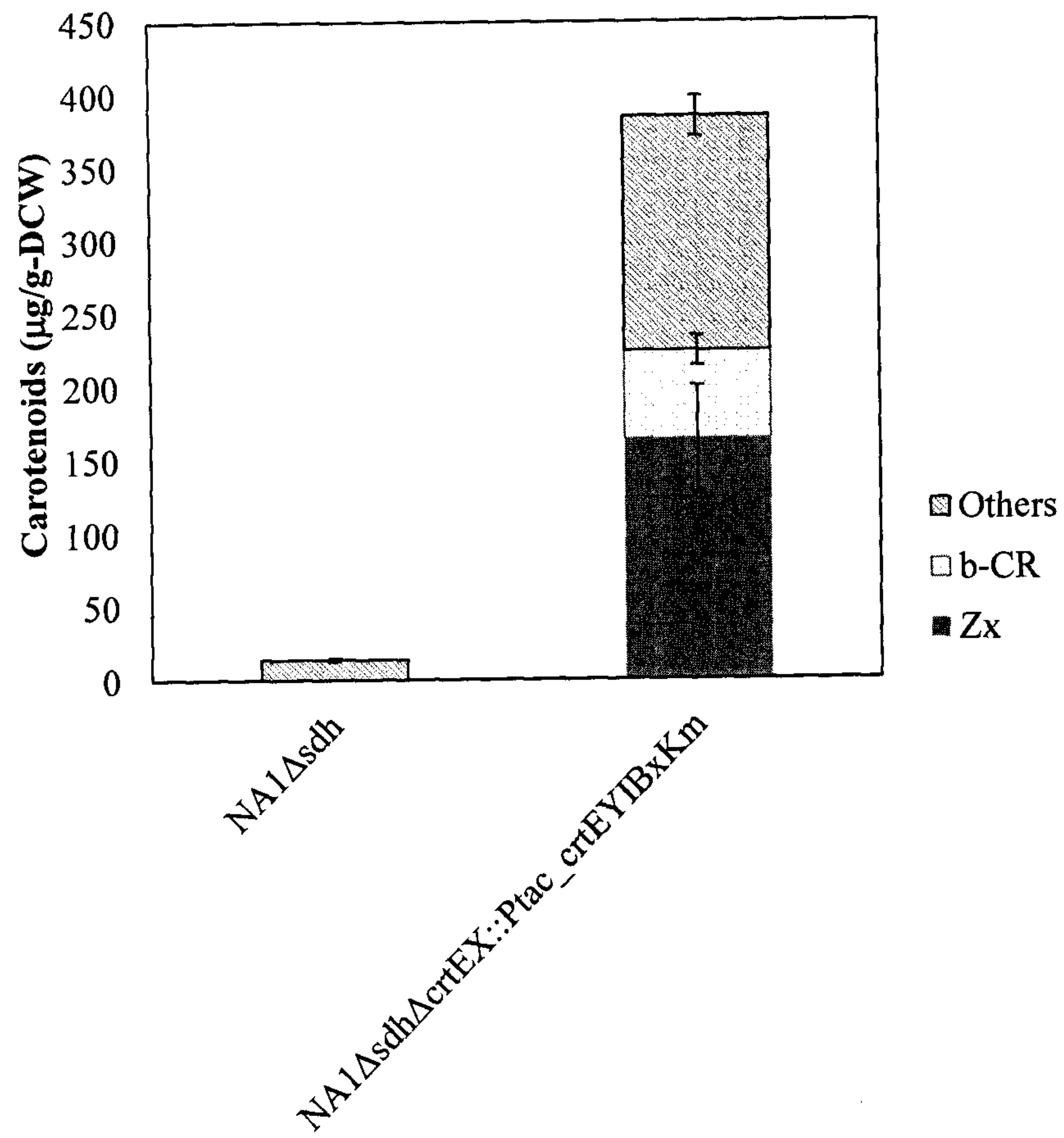
[Fig. 5]



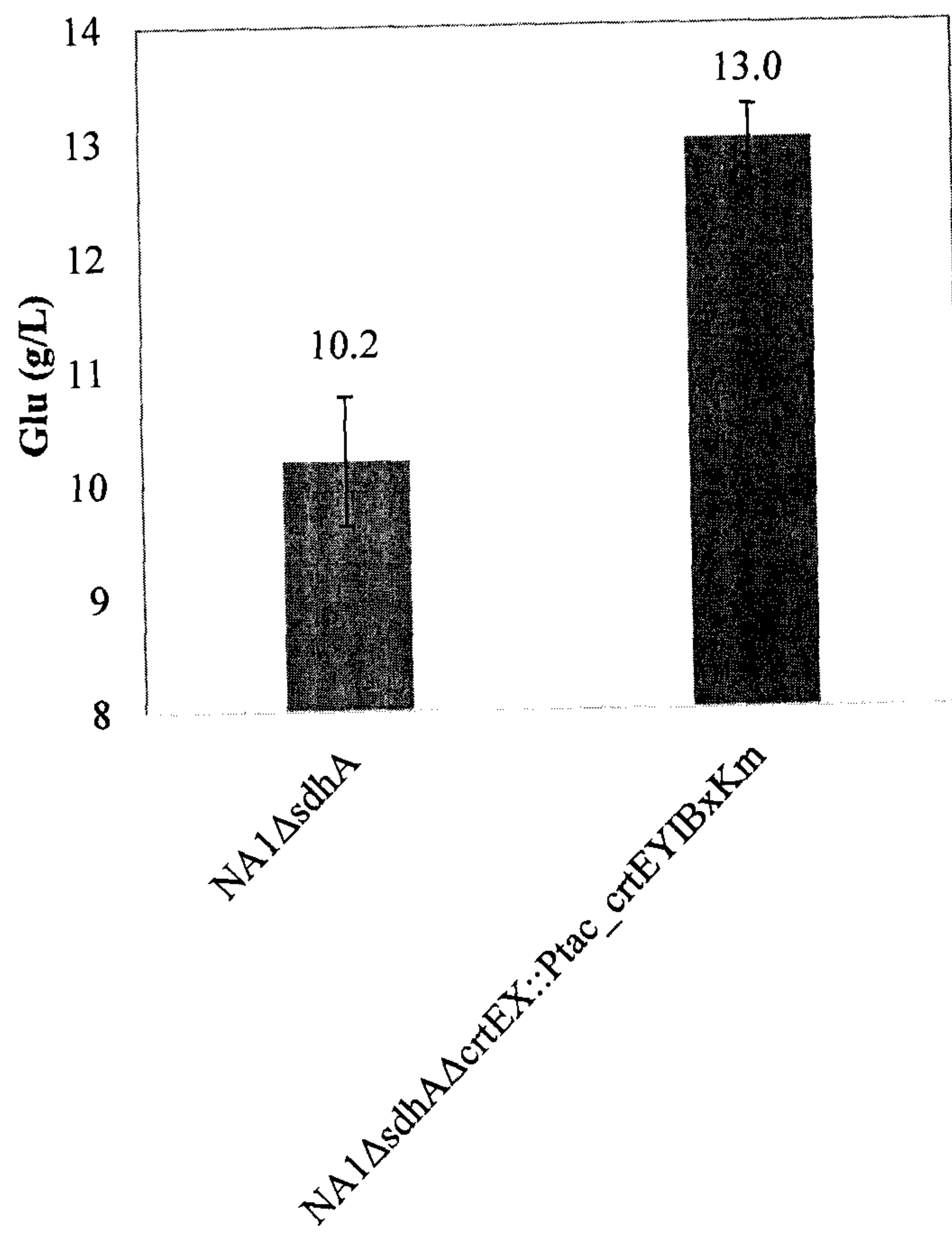
[Fig. 6]



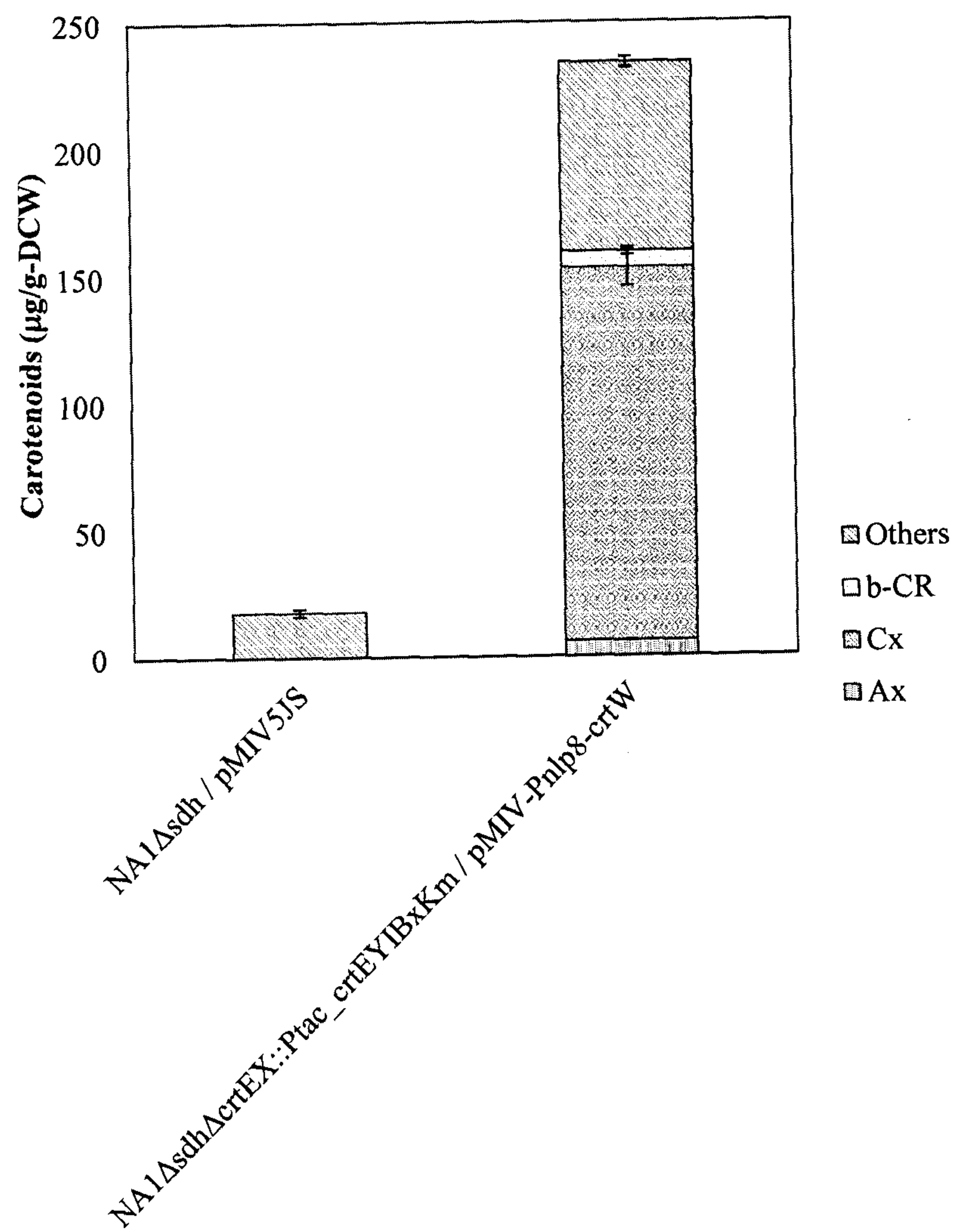
[Fig. 7]



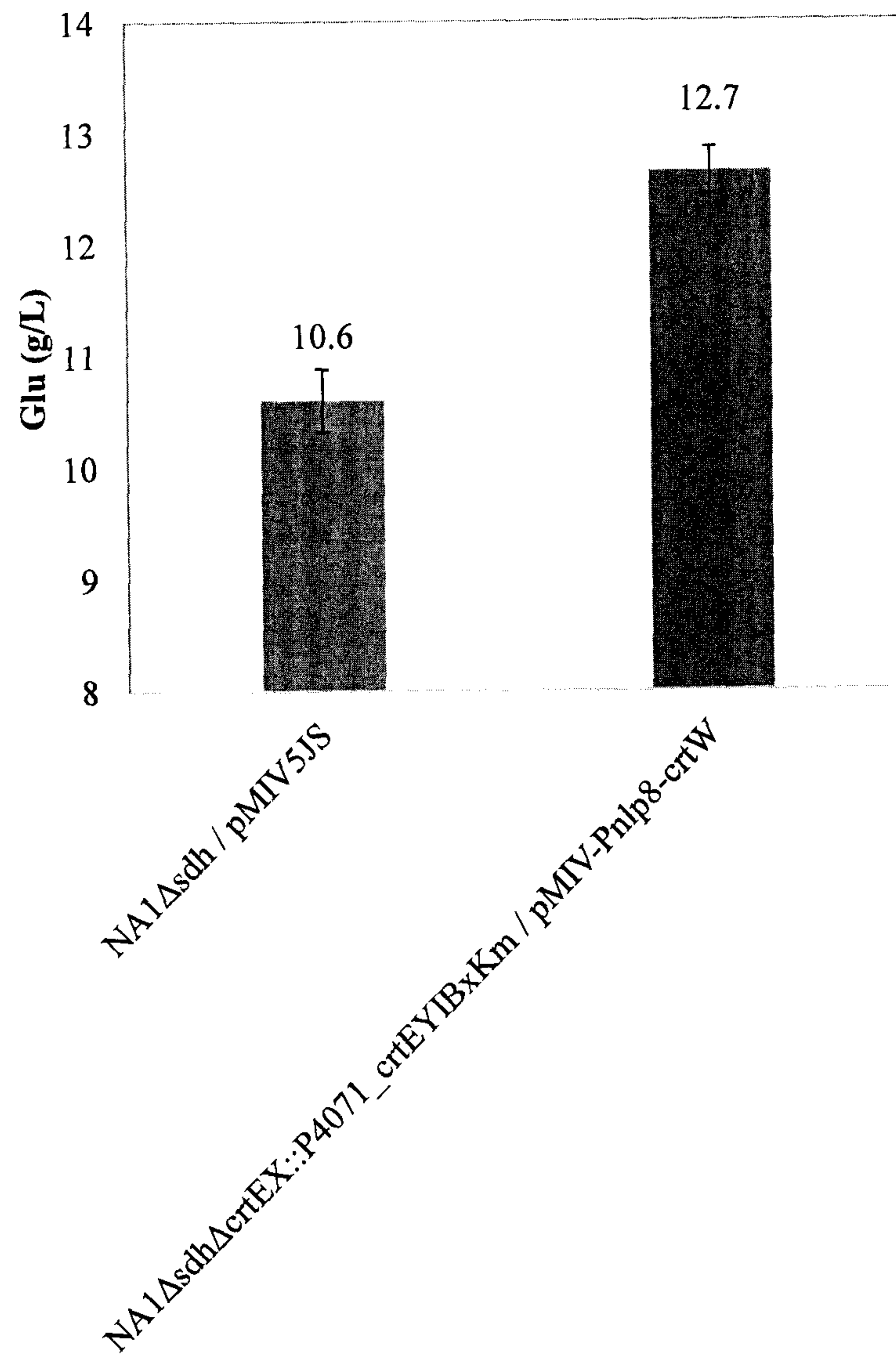
[Fig. 8]



[Fig. 9]



[Fig. 10]



[図1]

