<table>
<tr><td rowspan="2">Patent Right</td><td>Date</td><td>March 25, 2020</td><td rowspan="2">Court</td><td rowspan="2">Intellectual Property High Court, Second Division</td></tr>
<tr><td>Case number</td><td>2019 (Gyo-Ke) 10019, 2019 (Gyo-Ke) 10030</td></tr>
<tr><td colspan="5">- A case in which, with regard to an invention titled "L-GLUTAMIC ACID-PRODUCING BACTERIUM AND METHOD FOR PRODUCING L-GLUTAMIC ACID", there are no violations of support requirement and enablement requirement, and the invention does not lack an inventive step.</td></tr>
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Case type: Rescission of Trial Decision to Maintain

Result: Dismissed

References: Article 29, paragraph (2), Article 36, paragraph (4), item (i), and Article 36, paragraph (6), item (i) of the Patent Act

Related rights, etc.: Patent No. 5343303

Summary of the Judgment

1. The present case is a lawsuit for rescission of a trial decision to maintain with regard to a trial for invalidation of a patent concerning an invention titled "L-GLUTAMIC ACID-PRODUCING BACTERIUM AND METHOD FOR PRODUCING L-GLUTAMIC ACID." As grounds for rescission of the trial decision, the plaintiffs asserted that the findings and determinations made in the trial decision are erroneous on violations of support requirement and enablement requirement, lack of inventive step, and violation of clarity requirement.
2. The invention according to the patent of the present case (hereinafter referred to as "Present Invention") is an invention of modifying a gene encoding an osmoregulatory channel protein of coryneform bacterium which produces glutamic acid (yggB gene) and introducing various mutations to the coryneform bacterium, to thereby promote efflux of glutamic acid from the above channel protein and to increase an amount of glutamic acid produced. With regard to the support requirement and the enablement requirement on a 19-type mutation which is one of the above mutations, in summary, the present judgment determined as follows and concluded that the findings and determinations made in the trial decision are not erroneous.

 (1) The description states the 19-type mutation in Example 8, in which [Table 7] shows that ATCC13869-19 strain, which is the 19-type mutant strain, produces 0.2 g/L more L-glutamic acid than the wild-type strain. Based on this result, the description states in paragraph [0120] that "the ATCC13869-19 strain greatly

enhanced L-glutamic acid accumulation as compared with the ATCC13869 strain which is the parent strain." Thus, from these statements, a person ordinarily skilled in the art recognizes that the 19-type mutation can solve the problem of the Present Invention even if not under the condition of inducing the production of glutamic acid. In addition, the person ordinarily skilled in the art can work the Present Invention related to the 19-type mutation without undue trial-and-error.

(2) The plaintiffs asserted that in Example 8, the difference in an amount of glutamic acid produced was merely 0.2 g/L between the wild-type strain in which a mutation was not introduced and the 19-type mutant strain, and this difference is within the margin of error in light of values such as blank values in other examples, thus a person ordinarily skilled in the art cannot recognize that glutamic acid-producing ability of the 19-type mutant strain is enhanced from Example 8. However, the significance of the blank values in other examples and the culture conditions of glutamic acid-producing bacteria vary depending on each example or culture. Therefore, it cannot be deemed that the value of Example 8 was an error as compared to values of other examples.

3. The authors of the cited document of Exhibit Ko 8 (Susanne RUFFERT, Reinhard Krämer et al. "Efflux of compatible solutes in Corynebacterium glutamicum mediated by osmoregulated channel activity" European Journal of Biochemistry 247, pp 572-580, 1997) conducted experiments themselves and analyzed the results of the experiments to draw a conclusion that the efflux of glutamic acid is not due to an osmoregulatory channel protein but is due to carrier protein. In addition, at the time of the priority date, it had been clear that the efflux of lysine, which is a type of amino acid, as well as glutamic acid, is due to carrier protein. Further, one of the co-authors of Exhibit Ko 8 proposed that the efflux of glutamic acid is also due to carrier protein. In light of the above with regard to the findings of the cited invention, the present judgment determined that it is not sufficient to acknowledge that a person ordinarily skilled in the art who comes in contact with Exhibit Ko 8 recognizes that the efflux of glutamic acid occurs from the osmoregulatory channel protein of coryneform bacterium. In addition, the present judgment determined that it cannot be acknowledged that the person ordinarily skilled in the art would have been able to easily make the Present Invention based on Exhibit Ko 8.

Judgment rendered on March 25, 2020
2019 (Gyo-Ke) 10019 A case of seeking rescission of the JPO decision (hereinafter referred to as "the First Case")
2019 (Gyo-Ke) 10030 A case of seeking rescission of the JPO decision (hereinafter referred to as "the Second Case")
Date of conclusion of oral argument: February 3, 2020

Judgment

Plaintiff of the First Case: CJ Japan Corp.

Plaintiff of the Second Case: CJ CheilJedang Corp.

Defendant of the First and the Second Cases: Ajinomoto Co., Inc.
(hereinafter referred to as "Defendant")

Main text

1. The Plaintiffs' claims in both cases shall be dismissed.
2. The court costs shall be borne by the Plaintiffs.
3. With regard to the Plaintiff of the Second Case, an additional period for filing a final appeal and a petition for acceptance of the final appeal against this judgment shall be 30 days.

Facts and reasons

No. 1 Claim

The trial decision for Invalidation Trial No. 2017-800022 which the JPO made on January 8, 2019 shall be rescinded.

No. 2 Outline of the case

The present case is a lawsuit for rescission of the JPO decision to maintain with regard to a request for a trial for patent invalidation. The issues are whether or not the support requirement and the enablement requirement are violated, whether or not the invention of the present case has an inventive step, and whether or not the clarity requirement is violated.

1 History of Procedures

(1) The Defendant filed a patent application with regard to an invention titled "L-GLUTAMIC ACID-PRODUCING BACTERIA AND METHOD FOR PRODUCING L-GLUTAMIC ACID" on December 28, 2005 (hereinafter referred to as "the filing date of the present case") (Priority claimed: September 9, 2005 [hereinafter referred to as "the priority date of the present case"], Country where priority is claimed: Japan), and the establishment of a patent right was registered in connection with the patent application (Patent No. 5343303) on August 23, 2013 (hereinafter referred to as "the Present Patent").

(2) The Plaintiff of the First Case filed a request for a trial for invalidation of the present patent (Invalidation Trial No. 2017-800022) on February 24, 2017, and the Plaintiff of the Second Case intervened in the trial. In the trial procedures, the Defendant filed a request for correction (hereinafter referred to as "the present correction") including cancellation of Claim 3 in a written request for correction of July 7, 2017 (Exhibit Ko 39, Exhibit Ko 39-2-3).

The Japan Patent Office allowed the present correction (hereinafter both the description and the drawings after the correction are referred to as "the present description"), and made a trial decision that "As to the request for the trial with regard to the inventions according to Claims 1, 2 and 4 to 12 of Patent No. 5343303, the granted patent shall be maintained. The request for the trial with regard to the invention according to Claim 3 of Patent No. 5343303 shall be dismissed" on January 8, 2019 (hereinafter referred to as "the trial decision of the present case"). A certified copy of the trial decision of the present case was served to the Plaintiffs on January 18, 2019.

2 Summary of the Invention

Claims 1, 2, and 4 to 12 of the Present Patent after the present correction (Exhibit Ko 39-2, hereinafter referred to as "Present Invention 1", etc. in accordance with the number of each claim, and collectively referred to as the "Present Invention") are as follows.

[Claim 1] A coryneform bacterium having an L-glutamic acid-producing ability,

wherein a mutant-type yggB gene is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced as compared to a non-modified strain,

wherein any of the following mutations (i), (i'), (i''), and (ii) is introduced into the mutant-type yggB gene:

(i) deletion of a region of amino acid numbers 419 to 533 of an amino acid sequence of SEQ ID NOS: 6, 68, 84, or 85, or a region of amino acid numbers 419 to

529 of SEQ ID NO: 62;

(i') insertion of an insertion sequence or a transposon into a region of amino acid numbers 419 to 533 of an amino acid sequence of SEQ ID NOS: 6, 68, 84, or 85, or a region of amino acid numbers 419 to 529 of SEQ ID NO: 62;

(i'') a mutation in which proline in a region of amino acid numbers 419 to 533 of an amino acid sequence of SEQ ID NOS: 6, 68, 84, or 85, or a region of amino acid numbers 419 to 529 of SEQ ID NO: 62 is substituted with another amino acid;

and

(ii) substitution, deletion, or insertion of one to five amino acids in a region selected from the group consisting of amino acid numbers 1 to 23, 86 to 108, and 110 to 132 of an amino acid sequence of SEQ ID NOS: 6, 62, 68, 84, or 85.

[Claim 2] The coryneform bacterium according to Claim 1, wherein the mutation (i'') is a mutation in which proline at position 424 is substituted with one amino acid selected from the group consisting of alanine, glycine, valine, leucine, and isoleucine, and/or, proline at position 437 is substituted with one amino acid selected from the group consisting of threonine, serine, and tyrosine, in the amino acid sequence of SEQ ID NOS: 6, 62, 68, 84, or 85.

[Claim 4] A coryneform bacterium having an L-glutamic acid-producing ability,

wherein a mutant-type yggB gene is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced as compared to a non-modified strain,

wherein the mutation in the mutant-type yggB gene is a mutation in which alanine at position 100 is substituted with threonine, and/or alanine at position 111 is substituted with threonine or valine, in the amino acid sequence SEQ ID NOS: 6, 62, 68, 84, or 85.

[Claim 5] A coryneform bacterium having an L-glutamic acid-producing ability,

wherein a mutant-type yggB gene is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced as compared to a non-modified strain,

wherein the mutation in the mutant-type yggB gene is a mutation in which one to five amino acids are inserted between leucine at position 14 and tryptophan at position 15 in an amino acid sequence of SEQ ID NOS: 6, 62, 68, 84, or 85.

[Claim 6] The coryneform bacterium according to any one of Claims 1, 2, 4, and 5, wherein the mutant-type yggB gene is a mutant-type yggB gene selected from the group consisting of the following (a) to (n):

(a) a DNA encoding an amino acid sequence of SEQ ID NO: 8;

(b) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 8, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(c) a DNA encoding an amino acid sequence of SEQ ID NO: 20;

(d) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 20, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(e) a DNA encoding an amino acid sequence of SEQ ID NO: 22;

(f) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 22, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(g) a DNA encoding an amino acid sequence of SEQ ID NO: 24;

(h) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 24, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(i) a DNA encoding an amino acid sequence of SEQ ID NO: 64;

(j) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 64, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(k) a DNA encoding an amino acid sequence of SEQ ID NO: 70;

(l) a DNA encoding a protein having an amino acid sequence in which one to

five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 70, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(m) a DNA encoding an amino acid sequence of SEQ ID NO: 74; and

(n) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 74, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin.

[Claim 7] The coryneform bacterium according to any one of Claims 1, 2, and 4 to 6, wherein the coryneform bacterium enhances resistance to L-glutamic acid analogues by introducing the mutant-type yggB gene.

[Claim 8] The coryneform bacterium according to any one of Claims 1, 2, and 4 to 7, wherein the coryneform bacterium is further modified so as to inactivate a symA gene of (i):

(i) a DNA containing a nucleotide sequence of nucleotide numbers 585 to 1121 of SEQ ID NO: 86.

[Claim 9] The coryneform bacterium according to any one of Claims 1, 2, and 4 to 8, wherein the coryneform bacterium is further modified so as to decrease an activity of α-ketoglutarate dehydrogenase.

[Claim 10] The coryneform bacterium according to any one of Claims 1, 2, and 4 to 9, wherein the coryneform bacterium belongs to the genus Corynebacterium or the genus Brevibacterium.

[Claim 11] A method for producing L-glutamic acid, comprising culturing a coryneform bacterium according to any one of Claims 1, 2, and 4 to 10 in a medium so as to cause production and accumulation of L-glutamic acid in the medium, and collecting the L-glutamic acid from the medium.

[Claim 12] A mutant-type yggB gene which is selected from the following (a) to (n):

(a) a DNA encoding an amino acid sequence of SEQ ID NO: 8;

(b) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 8, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is

enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(c) a DNA encoding an amino acid sequence of SEQ ID NO: 20;

(d) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 20, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(e) a DNA encoding an amino acid sequence of SEQ ID NO: 22;

(f) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 22, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(g) a DNA encoding an amino acid sequence of SEQ ID NO: 24;

(h) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 24, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(i) a DNA encoding an amino acid sequence of SEQ ID NO: 64;

(j) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 64, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(k) a DNA encoding an amino acid sequence of SEQ ID NO: 70;

(l) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 70, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an

excess of biotin;

(m) a DNA encoding an amino acid sequence of SEQ ID NO: 74; and

(n) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 74, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin.

3 Outline of Reasons of the Trial Decision of the Present Case

Among Reasons 3 to 6 for Invalidation, only the portions concerning the issues are outlined below.

(1) Reason 3 for Invalidation (Lack of an Inventive Step of Present Inventions 1, 4, 6, 7, 9, and 12 over Exhibit Ko 8 [Susanne RUFFERT, Reinhard Krämer et al. "Efflux of compatible solutes in Corynebacterium glutamicum mediated by osmoregulated channel activity" European Journal of Biochemistry 247, pp 572-580, 1997] cited as the primary prior art)

A. Invention Disclosed in Exhibit Ko 8 (hereinafter referred to as "Invention of Exhibit Ko 8")

Exhibit Ko 8 discloses an invention of "Corynebacterium glutamicum having an osmoregulated channel which is suggested to be similar to the MscS in Escherichia coli." However, it cannot be found that Exhibit Ko 8 discloses that the osmoregulated channel in Corynebacterium glutamicum is involved in efflux of L-glutamic acid.

B. Comparison between Present Invention 1 and Invention of Exhibit Ko 8, and Determinations on Different Feature Therebetween.

(A) Common Feature

Coryneform bacterium having a gene of an osmoregulated channel.

(B) Different Feature

Present Invention 1 is "a coryneform bacterium having an L-glutamic acid-producing ability, wherein a mutant-type yggB gene is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced as compared to a non-modified strain, wherein any of the following mutations (i), (i'), (i''), or (ii) is introduced into the mutant-type yggB gene: (i) deletion of a region of amino acid numbers 419 to 533 of an amino acid sequence of SEQ ID NOS: 6, 68, 84, or 85, or a region of amino acid numbers 419 to 529 of SEQ ID NO: 62; (i') insertion of an insertion sequence or a transposon into a region of

amino acid numbers 419 to 533 of an amino acid sequence of SEQ ID NOS: 6, 68, 84, or 85, or a region of amino acid numbers 419 to 529 of SEQ ID NO: 62; (i'') a mutation in which proline in a region of amino acid numbers 419 to 533 of an amino acid sequence of SEQ ID NOS: 6, 68, 84, or 85, or a region of amino acid numbers 419 to 529 of SEQ ID NO: 62 is substituted with another amino acid; and (ii) substitution, deletion, or insertion of one to five amino acids in a region selected from the group consisting of amino acid numbers 1 to 23, 86 to 108, and 110 to 132 of an amino acid sequence of SEQ ID NOS: 6, 62, 68, 84, or 85." In contrast, the Invention of Exhibit Ko 8 does not specify such feature.

(C) Determinations on Different Feature

Exhibit Ko 8 does not disclose that the gene of the osmoregulated channel as disclosed in Exhibit Ko 8 is a yggB gene. In addition, Exhibit Ko 8 neither discloses nor suggests that the mutant-type yggB gene is produced and that the produced mutant-type yggB gene is introduced into a coryneform bacterium in order to enhance an L-glutamic acid-producing ability.

It cannot be deemed that the disclosures of Exhibit Ko 2 (International Publication No. WO95/34672), Exhibit Ko 10 (Reinhard Krämer et al. "Molecular and biochemical characterization of mechanosensitive channels in Corynebacterium glutamicum" FEMS Microbiology Letters 218 pp 305-309, 2003), Exhibit Ko 13 (Randal B. Bass et al. "Crystal Structure of Escherichia coli MscS, a Voltage-Modulated and Mechanosensitive Channel" Science 298 pp 1582-1587, 2002), Exhibit Ko 14 (Samantha Miller et al. "Domain organization of the MscS mechanosensitive channel of Escherichia coli" The EMBO Journal, Vol. 22 No. 1 pp 36-46, 2003), and Exhibit Ko 15 (Marcos Sotomayor et al. "Molecular Dynamics Study of Gating in the Mechanosensitive Channel of Small Conductance MscS" Biophysical Journal 87 pp 3050-3065, 2004) indicate that a yggB gene of Corynebacterium glutamicum is involved in efflux of L-glutamic acid. In addition, it cannot be deemed that those disclosures suggest that the mutant-type yggB gene is produced and that the produced mutant-type yggB gene is introduced into a coryneform bacterium in order to enhance an L-glutamic acid-producing ability.

The disclosures of Exhibit Ko 3 (Unexamined Patent Application Publication No. 1986-268185), Exhibit Ko 4 (Unexamined Patent Application Publication No. 1987-201585), Exhibit Ko 7 (E. H. Muslin et al. "The effect of proline insertions on the thermostability of a barely α-glucosidase" Protein Engineering Vol. 15 No. 1 pp 29-33, 2002), Exhibit Ko 9 (Kuniyuki Okada et al. "Functional Design of Bacterial Mechanosensitive Channels" The JOURNAL OF BIOLOGICAL CHEMISTRY Vol.

277 No. 31 pp 27682-27688, 2002), Exhibit Ko 12 (Pascale Lapujadae et al. "Glutamate Excretion as a Major Kinetic Bottleneck for the Thermally Triggered Production of Glutamic Acid by *Corynebacterium glutamicum*" Metabolic Engineering 1, pp 255-261, 1999), Exhibit Ko 17 (National Publication of International Patent Application No. 2002-508921), Exhibit Ko 18 (Unexamined Patent Application Publication No. 1987-166890), Exhibit Ko 19 (Unexamined Patent Application Publication No. 1988-214189), Exhibit Ko 20 (Unexamined Patent Application Publication No. 2004-313202), Exhibit Ko 21 (M. Marquet et al. "Glutamate excretion by *Corynebacterium glutamicum* : a study of glutamate accumulation during a fermentation course" Appl Microbiol Biotechnol 25 pp 220-223, 1986), Exhibit Ko 22 (Ralf Kelle et al. "Reaction Engineering Analysis of L-Lysine Transport by *Corynebacterium glutamicum*" Biotechnology and Bioengineering 51 pp 40-50, 1996), Exhibit Ko 23 (Susanne Morbach et al. "L-Isoleucine Production with *Corynebacterium glutamicum*: Further Flux Increase and Limitation of Export" APPLIED AND ENVIRONMENTAL MICROBIOLOGY pp 4345-4351, December 1996), Exhibit Ko 24 (Dieter J. Reinscheid et al. "Stable Expression of *hom-1-thrB* in *Corynebacterium glutamicum* and Its Effect on the Carbon Flux to Threonine and Related Amino Acids" APPLIED AND ENVIRONMENTAL MICROBIOLOGY pp 126-132, January 1994), Exhibit Ko 25-1 (International Publication No. WO2003/046123), Exhibit Ko 30 (Unexamined Patent Application Publication No. 1988-214189), and Exhibit Ko 35 (Abdul Haleem Shah et al. "Optimization of Culture Conditions for L-Lysine Fermentation by *Corynebacterium glutamicum*" Online Journal of Biological Science 2(3) pp 151-156, 2002), all of which are submitted as evidences which show well-known art or common technical knowledge, neither indicate that a yggB gene of Corynebacterium glutamicum is involved in efflux of L-glutamic acid, nor motivate production of the mutant-type yggB gene and introduction of the produced mutant-type yggB gene into a coryneform bacterium in order to an enhance an L-glutamic acid-producing ability.

Therefore, even based on the disclosures of Exhibits Ko 2, 10, and 13 to 15 and the well-known art before the priority date of the present case, it cannot be deemed that it would be easily conceivable to a person ordinarily skilled in the art to select a yggB gene as a gene of an osmoregulated channel in the Invention of Exhibit Ko 8, to further produce a mutant-type yggB gene, and to introduce the produced mutant-type yggB gene into a coryneform bacterium in order to enhance an L-glutamic acid-producing ability.

(D) Effect of Present Invention 1

From the statement of Working Examples 2 to 13 in the present description, it can be deemed that Present Invention 1 achieves an effect of being capable of providing a coryneform bacterium of which an L-glutamic acid-producing ability is enhanced, and that such effect of Present Invention 1 is remarkably excellent as compared to the Invention of Exhibit Ko 8.

(E) Therefore, it cannot be deemed that Present Invention 1 could have been easily made by a person ordinarily skilled in the art on the basis of the inventions disclosed in Exhibits Ko 8, 2, 10, and 13 to 15 and the well-known art.

C. Present Inventions 6, 7, and 9 to 11

Present Inventions 6, 7, 9, and 10 are inventions in which Present Invention 1 is subjected to further limitations. Present Invention 11 is an invention of a method for using the coryneform bacterium of Present Invention 1. Since it cannot be deemed that Present Invention 1 could have been easily made by a person ordinarily skilled in the art on the basis of the inventions disclosed in Exhibits Ko 8, 2, 10, and 13 to 15 and the well-known art, it also cannot be deemed that Present Inventions 6, 7, and 9 to 11 could have been easily made by a person ordinarily skilled in the art on the basis of the inventions disclosed in Exhibits Ko 8, 2, 10, and 13 to 15 and the well-known art.

D. Present Invention 4

Present Invention 4 is an invention which depended from Claim 1 before correction, and is made as an independent claim after correction. That is, Present Invention 4 is an invention of a coryneform bacterium which is modified by introducing a mutant-type yggB gene which has "a mutation in which alanine at position 100 is substituted with threonine, and/or alanine at position 111 is substituted with threonine or valine, in the amino acid sequence of SEQ ID NOS: 6, 62, 68, 84, or 85" which substantially corresponds to more specific concept of the mutation (ii) of Present Invention 1; i.e., "(ii) substitution, deletion, or insertion of one to five amino acids in a region selected from the group consisting of amino acid numbers 1 to 23, 86 to 108, and 110 to 132 of an amino acid sequence of SEQ ID NOS: 6, 62, 68, 84, or 85." Therefore, since it cannot be deemed that Present Invention 1 could have been easily made by a person ordinarily skilled in the art on the basis of the inventions disclosed in Exhibits Ko 8, 2, 10, and 13 to 15 and the well-known art, it also cannot be deemed that Present Invention 4 could have been easily made by a person ordinarily skilled in the art on the basis of the inventions disclosed in Exhibits Ko 8, 2, 10, and 13 to 15 and the well-known art.

E. Present Invention 12

(A) Common Feature with Invention of Exhibit Ko 8

A gene of an osmoregulated channel.

(B) Different Feature from Invention of Exhibit Ko 8

Present Invention 12 is "a mutant-type yggB gene which is selected from the following (a) to (n):

(a) a DNA encoding an amino acid sequence of SEQ ID NO: 8;

(b) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 8, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(c) a DNA encoding an amino acid sequence of SEQ ID NO: 20;

(d) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 20, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(e) a DNA encoding an amino acid sequence of SEQ ID NO: 22;

(f) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 22, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(g) a DNA encoding an amino acid sequence of SEQ ID NO: 24;

(h) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 24, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(i) a DNA encoding an amino acid sequence of SEQ ID NO: 64;

(j) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence

of SEQ ID NO: 64, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(k) a DNA encoding an amino acid sequence of SEQ ID NO: 70;

(l) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 70, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin;

(m) a DNA encoding an amino acid sequence of SEQ ID NO: 74; and

(n) a DNA encoding a protein having an amino acid sequence in which one to five amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NO: 74, wherein the DNA is introduced into the coryneform bacterium, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced when the coryneform bacterium is cultured in a medium containing an excess of biotin." In contrast, Invention of Exhibit Ko 8 does not specify such feature.

(C) Determinations on the Different Feature

Exhibit Ko 8 does not disclose that the gene of the osmoregulated channel as disclosed in Exhibit Ko 8 is a yggB gene. In addition, Exhibit Ko 8 neither discloses nor suggests that the mutant-type yggB gene is produced in order to enhance an L-glutamic acid-producing ability.

It cannot be deemed that the disclosures of Exhibits Ko 2, 10, and 13 to 15 indicate that a yggB gene of Corynebacterium glutamicum is involved in efflux of L-glutamic acid, or suggest that the mutant-type yggB gene is produced in order to enhance an L-glutamic acid-producing ability. It cannot be deemed that the disclosures of Exhibits Ko 3, 4, 7, 9, 12, 17 to 24, 25-1, 30, and 35, all of which are submitted as evidences which show well-known art or common technical knowledge, indicate that a yggB gene of Corynebacterium glutamicum is involved in efflux of L-glutamic acid, or motivate production of the mutant-type yggB gene in order to enhance an L-glutamic acid-producing ability. Therefore, even based on the disclosures of Exhibits Ko 2, 10, and 13 to 15 and the well-known art before the priority date of the present case, it cannot be deemed that it would be easily conceivable to a person ordinarily skilled in the art to select a yggB gene as a gene of

an osmoregulated channel in Invention of Exhibit Ko 8, and further, to produce a mutant-type yggB gene in order to enhance an L-glutamic acid-producing ability.

(D) Effect of Present Invention 12

From the statement of Working Examples 2 to 13 in the present description, it can be deemed that Present Invention 12 achieves an effect of being capable of providing a coryneform bacterium of which an L-glutamic acid-producing ability is enhanced, and that such effect of Present Invention 12 is remarkably excellent as compared to the Invention of Exhibit Ko 8.

(E) Therefore, it cannot be deemed that Present Invention 12 could have been easily made by a person ordinarily skilled in the art on the basis of the inventions disclosed in Exhibits Ko 8, 2, 10, and 13 to 15 and the well-known art.

(2) Reason 4 for Invalidation (Enablement Requirement) and Reason 5 for Invalidation (Support Requirement)

A. Problem of the Present Invention

The problem of the present invention is to provide a novel art to enhance an L-glutamic acid-producing ability of a coryneform bacterium when L-glutamic acid is produced by using the coryneform bacterium.

B.(A) Experimental Results of Working Example 8 of the present description

In the experimental results in Working Example 8 of the present description, it is shown that after culturing each strain, the glutamic acid concentration of the wild-type strain was 0.5 g/L, whereas the glutamic acid concentration of the 19-type yggB mutant strain was 0.7 g/L (Table 7). That is, it clearly states that by introducing 19-type YggB mutation, the glutamic acid-producing ability became 1.4-fold that of the wild-type. Thus, it can be deemed that the Detailed Description of the Invention is stated in a clear and sufficient manner to enable a person ordinarily skilled in the art to work the invention to enhance an L-glutamic acid-producing ability.

The experimental results in Exhibits Ko 28 and 34 are experimental results according to a stirring culture at low speed by using an "Erlenmeyer flask". However, in the first place, the present description does not state that the "Erlenmeyer flask" is used for the culture. Further, there is no submitted evidences that even if the experiment under the experimental condition of Working Example 8 in the present description was performed by using a "Sakaguchi flask" as stated in the present description, the experimental results of Working Example 8 of the present description could not be reproduced. Therefore, it cannot be deemed that the matters stated in Exhibits Ko 28 and 34 are grounds for the assertion that Present Invention 11 extends to the scope where the problem which is "to provide a novel means for enhancing an

L-glutamic acid-producing ability" cannot be solved.

(B) Culture Condition for Coryneform Bacterium

In the Present Invention, a means for solving the problem is that "It has been clarified that a yggB gene is involved in the production of L-glutamic acid by coryneform bacterium. It has been found that the L-glutamic acid-producing ability of coryneform bacterium can be greatly enhanced by modifying the coryneform bacterium by using the yggB gene." Preferable condition such as the culture condition for coryneform bacterium is not a matter which is necessary to solve the problem as a matter for defining the invention. In addition, Working Example 10 of the present description is an experiment for testing a condition which can induce L-glutamic acid production (hereinafter, such "a condition which can induce L-glutamic acid production" is referred to as an "inducing condition"; otherwise, referred to as a "non-inducing condition") such as a Tween 40-added condition or a biotin-limited condition. It can be deemed that Working Example 10 of the present description does not show that an amount of L-glutamic acid produced was enhanced only under such inducing condition, and that the experimental results in Working Example 8 show that under non-inducing conditions, the L-glutamic acid-producing ability of coryneform bacterium was enhanced by a 19-type YggB mutant. Therefore, it cannot be deemed that Present Invention 11 extends to the scope where the problem which is "to provide a novel means for enhancing L-glutamic acid-producing ability" cannot be solved, and it cannot be deemed that the Detailed Description of the Invention is not stated in a clear and sufficient manner to enable a person ordinarily skilled in the art to work the invention to enhance an L-glutamic acid-producing ability even if Tween40 is not added or biotin is not limited.

(C) In view of the foregoing, it can be deemed that Present Invention 11 is not beyond the scope which is stated in such a way that a person ordinarily skilled in the art can recognize that the problem of the invention can be solved, and that the detailed description of the Present Invention is stated in a clear and sufficient manner to enable a person ordinarily skilled in the art to work the Present Invention 11.

C. 2A-1-Type Mutation (Mutations (i) and (i') of the Present Invention 1)

The present description discloses a 2A-1 mutation in which five amino acids, which constitute an IS (insertion sequence) inserted by a transposon, were inserted into the C-terminal side portion positioned downstream from amino acid number 419 of amino acid sequence of SEQ ID NO: 6 in Working Examples 5 and 6, and states that it was confirmed that the glutamic acid-producing ability of the coryneform bacterium into which the 2A-1 mutation was introduced was enhanced. It can be

deemed that this 2A-1 mutation, in view of the embodiment of the mutation, is substantially a mutation in which C-terminal region positioned after amino acid number 419 of the YggB protein is deleted. Thus, a person ordinarily skilled in the art can recognize from the statement of Working Examples 5 and 6 that the results of Working Examples 5 and 6 arose by modifying a three-dimensional structure of the C-terminal region positioned after amino acid number 419 of the YggB protein, and that similar effects can be obtained with regard to a mutation in which the C-terminal region positioned after amino acid number 419 of the YggB protein is deleted. Therefore, in a coryneform bacterium into which a mutation in which an IS or a transposon was inserted into the C-terminal region positioned after amino acid number 419 of the YggB protein was introduced, when a three-dimensional structure of the C-terminal region positioned after amino acid number 419 is modified, the person ordinarily skilled in the art can recognize that effects similar to those by the mutations as stated in the above Working Examples can be obtained. For the foregoing reasons, it can be deemed that a person ordinarily skilled in the art can recognize from the statement of the above Working Examples and paragraphs [0069] to [0079] of the present description that the problem of enhancing an L-glutamic acid-producing ability can be solved by mutations (i) and (i'), and that the Detailed Description of the Invention is stated in a clear and sufficient manner to enable a person ordinarily skilled in the art to understand that an L-glutamic acid-producing ability is enhanced by mutations (i) and (i').

D. 66-Type Mutation and 22-type Mutation (Mutation (i'') of Present Invention 1)

(A) The present description states in Working Examples 12 and 13 that a coryneform bacterium into which 66-type mutation was introduced was constructed, where the 66-type mutation is a mutation in which proline of amino acid number 424 is substituted with leucine, and that a coryneform bacterium into which 22-type mutation was introduced was constructed, where the 22-type mutation is a mutation in which proline of amino acid number 437 is substituted with serine, and that the mutations enhanced an L-glutamic acid-producing ability. In addition, the position of proline which is present in the region of amino acid numbers 419 to 533 of the amino acid sequence of SEQ ID NO: 6, etc. is specifically stated in paragraph [0072] of the present description. Therefore, it cannot be found that it is necessary for a person ordinarily skilled in the art to perform a trial-and-error or a complex and highly-advanced experiment beyond what could be expected, according to the statement of the above Working Examples and the statement of paragraphs [0069] to

[0079] of the present description, in order to construct a mutation-introduced coryneform bacterium in which the mutation is one in which proline in the region of amino acid numbers 419 to 533 of the amino acid sequence of SEQ ID NO: 6, etc. is substituted with another amino acid, and among the constructed mutation-introduced coryneform bacteria, to obtain the coryneform bacterium of which an L-glutamic acid-producing ability is enhanced.

(B) In the present description, there is at least one Working Example in which the mutation-introduced coryneform bacterium in which the mutation is one in which proline in the region of amino acid numbers 419 to 533 of the amino acid sequence of SEQ ID NO: 6, etc. is substituted with another amino acid can enhance an L-glutamic acid-producing ability, as mentioned in the above (A). In addition, the present description states in paragraph [0072] that it is considered that proline in the C-terminal side of the yggB gene plays an important role in maintaining a three-dimensional structure of the YggB protein. Therefore, it can be deemed that from the statement of the above Working Examples 12 and 13 and the statement of paragraphs [0069] to [0079] of the present description, and the statement on a role of proline in a protein structure in Exhibit Otsu 19, etc., with regard to the mutation-introduced coryneform bacterium in which the mutation is one in which proline in the region of amino acid numbers 419 to 533 of the amino acid sequence of SEQ ID NO: 6, etc. is substituted with another amino acid, when the three-dimensional structure in the C-terminal region positioned after amino acid number 419 was modified, a person ordinarily skilled in the art can recognize that effects similar to those achieved by the mutations stated in each of the above Working Examples of the present description can be obtained, and that the problem of enhancing an L-glutamic acid-producing ability can be solved.

(C) For the foregoing reasons, with regard to mutation (i'') of Present Invention 1, it can be deemed that Present Invention 1 is not beyond the scope which is stated in such a way that a person ordinarily skilled in the art can recognize that the problem of the invention can be solved, and that the detailed description of the Present Invention is stated in a clear and sufficient manner to enable a person ordinarily skilled in the art to work Present Invention 1.

E. Mutation in Transmembrane Region (Mutation (ii) of Present Invention 1)

(A) The present description states in Working Examples 7 to 9 and 11 that an A1-type mutation-introduced coryneform bacterium in which the A1-type mutation was one in which an IS consisting of three amino acids (Cys-Ser-Leu) was inserted between leucine at position 14 and tryptophan at position 15 in the amino acid

sequence of SEQ ID NO: 6 was constructed, to thereby enhance the L-glutamic acid-producing ability (Working Example 7), and that a 19-type mutation-introduced coryneform bacterium in which the 19-type mutation was one in which alanine at position 100 in the amino acid sequence of SEQ ID NO: 6 was substituted with threonine was constructed, to thereby enhance the L-glutamic acid-producing ability (Working Example 8), and that an L30-type mutation-introduced coryneform bacterium in which the L30-type mutation was one in which alanine at position 111 in the amino acid sequence of SEQ ID NO: 6 was substituted with valine was constructed, to thereby enhance the L-glutamic acid-producing ability (Working Example 9), and that an 8-type mutation-introduced coryneform bacterium in which the 8-type mutation was one in which alanine at position 111 of SEQ ID NO: 62 was substituted with threonine (Working Example 11). In addition, the regions of amino acid numbers 1 to 23, 86 to 108, and 110 to 132 of the amino acid sequence of SEQ ID NO: 6, etc. are regions which are limited to an amino acid sequence consisting of 23 amino acids. Further, by the present correction, the number of substitutions, deletions, or insertions of amino acids which are introduced into the regions of amino acid numbers 1 to 23, 86 to 108, and 110 to 132 is limited to one to five. Therefore, it cannot be found that it is necessary for a person ordinarily skilled in the art to perform a trial-and-error or a complex and highly-advanced experiment beyond what could be expected, according to the statement of the above Working Examples and the statement of paragraphs [0069] to [0079] of the present description, in order to construct a mutation-introduced coryneform bacterium in which the mutation is one in which one to five amino acids are substituted, deleted, or inserted in the region selected from the group consisting of amino acid numbers 1 to 23, 86 to 108, and 110 to 132 of the amino acid sequence of SEQ ID NO: 6, etc., and among these, to obtain the coryneform bacterium of which an L-glutamic acid-producing ability is enhanced.

(B) In the present description, there is at least one Working Example in which a mutation-introduced coryneform bacterium in which the mutation is one in which one to five amino acids are mutated in the regions of amino acid numbers 1 to 23, 86 to 108, and 110 to 132 of the amino acid sequence of SEQ ID NO: 6, etc. could enhance the L-glutamic acid-producing ability. In addition, the regions of amino acid numbers 1 to 23, 86 to 108, 110 to 132 of the amino acid sequence of SEQ ID NO: 6, etc. are regions which are limited to an amino acid sequence consisting of 23 amino acids and the regions near the positions of the mutations in the above Working Examples. Further, by the present correction, the number of substitutions, deletions, or insertions of amino acids which are introduced into the regions of amino acid

numbers 1 to 23, 86 to 108, and 110 to 132 is limited to one to five. Therefore, it can be deemed that from the statement of the above Working Examples and the statement of paragraphs [0069] to [0079] of the present description, with regard to a mutation-introduced coryneform bacterium in which the mutation is one in which one to five amino acids are substituted, deleted, or inserted in the region selected from the group consisting of amino acid numbers 1 to 23, 86 to 108, and 110 to 132 of the amino acid sequence of SEQ ID NO: 6, etc., a person ordinarily skilled in the art can recognize that the problem of enhancing an L-glutamic acid-producing ability can be solved.

(C) For the foregoing reasons, with regard to mutation (ii) of Present Invention 1, it can be deemed that Present Invention 1 is not beyond the scope which is stated in such a way that a person ordinarily skilled in the art can recognize that the problem of the invention can be solved, and that the detailed description of the Present Invention is stated in a clear and sufficient manner to enable a person ordinarily skilled in the art to work Present Invention 1.

F. Amino Acid Sequence of SEQ ID NO: 85

The amino acid sequence of SEQ ID NO: 85 shows an amino acid sequence of a YggB protein conserved in a coryneform bacterium. Among the amino acid sequences of the YggB protein shown in SEQ ID NO: 6, etc., Xaa indicates a location where an amino acid may be substituted or deleted (paragraphs [0035] to [0036] and [0078] to [0079], etc. of the present description). Therefore, a person ordinarily skilled in the art can recognize that the amino acid sequence of SEQ ID NO: 85 also has a function as a YggB protein as well as the amino acid sequence of other SEQ ID NOS. In addition, in case where the mutation (i), (i'), (i'') or (ii) of Present Invention 1 is introduced, it can be deemed that a person ordinarily skilled in the art can also recognize that the problem of enhancing an L-glutamic acid-producing ability can be solved, and that the Detailed Description of the Invention is stated in a clear and sufficient manner to enable a person ordinarily skilled in the art to understand that an L-glutamic acid-producing ability is enhanced.

For the foregoing reasons, with regard to the amino acid sequence of SEQ ID NO: 85, it can be deemed that Present Invention 1 is not beyond the scope which is stated in such a way that a person ordinarily skilled in the art can recognize that the problem of the invention can be solved, and that the Detailed Description of the Present Invention is stated in a clear and sufficient manner to enable a person ordinarily skilled in the art to work Present Invention 1.

(3) Reason 6 for Invalidation (Violation of the Clarity Requirement for Present

Inventions 6 to 12)

A. Statement of Present Inventions 6 and 12

Taking into consideration the statement of paragraph [0032] of the present description and the common technical knowledge at the time of filing the application of the present case, the meaning and the contents of the phrase "excess of biotin" can be understood. Therefore, Present Invention 6 and 12 are clearly stated.

B. Statement of Present Invention 7

Taking into consideration the statement of paragraph [0068] of the present description and the common technical knowledge at the time of filing the application of the present case, the specific substance which is included in the term "L-glutamic acid analogues" can be understood. Therefore, Present Invention 7 is clearly stated.

C. Statement of Present Invention 8

It is clear that Present Invention 8 relates to a "coryneform bacterium." In addition, it is clear that "a symA gene in (i)" in Present Invention 8 is not (i) in corrected Claim 1, but "(i) a DNA containing nucleotide sequence of nucleotide numbers 585 to 1121 of SEQ ID NO: 86" in corrected Claim 8. Therefore, Present Invention 8 is clearly stated.

D. Statement of Present Invention 9

Taking into consideration the statement of paragraph [0083] of the present description and the common technical knowledge at the time of filing the application of the present case, the meaning and the contents of the phrase "decrease an activity of α-ketoglutarate dehydrogenase" can be understood. Therefore, Present Invention 9 is clearly stated.

E. Statement of Present Inventions 10 and 11

Present Inventions 10 and 11 depend from corrected Claims 6 to 9. As mentioned in the above A to D, it can be deemed that Present Inventions 6 to 9 are clearly stated. Therefore, Present Inventions 10 and 11 are also clearly stated.

F. Statement of a Portion of Present Invention 6 which Depends from Claims 4 and 5

Taking into consideration the statement of the present description and the common technical knowledge at the time of filing the application, it is clear that a portion of Present Invention 6 which depends from Claim 4 corresponds to the mutant-type yggB genes of (e) to (j), and a portion of Present Invention 6 which depends from Claim 5 corresponds to the mutant-type yggB genes of (c) and (d). Therefore, Present Invention 6 and Present Inventions 7 to 11 which depend from Claim 6 are clearly stated.

4 Reasons for Rescission of the Trial Decision as Asserted by the Plaintiffs

(1) Erroneous Findings and Determinations on the Violation of the Enablement Requirement and the Support Requirement Concerning 19-Type Mutation (Reason 1 for Rescission)

(2) Erroneous Findings and Determinations on an Inventive Step (Reasons 2 and 3 for Rescission)

(3) Erroneous Findings and Determinations on the Violation of the Enablement Requirement and the Support Requirement Concerning Mutation Other Than 19-Type Mutation (Reason 4 for Rescission)

(4) Erroneous Findings and Determinations on the Clarity Requirement (Reason 5 for Rescission)

(omitted)

No. 4 Judgment of this court

1 Present Invention

(1) Statement of the Present Description (Exhibits Ko 1 and 39-3)

A. Technical Field

[0001]

The present invention relates to the fermentation industry. In detail, the present invention relates to a method for producing L-glutamic acid and a bacterium for using this method. L-glutamic acid is widely used as a raw material in the production of seasonings and the like.

B. Background Art

[0002]

L-glutamic acid has been conventionally produced on an industrial scale by a fermentation method using coryneform bacteria which have L-glutamic acid-producing ability, such as bacteria belonging to the genus Brevibacterium or the genus Corynebacterium. As for these coryneform bacteria, strains isolated from nature or artificial mutants of such strains are used in order to enhance productivity.

[0003]

Generally, a wild-type strain of coryneform bacteria does not produce L-glutamic acid under the condition of the presence of biotin. Accordingly, the production of L-glutamic acid by coryneform bacteria is typically performed under glutamic acid production-inducing condition such as biotin-limited condition, a surfactant-added condition, and penicillin-added condition (Non Patent Document 1).

Further, as strains which can produce L-glutamic acid in the presence of sufficient biotin without applying these methods, a surfactant-temperature-sensitive strain (Patent Document 1), a penicillin-sensitive strain (Patent Document 2), a cerulenin-sensitive strain (Patent Document 3), and a lysozyme-sensitive strain (Patent Document 4), etc. have been developed.

[0004]

However, such L-glutamic acid-producing bacteria which have been developed by these methods may often show a decrease in fatty acid-producing ability or a decrease in cell wall synthesis ability. Thus, use of these methods is very likely to cause reduced adaptation to environmental changes in exchange for L-glutamic acid production. Therefore, considerable efforts have been required for the development of a strain capable of accumulating a significant amount of L-glutamic acid by using these methods.

[0005]

On the other hand, a strain which produces L-glutamic acid in the presence of sufficient biotin can be achieved by deleting a gene encoding α-ketoglutarate dehydrogenase (Patent Document 5). However, the α-ketoglutarate dehydrogenase gene-deficient strain blocks TCA cycle in the midway and thus grows slowly. Therefore, it was difficult to surely obtain a sufficient amount of bacterial cells, which was one of the problems.

[0006]

A yggB gene of coryneform bacteria is a homologue of a yggB gene of Escherichia coli (Non Patent Documents 2 and 3), and has been analyzed as a kind of mechanosensitive channel (Non Patent Document 4). However, an effect of the yggB gene on L-glutamic acid production was not known.

C. Problem to be Solved by the Invention

[0007]

A problem of the present invention is to provide a novel art to enhance an L-glutamic acid-producing ability of a coryneform bacterium when L-glutamic acid is produced by using the coryneform bacterium.

D. Means for Solving the Problem

[0008]

The present researchers have made extensive studies to solve the above problem. As a result, the present researchers have clarified that a yggB gene is involved in the production of L-glutamine (Note by the Judgment: This is acknowledged to be a typographical error of "L-glutamic acid") by a coryneform

bacterium. In addition, the present researchers have found that an L-glutamic acid-producing ability can be greatly enhanced by modifying the coryneform bacterium by using the yggB gene. Finally, the present researchers have completed the present invention.

E. Advantageous Effect of the Invention

[0010]

L-glutamic acid can be efficiently produced by using a coryneform bacterium which is modified by using a yggB gene of the present invention.

F. Mode for Carrying Out the Invention

(A) Coryneform Bacterium of the Present Invention

[0011]

The coryneform bacterium of the present invention has an L-glutamic acid-producing ability, and is modified by using a yggB gene, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced as compared to a non-modified strain.

[0012]

In the present invention, the "coryneform bacterium" includes a bacterium which has been conventionally classified into the genus Brevibacterium, but is currently classified into the genus Corynebacterium (Int. J. Syst. Bacteriol., 41, 255(1991)), and also includes the Brevibacterium bacteria which are very closely related to Corynebacterium bacteria. Examples of such coryneform bacterium include the following. ...

[0015]

In the present invention, the phrase "L-glutamic acid-producing ability" means an ability to cause accumulation of L-glutamic acid in a medium or a bacterial cell when the coryneform bacterium of the present invention is cultured in the medium. The "L-glutamic acid-producing ability" may be a property of a wild-type strain of the coryneform bacterium, because most of the wild-type strains of coryneform bacterium can produce L-glutamic acid under the "L-glutamic acid-producing condition" as mentioned below. However, the L-glutamic acid-producing ability may be imparted or enhanced by breeding, or may be imparted by modification by using a yggB gene, as mentioned below.

The phrase "an L-glutamic acid-producing ability of a coryneform bacterium is enhanced" means that the L-glutamic acid-producing ability of the coryneform bacterium is enhanced as compared to a non-modified strain such as a wild-type strain. In this regard, examples of the wild-type strain of the coryneform bacterium include

Corynebacterium glutamicum ATCC13032 strain, 13869 strain, 14067 strain, and Corynebacterium melassecola ATCC 17965 strain. The "non-modified strain" may also include a strain which expresses the wild-type yggB gene at the same level as the wild-type strains or a strain in which a mutation is not introduced into the coding region of a yggB gene.

(B) Enhancing Expression Level of yggB Gene

[0030]

A yggB gene encodes a protein which is known as a kind of mechanosensitive channel, and which is also referred to as mscS (FEMS Microbiol Lett. 2003 Jan. 28; 218(2): 305-9.).

Enhancing an expression level of the yggB gene leads to improvement of an L-glutamic acid-producing ability of a coryneform bacterium as compared to a non-modified strain. That is, by culturing a coryneform bacterium which has been modified so that an expression level of the yggB gene is increased, the coryneform bacterium causes accumulation of a greater amount of L-glutamic acid than a non-modified strain, or alternatively, the coryneform bacterium produces L-glutamic acid at a higher rate than a non-modified strain. It is preferable that a yggB gene expression-enhancing strain increases an L-glutamic acid yield (a yield per consumed sugar) by 2% or more, more preferably 4% or more, and particularly preferably 6% or more, as compared to a parent strain or a non-modified strain. The yggB gene expression-enhancing strain may increase a bacterial cell production-subtracted yield as compared to a non-modified strain. The "bacterial cell production-subtracted yield" means a value calculated by subtracting a carbon yield which is used for production of bacterial cells from a yield per consumed sugar.

[0031]

Whether the expression level of the yggB gene has been increased can be determined by comparing an amount of m-RNA of the yggB gene with that of a wild-type or that of a non-modified strain. Methods of determining the expression level include Northern hybridization and RT-PCR (Molecular cloning (Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001)). The expression level of the yggB gene may increase as compared to a wild-type or a non-modified strain. For example, it is preferable that the expression level of the yggB gene increases 1.5-fold or more, more preferably 2-fold or more, still more preferably 3-fold or more, as compared to a wild-type or a non-modified strain.

[0032]

The L-glutamic acid-producing ability of the coryneform bacterium which has

been modified so that the expression level of the yggB gene increases may be enhanced as compared to a non-modified strain under at least one condition of an L-glutamic acid-producing condition and an excess of biotin-containing condition.

In this regard, the "L-glutamic acid producing condition" means a condition that a substance which induces L-glutamic acid production is added to a medium which contains carbon sources, nitrogen sources, inorganic salts, and, if necessary, contains organic micronutrients such as amino acids and vitamins, or alternatively a condition that an amount of a substance which inhibits L-glutamic acid production is limited in a medium. Examples of the substance which induces L-glutamic acid production include penicillin G and surfactants containing saturated fatty acid such as Tween 40. Examples of the substance which is limited in order to inhibit L-glutamic acid production include biotin ("Amino San Hakko (Amino Acid Fermentation)", Gakkai Syuppan Senta (Academic Society Publishing Center), 1986 (in Japanese)). With regard to a concentration of these substances which are added to the medium under the L-glutamic acid-producing condition, the concentration of biotin is less than 15 μg/L, preferably less than 10 μg/L, and more preferably less than 5 μg/L, and it is not necessary to contain any biotin in the medium at all. The concentration of penicillin which is added to the medium is 0.1 U/ml or more, preferably 0.2 U/ml or more, and more preferably 0.4 U/ml or more. The concentration of surfactants which are added to the medium is 0.5 g/L or more, preferably 1 g/L or more, and more preferably 2 g/L or more.

On the other hand, the "excess of biotin-containing condition" means, for example, a condition containing biotin of 30 μg/L or more, preferably 40 μg/L, and more preferably 50 μg/L, in the medium.

[0033]

Examples of the yggB gene of the coryneform bacterium include a gene encoding a protein having an amino acid sequence of SEQ ID NOS: 6, 62, 68, or 84. More specifically, examples of the yggB gene of the coryneform bacterium include: a gene having a sequence of nucleotide numbers 1437 to 3035 of SEQ ID NO: 5; a gene having a sequence of nucleotide numbers 507 to 2093 of SEQ ID NO: 61; a gene having a sequence of nucleotide numbers 403 to 2001 of SEQ ID NO: 67; and a gene having a sequence of nucleotide numbers 501 to 2099 of SEQ ID NO: 83. The gene having a sequence of nucleotide numbers 1437 to 3035 of SEQ ID NO: 5 is a yggB gene of Corynebacterium glutamicum ATCC13869 strain. The gene having a sequence of nucleotide numbers 507 to 2093 of SEQ ID NO: 61 is a yggB gene of Corynebacterium glutamicum (Brevibacterium flavum) ATCC14967 strain. The

gene having a sequence of nucleotide numbers 403 to 2001 of SEQ ID NO: 67 is a yggB gene of Corynebacterium melassecola ATCC17965 strain. The gene having a sequence of nucleotide numbers 501 to 2099 of SEQ ID NO: 83 is encoded to nucleotide numbers 1336092 to 1337693 in the genome sequence registered as GenBank Accession No. NC_003450 of Corynebacterium glutamicum ATCC13032, and is registered as NCgl 1221 (NP_600492. Reports small-conductance ... [gi:19552490]).

Further, since the nucleotide sequence of the yggB gene may differ depending on the species and the strain of the coryneform bacterium, the yggB gene may be a variant of the nucleotide sequence consisting of nucleotide numbers 1437 to 3035 of SEQ ID NO: 5. A variant of the yggB gene can be searched by referring to the nucleotide sequence consisting of nucleotide numbers 1437 to 3035 of SEQ ID NO: 5; for example, by means of BLAST and the like (http:// (remainder omitted)). In addition, the variant of the yggB gene includes a gene which can be amplified by PCR using yggB gene homologues, for example, synthetic oligonucleotides of SEQ ID NOS: 75 and 76, for example, by using coryneform bacterium chromosome as templates. Further, as the gene of the present invention, a yggB gene of coryneform bacterium is preferable. However, a gene derived from other microorganisms may be used so far as the gene has a function in a coryneform bacterium. A mutant-type yggB gene mentioned below may be used as a gene of the present invention.

[0034]

The yggB gene may be a gene having an amino acid sequence in which one or several amino acids are substituted, deleted, inserted, or added in an amino acid sequence of SEQ ID NOS: 6, 62, 68, or 84 so far as the yggB gene enhances L-glutamic acid-producing ability of the coryneform bacterium. The number of "several" as referred to herein means, for example, 2 to 20, preferably 2 to 10, and more preferably 2 to 5.

The above substitution is preferably a conservative substitution. Examples of the conservative substitution include: substitution of ala with ser or thr; substitution of arg with gln, his, or lys; substitution of asn with glu, gln, lys, his, or asp; substitution of asp with asn, glu, or gln; substitution of cys with ser or ala; substitution of gln with asn, glu, lys, his, asp, or arg; substitution of glu with gly, asn, gln, lys, or asp; substitution of gly with pro; substitution of his with asn, lys, gln, arg, or tyr; substitution of ile with leu, met, val, or phe; substitution of leu with ile, met, val, or phe; substitution of lys with asn, glu, gln, his, or arg; substitution of met with ile, leu, val, or phe; substitution of phe with trp, tyr, met, ile, or leu; substitution of ser with

thr or ala; substitution of thr with ser or ala; substitution of trp with phe or tyr; substitution of tyr with his, phe, or trp; and substitution of val with met, ile, or leu.

The yggB gene of the present invention include a gene which encodes a protein which is at least 80% homology, preferably at least 90% homology, more preferably at least 95% homology, and particularly preferably at least 97% homology, to the entire amino acid sequence of SEQ ID NOS: 6, 62, 68, or 84, and which enhances an L-glutamic acid-producing ability of the coryneform bacterium. In this regard, the homology can be calculated by BLAST developed by Karlin and Altschul (Pro. Natl. Acad. Sci. USA, 90, 5873 (1993)) and FASTA developed by Pearson (Methods Enzymol., 183, 63 (1990)). The homology search programs (such as BLASTN and BLASTP) based on these algorithms are available from NCBI and others (remainder omitted).

The above substitutions, deletions, insertions, additions, or inversions of amino acids as mentioned above include those caused by naturally occurring mutants or variants, such as those caused by individual differences or by species differences in microorganisms which maintain the yggB gene.

[0035]

Especially, the amino acids at the following positions in the amino acid sequence of SEQ ID NO: 6 may be substituted or deleted. The amino acid sequence of the YggB protein which is conserved among coryneform bacteria is shown in SEQ ID NO: 85, in which Xaa indicates a location where an amino acid may be substituted or deleted.

Glutamine residue at position 48 (preferably substituted with arginine residue)

Asparagine residue at position 275 (preferably substituted with serine residue)

Glutamic acid residue at position 298 (preferably substituted with alanine residue)

Alanine residue at position 343 (preferably substituted with valine residue)

Phenylalanine residue at position 396 (preferably substituted with isoleucine residue)

Serine residue at position 438 (preferably substituted with glycine residue)

Valine residue at position 445 (preferably substituted with alanine residue)

Alanine residue at position 454 (preferably substituted with valine residue)

Proline residue at position 457 (preferably substituted with serine residue)

Serine residue at position 474 (preferably substituted with asparagine residue)

Valine residue at position 517 (preferably deleted)

Glutamic acid residue at position 518 (preferably deleted)

Alanine residue at position 519 (preferably deleted)

Proline residue at position 520 (preferably deleted)

[0036]

The above yggB gene homologue can be obtained by modifying the gene having a sequence of nucleotide numbers 1437 to 3035 of the nucleotide sequence of SEQ ID NO: 5, the gene having a sequence of nucleotide numbers 507 to 2093 of the nucleotide sequence of SEQ ID NO: 61, the gene having a sequence of nucleotide numbers 403 to 2001 of the nucleotide sequence of SEQ ID NO: 67, or the gene having a sequence of nucleotide numbers 501 to 2099 of the nucleotide sequence of SEQ ID NO: 83 by, for example, the site-directed mutagenesis method, so that the amino acid residue at the specific site in the encoded protein is substituted, deleted, inserted, or added.

Further, such genes can be also obtained by a conventionally known mutagenesis treatment. Examples of the mutagenesis treatment include: a method of treating a gene having a sequence of nucleotide numbers 1437 to 3035 of the nucleotide sequence of SEQ ID NO: 5, a gene having a sequence of nucleotide numbers 507 to 2093 of the nucleotide sequence of SEQ ID NO: 61, a gene having a sequence of nucleotide numbers 403 to 2001 of the nucleotide sequence of SEQ ID NO: 67, and a gene having a sequence of nucleotide numbers 501 to 2099 of the nucleotide sequence of SEQ ID NO: 83 in vitro with hydroxylamine and the like; and treating a microorganism, for example, an Escherichia bacterium, harboring the gene with ultraviolet ray or a mutagenesis agent typically used in mutagenesis treatments, such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or ethyl methanesulfonate (EMS). The above substitutions, deletions, insertions, additions, inversions, etc. of amino acids include those caused by naturally occurring mutants or variant, such as those caused by individual differences or by species differences in microorganisms harboring the yggB gene. When these genes are introduced into a coryneform bacterium, whether these genes enhance the L-glutamic acid-producing ability of coryneform bacterium can be confirmed by, for example, introducing the genes into a wild-type strain of the coryneform bacterium and determining if the L-glutamic acid-producing ability of the obtained coryneform bacterium is enhanced under the above condition.

(C) Introduction of Mutant-Type yggB Gene

[0050]

The modification using the yggB gene also includes introduction of a mutant-type yggB gene into a coryneform bacterium. "Introduction of a mutant-type yggB

gene" as used herein includes: introduction of a mutation into a chromosomal yggB gene of a coryneform bacterium; introduction of a plasmid containing a mutant-type yggB gene into a coryneform bacterium; and substitution of a chromosomal yggB gene with a mutant-type yggB gene.

In the present invention, the "mutant-type yggB gene" means a yggB gene comprising a mutation in a coding region of the yggB gene, in which the mutation imparts a function of enhancing an L-glutamic acid-producing ability of a coryneform bacterium under an excess of biotin-containing condition to the yggB gene. In this regard, a mutant-type yggB gene may be a gene which enhances an L-glutamic acid-producing ability of a coryneform bacterium not only under an excess of biotin-containing condition when the gene is introduced into the coryneform bacterium, but also even under the L-glutamic acid-producing condition as mentioned above.

The phrase "enhance an L-glutamic acid-producing ability of a coryneform bacterium under an excess of biotin-containing condition" means that, when the coryneform bacterium of the present invention is cultured in a medium containing biotin at a concentration in which a non-modified strain of the coryneform bacterium cannot cause accumulation of L-glutamic acid, for example, in a medium containing 30 μg/L or more of biotin, the coryneform bacterium of the present invention causes accumulation of more L-glutamic acid in the medium than that of a non-modified strain, or the coryneform bacterium of the present invention produces L-glutamic acid at a higher rate than that of the non-modified strain.

[0051]

Hereinafter, a method for obtaining a mutant-type yggB gene of the present invention and a method for introducing a mutation into the yggB gene are stated. However, a method for obtaining a mutant-type yggB gene of the present invention and a method for introducing a mutation into the yggB gene are not limited to the following methods.

a. Method of Utilizing odhA Gene-Deleted Strain

[0051]

The inventors of the present invention have found that an odhA gene (a sucA gene)-deleted strain (hereinafter referred to as "odhA-disrupted strain") can be utilized for a method for obtaining a mutant-type yggB gene, in which the odhA gene encodes an E1o subunit of α-ketoglutarate dehydrogenase. The construction of the odhA-disrupted strain can be performed by a method using the sacB gene as mentioned above.

b. Method of Utilizing a Transposable Element

[0058]

A coryneform bacterium having a mutant-type yggB gene may be also screened by using a transposable element which functions in the coryneform bacterium. The transposable element includes an insertion sequence (IS element) and a transposon. The mutant-type yggB gene may be a gene which can be obtained by allowing an insertion sequence (IS element) and/or a transposon to be accidentally inserted into the wild-type yggB gene. Alternatively, the mutant-type yggB gene may be a gene which is constructed artificially by using an artificial transposon. A strain into which a transposable element is inserted can be selected by reference to an index, such as a decrease in sensitivity to L-glutamic acid analogues. As an L-glutamic acid analogue, 4-fluoroglutamic acid can be utilized. Further, a strain into which a transposable element is inserted can be selected by selecting antibiotics-resistant strains at random with the use of an artificial transposon containing an antibiotics-resistant gene, and confirming a length of a yggB gene of the antibiotics-resistant strains by PCR.

c. Method of Introducing a Mutation into the yggB Gene In Vitro at Random

[0064]

A mutation can be introduced into the yggB gene in vitro at random, among which the mutant-type yggB gene can be selected from a clone which can produce L-glutamic acid without the addition of surfactants. The parent strain useful for screening is preferably a strain which cannot cause accumulation of L-glutamic acid under the condition containing an excess of biotin; for example, C. glutamicum wild-type ATCC13869 strain, ATCC13032 strain, ATCC14067 strain, and C. melasecola wild-type ATCC17965 strain.

d. Method for Obtaining L-glutamic Acid Analogue-Resistant Strains

[0068]

Mutant-type yggB genes can be also obtained by culturing a coryneform bacterium having a wild-type yggB gene in a medium containing an L-glutamic acid analogue, and obtaining L-glutamic acid analogue-resistant strains which can grow in the same medium. A parent strain used for screening is preferably the wild-type strain of the coryneform bacterium as mentioned above, and may be any strains having the wild-type yggB gene. In addition, a coryneform bacterium having a plasmid containing the wild-type yggB gene can be also used.

Examples of L-glutamic acid analogues include γ-methyl L-glutamate, α-methyl glutamic acid, β-hydroxyglutamic acid, methioninesulfoximine, glutamic acid-γ monohydroxamate, 2-amino-4-phosphonobutyric acid, γ-monoethyl L-glutamate,

dimethyl L-glutamate, di-t-butyl L-glutamate, monofluoroglutamic acid, diethyl L glutamate, D-glutamic acid, and 4-fluoroglutamic acid, and among these, 4-fluoroglutamic acid analogue is preferably used.

L-glutamic acid analogue-resistant strains can be obtained as follows. A coryneform bacterium is inoculated on a minimum medium containing an L-glutamic acid analogue, and colonies which have appeared after 24 to 48 hours are collected. Concentration of the L-glutamic acid analogue contained in the medium is preferably a concentration at which a coryneform bacterium having a non-modified yggB gene cannot grow and a coryneform bacterium having a mutated yggB gene can grow. More specifically, when 4-fluoroglutamic acid is used as L-glutamic acid analogue, the concentration of 4-fluoroglutamic acid is 1.25 mM, preferably 2.5 mM, and more preferably 5 mM. In the present invention, the L-glutamic acid analogue-resistance means that when 4-fluoroglutamic acid is added to a minimum medium and the strain is cultured on the minimum medium for a whole day and night, in a concentration where a viable cell count (number of cells capable of forming colonies) of a parent strain can be suppressed to 1/100 or less, the strain still exhibits 1/10 or more growth.

The obtained L-glutamic acid analogue-resistant strains are inoculated into an L-glutamic acid producing liquid medium containing an excess of biotin and cultured with shaking, followed by measurement of the concentration of L-glutamic acid. In the wild-type strain, L-glutamic acid increases very little. In contrast, in some of the L-glutamic acid analogue-resistant strains, L-glutamic acid significantly accumulates. From such strain, a yggB gene is amplified by PCR, and the nucleotide sequence thereof is determined, and thereby, a novel mutant-type yggB gene can be obtained.

(D) Mutant-Type yggB Gene of the Present Invention

[0069]

Hereinafter, specific examples of the mutant-type yggB gene are stated. However, the mutant-type yggB gene of the present invention is not particularly limited so far as mutant-type yggB gene has a mutation to enhance an L-glutamic acid producing ability of a coryneform bacterium in the presence of an excess of biotin.

a. Mutation in C-Terminal Side

[0070]

This mutation is a mutation which is introduced into a portion of a nucleotide sequence of a region encoding a sequence of amino acid numbers 419 to 533 of SEQ ID NOS: 6, 68, 84, or 85, or a sequence of amino acid numbers 419 to 529 of SEQ ID NO: 62. For example, in the nucleotide sequence of SEQ ID NO: 5, this region corresponds to the region consisting of nucleotide numbers 2692 to 3035. This

mutation may be of any type so far as the mutation is introduced into at least a portion of the nucleotide sequence of the region. Among mutations, insertion of an insertion sequence (hereinafter referred to as IS) or a transposon is preferable. The mutation may be accompanied by an amino acid substitution (missense mutation), or a frame-shift mutation by insertion of the above IS may be introduced, or a nonsense mutation may be introduced, any of which is preferable.

(a) Mutation by Insertion of Transposable Element (2A-1-Type Mutation)

[0071]

Examples of a mutation in the C-terminal side include a mutation in which an IS is inserted next to G at position 2691 of SEQ ID NO: 5. The nucleotide sequence of the mutant-type yggB gene in which this mutation is introduced is shown in SEQ ID NO: 7, and the amino acid sequence of the mutant-type YggB protein encoded by this mutant-type yggB gene is shown in SEQ ID NO: 8. In SEQ ID NO: 8, the region positioned downstream from valine at position 419 of SEQ ID NO: 6 is substituted with a sequence which is derived from a short IS. In this regard, the IS inserted into SEQ ID NO: 7 has high homology to IS1207 (GenBank accession No. X96962) and IS719 (GenBank accession No. E12759).

The above mutant-type yggB gene is called a 2A-1-type mutation. The 2A-1-type mutation includes mutation in which the above region of the C-terminal side in SEQ ID NOS: 6, 62, 68, 84 and 85 is deleted or substituted.

Further, the scope of the present invention includes a mutation in which another IS is inserted into the above region, for example, a mutation in which an IS encoding transposase as mentioned above is inserted into the above region. The position into which transposase is introduced may be any position in the above region, and a location which each transposase can easily recognize and a location of a hot spot into which an IS can be easily inserted are preferable.

(b) Mutation in which Proline Residue is Substituted with Another Amino Acid (66-Type Mutation, 22-Type Mutation)

[0072]

In addition, examples of a mutation in the C-terminal side include a mutation in which proline which is present in a region in a sequence of amino acid numbers 419 to 533 of SEQ ID NOS: 6, 68, 84, or 85, or a region of amino acid numbers 419 to 529 of SEQ ID NO: 62 is substituted with another amino acid. The proline which may be substituted is present in the following positions of SEQ ID NO: 6:

Proline residue at position 424 (424th in SEQ ID NOS: 62, 68, 84, 85);

Proline residue at position 437 (437th in SEQ ID NOS: 62, 68, 84, 85);

Proline residue at position 453 (453rd in SEQ ID NOS: 62, 68, 84, 85);

Proline residue at position 457 (457th in SEQ ID NOS: 62, 68, 84, 85);

Proline residue at position 462 (462nd in SEQ ID NOS: 62, 68, 84, 85);

Proline residue at position 469 (469th in SEQ ID NOS: 62, 68, 84, 85);

Proline residue at position 484 (484th in SEQ ID NOS: 62, 68, 84, 85);

Proline residue at position 489 (489th in SEQ ID NOS: 62, 68, 84, 85);

Proline residue at position 497 (497th in SEQ ID NOS: 62, 68, 84, 85);

Proline residue at position 515 (515th in SEQ ID NOS: 62, 68, 84, 85);

Proline residue at position 529 (529th in SEQ ID NOS: 68, 84, 85; and 525th in SEQ ID NO: 62); and

Proline residue at position 533 (533rd in SEQ ID NOS: 68, 84, 85; 529th in SEQ ID NO 62).

It is considered that proline residues in the C-terminal side of the yggB gene play an important role in maintaining a three-dimensional structure of the YggB protein (Protein Eng. 2002 Jan; 15(1): 29-33, J Biol Chem. 1991 Dec 25; 266(36): 24287-94.).

Particularly, proline at positions 424 and 437 is preferably substituted with another amino acid.

In this regard, another amino acid may be any amino acid so far as such other amino acid is a natural amino acid other than proline. Proline is preferably substituted into a residue selected from Lys, Glu, Thr, Val, Leu, Ile, Ser, Asp, Asn, Gln, Arg, Cys, Met, Phe, Trp, Tyr, Gly, Ala, and His. Particularly, proline at position 424 is preferably substituted with a hydrophobic amino acid Ala, Gly, Val, Leu, Ile. Among these hydrophobic amino acids, proline at position 424 is more preferably substituted with a branched amino acid Leu, Val, Ile (66-Type Mutation). Examples of a mutation in which proline at position 424 is substituted with leucine includes a mutation in which "C" at position 1673 is substituted with "T" in SEQ ID NO: 67. This mutant-type yggB gene is shown in SEQ ID NO: 69, and the amino acid sequence of the mutant-type YggB protein encoded by this mutant-type yggB gene is shown in SEQ ID NO: 70.

Further, proline at position 437 is preferably substituted with an amino acid having a hydroxyl group in its side chain (Thr, Ser, Tyr). Among these, proline at position 437 is most preferably substituted with Ser (22-Type Mutation). Examples of a mutation in which proline at position 437 is substituted with serine include a mutation in which C at position 2745 is substituted with T in SEQ ID NO: 5. In addition, this mutation may be accompanied with a mutation in which C at position

3060 is substituted with T in SEQ ID NO: 5. This mutant-type yggB gene is shown in SEQ ID NO: 73, and the amino acid sequence of the mutant-type YggB protein encoded by this mutant-type yggB gene is shown in SEQ ID NO: 74.

b. Mutation in Transmembrane Region

[0073]

The YggB protein encoded by the yggB gene is presumed to have five transmembrane regions. In the amino acid sequence of the wild-type YggB protein shown in SEQ ID NOS: 6, 62, 68, 84, 85, the transmembrane regions correspond to amino acid numbers 1 to 23 (the first transmembrane region), amino acid numbers 25 to 47 (the second transmembrane region), amino acid numbers 62 to 84 (the third transmembrane region), amino acid numbers 86 to 108 (the fourth transmembrane region), and amino acid numbers 110 to 132 (the fifth transmembrane region). In SEQ ID NO: 5, DNA encoding these regions corresponds to nucleotide numbers 1437 to 1505, nucleotide numbers 1509 to 1577, nucleotide numbers 1620 to 1688, nucleotide numbers 1692 to 1760, and nucleotide numbers 1764 to 1832, respectively. The mutation of the Present Invention is preferably introduced into the DNA encoding these transmembrane regions. The mutation which is introduced into these regions is preferably mutation including substitution, deletion, addition, insertion, or inversion of one or several amino acids without involving a frame-shift mutation and a nonsense mutation. Accordingly, in case of substitution in an amino acid sequence, a missense mutation involving amino acid substitution is preferable. The term "several" in the above-mentioned "substitution, deletion, addition, insertion, or inversion of one or several amino acids" means 2 to 20, preferably 2 to 10, more preferably 2 to 5, and still more preferably 2 or 3. The mutation of insertion and deletion of one or several amino acids includes introduction of point mutations of one to several nucleotides and introduction of nucleotide sequence without involving frame-shift mutation, and is, for example, insertion or deletion of 3, 6, 9, 12, 15, 18, or 21 nucleotides, preferably deletion or insertion of 3, 6, or 9 nucleotides, and more preferably deletion or insertion of 3 nucleotides.

[0074]

For example, the following mutations are listed.

(a) Mutation in the First Transmembrane Region (A1-Type Mutation)

[0074]

This mutation is a mutation in which one to several amino acids are inserted between leucine residue at position 14 and tryptophan residue at position 15 in an amino acid sequence shown in any of SEQ ID NOS: 6, 62, 68, 84, 85.

Specific examples of the above mutation include a mutation in which three amino acids (for example, Cys-Ser-Leu) are inserted between leucine residue at position 14 and tryptophan residue at position 15. Examples of this mutation include a mutation in which TTCATTGTG is inserted next to G at position 1480 in SEQ ID NO: 5. The nucleotide sequence of the mutant-type yggB gene into which this mutation is introduced is shown in SEQ ID NO: 19, and the amino acid sequence of the mutant-type YggB protein encoded by this mutant-type yggB gene is shown in SEQ ID NO: 20.

(b) Mutation in the Fourth Transmembrane Region (19-Type Mutation)

[0075]

This mutation is a mutation in which alanine residue at position 100 is substituted with another amino acid in an amino acid sequence shown in any of SEQ ID NOS: 6, 62, 68, 84, and 85. "Another amino acid" may be any amino acid residue other than alanine, and means any of arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, methionine, leucine, lysine, phenylalanine, proline, serine, tryptophan, tyrosine, valine, and threonine. Among these, alanine residue at position 100 is preferably substituted with an amino acid having a hydroxyl group in its side chain (threonine, serine, tyrosine), and is particularly preferably substituted with threonine residue. Examples of such mutation include a mutation in which G at position 1734 is substituted with A in SEQ ID NO: 5.

The nucleotide sequence of the mutant-type yggB gene into which this mutation is introduced is shown in SEQ ID NO: 21, and the amino acid sequence of the mutant-type YggB protein encoded by this mutant-type yggB gene is shown in SEQ ID NO: 22.

(c) Mutation in the Fifth Transmembrane Region (L30-Type Mutation, 8-Type Mutation)

[0076]

This mutation is a mutation in which alanine residue at position 111 is substituted with another amino acid in an amino acid sequence shown in any of SEQ ID NOS: 6, 62, 68, 84, and 85. "Another amino acid" may be any amino acid residue other than alanine, and means any of arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, methionine, leucine, lysine, phenylalanine, proline, serine, tryptophan, tyrosine, valine, and threonine. Among these, alanine residue at position 111 is preferably substituted with a branched amino acid (valine, isoleucine, leucine), particularly preferably valine residue, or with an

amino acid having hydroxyl group in its side chain (threonine, serine, tyrosine), particularly preferably threonine residue. Examples of this mutation include a mutation in which C at position 1768 is substituted with T in SEQ ID NO: 5 (L30-type mutation) or a mutation in which G at position 837 is substituted with A in SEQ ID NO: 61 (8-type mutation). The nucleotide sequence of the mutant-type yggB gene into which L30-type mutation is introduced is shown in SEQ ID NO: 23, and the amino acid sequence of the mutant-type YggB protein encoded by this mutant-type yggB gene is shown in SEQ ID NO: 24. The nucleotide sequence of the mutant-type yggB gene into which 8-type mutation is introduced is shown in SEQ ID NO: 63, and the amino acid sequence of the mutant-type YggB protein encoded by this mutant-type yggB gene is shown in SEQ ID NO: 64.

[0078]

As a protein which is encoded by the mutant-type yggB gene, so far as an activity of the protein has a function of capable of enhancing L-glutamic acid-producing ability under the condition of the presence of an excess of biotin, the protein may be a functionally equivalent protein which has, for example, an amino acid sequence in which one or several amino acids in addition to amino acids of the above mutation point are substituted, deleted, inserted, or added in an amino acid sequence selected from SEQ ID NOS: 8, 20, 22, 24, 64, 70, and 74. The number of "several" as referred to herein means, for example, 2 to 20, preferably 2 to 10, more preferably 2 to 5. The above substitution is preferably a conservative substitution (neutral mutation). Examples of the conservative substitution include: substitution of ala with ser or thr; substitution of arg with gln, his, or lys; substitution of asn with glu, gln, lys, his, or asp; substitution of asp with asn, glu, or gln; substitution of cys with ser or ala; substitution of gln with asn, glu, lys, his, asp, or arg; substitution of glu with gly, asn, gln, lys, or asp; substitution of gly with pro; substitution of his with asn, lys, gln, arg, or tyr; substitution of ile with leu, met, val, or phe; substitution of leu with ile, met, val, or phe; substitution of lys with asn, glu, gln, his, or arg; substitution of met with ile, leu, val, or phe; substitution of phe with trp, tyr, met, ile, or leu; substitution of ser with thr or ala; substitution of thr with ser or ala; substitution of trp with phe or tyr; substitution of tyr with his, phe, or trp; and substitution of val with met, ile, or leu. As mentioned above, the amino acid which is present in Xaa in SEQ ID NO: 85 may be substituted. The yggB gene of the present invention includes a homologue which encodes a protein which has at least 80% homology, preferably at least 90% homology, more preferably at least 95% homology, and particularly preferably at least 97% homology, to the entire amino acid

sequence of SEQ ID NOS: 8, 20, 22, 24, 64, 70, and 74, and which encodes a protein having an activity which is equivalent to that of a protein encoded by the mutant-type yggB gene in the coryneform bacterium.

(E) Method of Introducing Mutant-Type yggB Gene into Coryneform Bacterium

[0080]

The mutant-type yggB gene having the above mutation can be obtained by, for example, the site-directed mutagenesis method. More specifically, cloning can be performed by using an overlap extension PCR method, etc. which amplifies the relevant locations using a PCR primer having a sequence containing a mutation in the corresponding region of the yggB gene (Urban, A., Neukirchen, S. and Jaeger, K. E., A rapid and efficient method for site-directed mutagenesis using one-step overlap extension PCR. Nucleic Acids Res, 25, pp 2227-8. (1997).).

The coryneform bacterium of the present invention can be obtained by introducing the obtained mutant-type yggB gene into a coryneform bacterium. Examples of a method of introduction include a method of substituting a wild-type yggB gene on a chromosome with the mutant-type yggB gene. The mutant-type yggB gene may be introduced into a strain in which a wild-type yggB gene on a chromosome is disrupted. In this regard, the mutant-type yggB gene may be introduced while a wild-type yggB gene is left on a chromosome as a single recombinant strain. One copy of the incorporated wild-type gene and one copy of the incorporated mutant-type gene may be present on a chromosome. The substitution of the yggB gene on the chromosome can be performed by using, for example, a temperature-sensitive plasmid containing a sacB gene encoding the above levan sucrase.

[0082]

A bacterium which is further modified so as to inactivate a gene which suppresses a function of the above mutant-type yggB gene may be used. In this regard, the phrase "suppress a function of yggB gene" means that glutamic acid production of a mutant-type yggB gene-introduced strain is suppressed by amplifying the gene. Examples of the gene which suppresses a function of a mutant-type yggB gene include a symA gene (supresser of yggB mutation). The symA gene is encoded in nucleotide numbers 2051306 to 2051845 of the genome sequence registered as Genbank Accession No. NC_003450 of Corynebacterium glutamicum ATCC13032, and is registered as NCgl 1867 (NP_601149. hypothetical prot...[gi:19553147]). The symA gene of C. glutamicum ATCC13869 is shown in nucleotide numbers 585 to

1121 of SEQ ID NO: 86. Gene inactivation can be achieved by disrupting the gene, or by modifying the gene so as to decrease an expression level. Disruption of the symA gene and introduction of the mutation so as to decrease an expression level can be performed using a method similar to the above methods for decreasing enzymatic activity.

[0083]

In the present invention, in addition to the modification using a yggB gene, a bacterium which is modified so as to decrease an activity of α-ketoglutarate dehydrogenase (hereinafter referred to as α-KGDH) may be used. The phrase "decrease an activity of α-ketoglutarate dehydrogenase" means that the α-KGDH activity is decreased as compared to that of a wild-type strain or a non-modified strain such as a parent strain. The α-KGDH activity can be measured according to the method of Shiio et al. (Isamu Shiio and Kyoko Ujigawa-Takeda, Agric. Biol. Chem., 44(8), 1897-1904, 1980). The α-KGDH activity may be decreased as compared to a non-modified strain such as a wild-type strain or a parent strain. The α-KGDH activity is preferably decreased to about 1/2 or less, more preferably about 1/4 or less, and still more preferably about 1/10 or less as compared to a parent strain. It may be the case that the bacterium has no α-KGDH activity at all.

(F) Method of Producing L-glutamic Acid of the Present Invention

[0085]

L-glutamic acid can be produced by obtaining a coryneform bacterium in the above manner, then culturing the obtained coryneform bacterium in a medium, to thereby cause production and accumulation of L-glutamic acid in the medium, and collecting the L-glutamic acid from the medium.

[0086]

As the medium for the culture, an ordinary medium can be used, in which the ordinary medium contains carbon sources, nitrogen sources, and inorganic salts, and, if necessary, organic micronutrients such as amino acids and vitamins. Both synthetic medium and natural medium can be used. Any kind of carbon sources and nitrogen sources may be used so far as such sources can be utilized by a strain to be cultured.

[0087]

As the carbon sources, saccharides such as glucose, glycerol, fructose, sucrose, maltose, mannose, galactose, starch hydrolysate, and molasses can be used. In addition, organic acids such as acetic acid and citric acid, and alcohols such as ethanol may also be used alone or in combination with other carbon sources. As the nitrogen

sources, ammonia, ammonium salts such as ammonium sulfate, ammonium carbonate, ammonium chloride, ammonium phosphate, and ammonium acetate, nitrates, and the like can be used. As the organic micronutrients, amino acids, vitamins, fatty acids, nucleic acids, peptone containing these substances, casamino acid, yeast extract, and soybean protein decomposition products can be used. When an auxotrophic mutant strain which requires an amino acid and the like for growth is used, such a required nutrient is preferably added. As the inorganic salts, phosphates, magnesium salts, calcium salts, iron salts, manganese salts, and the like can be used.

Further, surfactants and penicillin having appropriate concentrations may be added according to properties of the yggB gene-modified strain to be used. Furthermore, a concentration of biotin may be adjusted according to properties of the yggB gene-modified strain to be used.

[0088]

As to the culture, aerobic culture is performed by preferably controlling a fermentation temperature to 20 to 45°C and pH to 3 to 9. When the pH decreases during the culture, neutralization should be performed by, for example, adding calcium carbonate or neutralizing with alkali such as ammonia gas. Under these conditions, culturing is performed for preferably about 10 to about 120 hours, to thereby accumulate a considerable amount of L-glutamic acid in the culture broth.

[0089]

Further, the culture can also be performed while precipitating L-glutamic acid in the medium by using a liquid medium adjusted to conditions such that the produced L-glutamic acid precipitates. Examples of the conditions such that L-glutamic acid precipitates include a pH of 5.0 to 4.0, preferably a pH of 4.5 to 4.0, more preferably a pH of 4.3 to 4.0, particularly preferably a pH of 4.0 (Specification of European Patent Application Publication No. 1078989).

[0090]

Collection of L-glutamic acid from the culture broth after completion of the culture may be performed by conventional methods. For example, L-glutamic acid may be collected by removing bacterial cells from the culture broth, followed by concentrating and crystallizing L-glutamic acid, or by using ion exchange chromatography. When the culture is performed under conditions such that L-glutamic acid precipitates, the L-glutamic acid which is precipitated in the culture broth can be collected by centrifugation, filtration, etc. In this case, L-glutamic acid which is dissolved in the medium may also be isolated after crystallization of the dissolved L-glutamic acid.

G. Examples

(A) [Working Example 1]

[0092]

<Construction of Vector for Gene Disruption Carrying sacB Gene>

(A) Construction of pBS3

Construction of a vector for gene disruption carrying a sacB gene was performed with reference to the method disclosed in International Publication Nos. WO 2005/113745 and WO 2005/113744. An sacB gene (SEQ ID NO: 11) was obtained by PCR using a chromosomal DNA of Bacillus subtilis as a template and SEQ ID NOS: 13 and 14 as primers. The PCR was performed using LA taq (TaKaRa) as follows: one cycle of heat retention at 94°C for 5 minutes; and 25 cycles of denaturing at 94°C for 30 seconds, annealing at 49°C for 30 seconds, and elongation at 72°C for 2 minutes. The obtained PCR product was purified by a conventional method, and then digested with BglII and BamHI and blunt-ended. This fragment was digested with AvaII, and then was inserted into a blunt-ended site. The obtained DNA was used to transform competent cells of Escherichia coli JM109 (manufactured by Takara Bio Inc.). Then, the transformed bacterial cells were spread on LB medium containing 25 μg/ml Kanamycin (hereinafter, abbreviated as Km), and were incubated for one night. Thereafter, colonies that appeared were selected, single colonies were isolated to obtain transformants. Plasmids were extracted from the obtained transformants, and the plasmid having an insert of the objective PCR product was named pBS3. Figure 1 shows the procedure for constructing pBS3.

[0093]

(B) Construction of pBS4S

The SmaI site in the kanamycin-resistant gene sequence on pBS3 was modified by nucleotide substitution (a mutation which is not cut by SmaI) without causing amino acid substitution using cross-over PCR, to thereby obtain a plasmid in which kanamycin-resistant gene was disrupted. First, PCR was performed using pBS3 as a template and synthetic DNAs of SEQ ID NOS: 15 and 16 as primers, to thereby obtain an N-terminal amplified product of the kanamycin-resistant gene. On the other hand, in order to obtain a C-terminal side amplified product of the Km-resistant gene, PCR was performed using pBS3 as a template and synthetic DNAs of SEQ ID NOS: 17 and 18 as templates. PCR was performed using Pyrobest DNA Polymerase (manufactured by Takara Bio Inc.) as follows: one cycle of heat retention at 98°C for 5 minutes; and 25 cycles of denaturing at 98°C for 10 seconds, annealing at 57°C for

30 seconds, and elongation at 72°C for 1 minute, to obtain the objective PCR product. SEQ ID NOS: 16 and 17 are partially complementary to each other. Further, the SmaI site in these sequences was disrupted by nucleotide substitution without causing amino acid substitution. Then, in order to obtain a fragment of the mutant-type kanamycin resistant gene in which the SmaI site was disrupted, the above N-terminal and C-terminal gene products were mixed together in substantially equimolar amounts. Thereafter, PCR was performed using the gene products as a template and synthetic DNAs of SEQ ID NOS: 15 and 18 as primers to obtain a Km-resistant gene amplified product in which a mutation was introduced. The PCR was performed using Pyrobest DNA Polymerase (manufactured by Takara Bio Inc.) as follows: one cycle of heat retention at 98°C for 5 minutes; and 25 cycles of denaturing at 98°C for 10 seconds, annealing at 57°C for 30 seconds, and elongation at 72°C for 1.5 minutes, to thereby obtain the objective PCR product.

[0094]

The PCR product was purified by a conventional method, and then digested with BanII, and thereafter inserted into the above BanII site of pBS3. This DNA was used to transform competent cells of Escherichia coli JM109 (manufactured by Takara Bio Inc.). That is, the transformed bacterial cells were spread on LB medium containing 25 μg/ml of kanamycin, and incubated for one night. Thereafter, colonies that appeared were selected, and single colonies were isolated to obtain transformants. Plasmids were extracted from the obtained transformants, and the plasmid having an insert of the objective PCR product was named pBS4S. Figure 2 shows the procedure for constructing pBS4S.

(B) [Working Example 2]

[0095]

<Construction of odhA Mutant Strain Derived from C. glutamicum ATCC13869 Strain> The sequence of the odhA gene encoding the α-ketoglutarate dehydrogenase of coryneform bacterium has already been identified (Microbiology 142, 3347-3354, (1996), GenBank accession No. D84102). Based on the published sequence of the odhA gene, the primers stated in SEQ ID NOS: 1 and 2 were designed, and PCR was performed using the primers and the chromosomal DNA of ATCC13869 strain as a template to amplify only the internal sequence of the odhA gene. The amplified PCR fragment was completely digested with BamHI and inserted to the BamHI site of pBS4S constructed in Working Example 1, whereby the plasmid pBS4SΔsucAint was constructed (Figure 3 shows the procedure for constructing).

[0096]

pBS4SΔsucAint was introduced into C. glutamicum ATCC13869 strain by the electric pulse method (Unexamined Patent Application Publication No. 1990-207791) and was spread over CM-Dex agar medium (5g/l glucose, 10 g/l polypeptone, 10 g/l yeast extract, 1 g/l KH_2PO_4, 0.4 g/l $MgSO_4$-$7H_2O$, 0.01 g/l $FeSO_4$-$7H_2O$, 0.01 g/l $MnSO_4$-4-$5H_2O$, 3 g/l urea, 1.2 g/l soybean protein hydrolysate solution, and 20 g/l agar, adjusted to pH 7.5 with NaOH: autoclaved at 120°C for 20 minutes) containing 25 μg/ml kanamycin. After culturing at 31.5°C, PCR was performed using each of the chromosomes extracted from strains which appeared to confirm that these strains were single cross-over recombinants in which pBS4SΔsucAint was incorporated by homologous recombination into the chromosome. Whether the strain is single cross-over recombinant can be easily confirmed by performing PCR using the chromosome of candidate strain as a template, and a sequence (SEQ ID NO: 3) specific to pBS4S plasmid and a sequence (SEQ ID NO: 4) complementary to a chromosomal sequence as primers (the sequence of pBS4S is absent on chromosome of a non-recombinant strain, and thus, no fragment is amplified from the non-recombinant strain, thereby enabling determination on whether the strain is single cross-over recombinant).

[0097]

The single cross-over recombinant thus obtained was named 2A-1 strain. The wild-type ATCC13869 strain and the 2A-1 strain were inoculated in 20 ml of a flask medium (30 g/l glucose, 15 g/l ammonium sulfate, 1 g/l KH_2PO_4, 0.4 g/l $MgSO_4$-$7H_2O$, 0.01 g/l $FeSO_4$-$7H_2O$, 0.01 g/l $MnSO_4$-4-$5H_2O$, 200 μg/l VB1, 300 μg/l Biotin, and 0.48 g/l soybean hydrolysates (T-N), adjusted to pH 8.0 with KOH: autoclaved at 115°C for 10 minutes), followed by addition of 1 g of calcium carbonate which had been dry heat-sterilized beforehand, and each of the strains was cultured with shaking at 31.5°C. After the sugar was completely consumed, the concentration of L-glutamic acid in the medium was determined. The results are shown in Table 1 (OD620 is a turbidity diluted to 101 times, and indicates an amount of the bacterial cells; Glu (g/L) indicates an amount of accumulated L-glutamic acid). It was confirmed that the 2A-1 strain has an L-glutamic acid-producing ability under the condition that the parent ATCC13869 strain did not produce L-glutamic acid at all.

[0098]

[Table 1]

<Table 1 Amount of L-glutamic Acid Produced by 2A-1 Strain>

	OD620(x101)	Glu(g/L)
ATCC13869	0.658	0.2
2A-1	0.315	17.7
Blank	0.002	0.4

(C) [Working Example 3]

[0099]

<Construction of odhA Revertant Strain Derived from 2A-1 Strain>

In the obtained 2A-1 strain, the odhA gene on the chromosome was disrupted by pBS4SΔT¥sucAint. By curing the plasmid on the chromosome from this strain, the odhA gene was reverted to the wild-type strain. On the other hand, the odhA-deleted strain grows very slowly in a medium containing no sugar. However, it is presumed that the odhA-revertant strain in which the odhA reverted to the wild-type grows well in a medium containing no sugar such as CM2B medium (10 g/l polypeptone, 10 g/l yeast extract, 5 g/l NaCl, 10 μg/L Biotin, 20 g/l agar, adjusted to pH 7.0 with KOH). To obtain such a revertant strain, the 2A-1 strain was spread over a CM2B plate to select growth-improved strains. The growth-improved 2A-1R strain which thus appeared was purified on the CM2B plate, and the kanamycin-sensitivity of the 2A-1R strain was examined. As a result, it was found that all of the selected strains were kanamycin-sensitive and sucrose-resistant.

[0100]

Since the pBS4SΔsucAint contains a kanamycin-resistant gene and the sacB gene encoding levan sucrase, strains harboring pBS4SΔsucAint exhibit kanamycin-resistance and sucrose-sensitivity, while strains from which pBS4SΔsucAint was removed exhibit kanamycin-sensitivity and sucrose-resistance. From these results, it was considered that the odhA had reverted to the wild-type one in the 2A-1R strain. Further, the sequence of the odhA gene was confirmed. As a result, it was confirmed that the odhA gene had no mutations. Therefore, it was concluded that the strain had reverted to the wild-type odhA gene. The L-glutamic acid-producing ability of 2A-1R strain was confirmed similarly to the method in Working Example 2. The results are shown in Table 2 (OD620 indicates an amount of the bacterial cells diluted to 101 times; Glu (g/L) indicates an amount of accumulated L-glutamic acid). Although the accumulation of L-glutamic acid by the 2A-1R strain was inferior to that of the 2A-1

strain, the 2A-1R strain accumulated a much higher amount of L-glutamic acid than the wild-type ATCC13869 strain (Table 2). In addition, when the shaking culture was continued after the sugar was completely consumed, decomposition of L-glutamic acid was observed in the 2A-1R strain, which has proved that the odhA gene had reverted to the wild-type in this strain (Figure 4).
[0101]
[Table 2]

<Table 2 Amount of L-glutamic Acid Produced by odhA Revertant Strain>

	OD620(x101)	Glu(g/L)
ATCC13869	0.696	0.5
2A-1	0.332	17.1
2A-1R	0.327	14.3
Blank	0.002	0.6

(D) [Working Example 4]
[0102]
<Isolation of Gene which is Involved in L-glutamic Acid Production by 2A-1R Strain>

On the CM2B plate medium, 2A-1R strain could form colonies at substantially the same rate as that of the wild-type strain ATCC13869. However, on the minimum medium (20 g/l glucose, 2.64 g/L ammonium sulfate, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.25 g/L $MgSO_4$-$7H_2O$, 0.01 g/L $FeSO_4$-$7H_2O$, 0.01 g/L $MnSO_4$-$7H_2O$, 0.01 g/L $CaCl_2$, 0.02 mg/L $CuSO_4$, 40 g/L MOPS, 30 mg/L protocatechuic acid, 200 μg/L VB_1-HCl, 300 μg/L Biotin, 20 g/L agar, adjusted to pH 6.7 with NaOH) plate, 2A-1R strain was found to show a considerably decreased colony-forming rate as compared to the wild-type strain ATCC13869. Accordingly, a gene which can recover the growth of the 2A-1R strain in the minimum medium was searched.
[0103]
The chromosomal DNA of ATCC13869 strain was partially digested with Sau3AI and ligated to the shuttle vector pVK9 which had been digested with BamHI. The obtained plasmid was precipitated with ethanol and used to transform electrocompetent cell of E. coli DH5α (Takara Bio Inc.) by the electric pulse method. pVK9 is a shuttle vector obtained by blunt-ending the AvaII site of pHSG299 (Takara Bio Inc.) and inserting therein a fragment comprising a sequence automatically replicable in coryneform bacteria excised with BamHI and KpnI from pHK4

(Unexamined Patent Application Publication No. 1993-007491). The transformed bacterial cells were spread over an LB plate medium (10 g/l polypeptone, 5 g/l yeast extract, 5 g/l NaCl, 20 g/l agar, adjusted to pH 7.0 with NaOH) containing 25 μg/ml kanamycin, and cultured at 37°C for one night. On the next day, all of the colonies which had appeared were collected from the plate with a platinum loop and plasmids were extracted to construct a library of ATCC13869. This library was transformed to the 2A-1R strain obtained in Working Example 3 by the electric pulse method, and the transformed cells were spread over a minimum medium plate containing 25 μg/ml kanamycin. The strains which showed an increased colony-forming rate were screened. By extracting a plasmid from the selected strains showing the increased colony-forming rate, it was found that the fragment having a sequence shown in SEQ ID NO: 5 was inserted into the BamHI site of pVK9. The obtained plasmid was named pL5k.

[0104]

Comparison of the nucleotide sequence inserted in the pL5k with the already published genome sequence of Corynebacterium glutamicum ATCC13032 (Acc. No. NC_003450) showed that pL5k contained only one ORF having the amino acid sequence shown in SEQ ID NO: 6 as a full-length ORF. Whether the ORF is a membrane protein can be predicted by the program "SOSUI" available on the internet (linked to http:// (remainder omitted) as of October 7, 2004). Results of analysis of the ORF by using "SOSUI" suggested that five transmembrane regions are present. In the amino acid sequence of SEQ ID NO: 6, the transmembrane regions correspond to the regions of amino acid numbers 1 to 23, amino acid numbers 25 to 47, amino acid numbers 62 to 84, amino acid numbers 86 to 108, and amino acid numbers 110 to 132. DNA sequences encoding these regions correspond the nucleotide numbers 1437 to 1505, nucleotide numbers 1509 to 1577, nucleotide numbers 1620 to 1688, nucleotide numbers 1692 to 1760, and nucleotide numbers 1764 to 1832 of SEQ ID NO: 5. Each of the amino acid sequences of these regions is shown in SEQ ID NOS: 25 to 29 and Table 3.

[0105]

[Table 3]

<Table 3 Predicted Transmembrane Regions of YggB Protein>

No.	N terminal	transmembrane region	C terminal	type	length	Seq ID
1	1	MILGVPIQYLLYSLWNWIVDTGF	23	SECONDARY	23	25
2	25	VAIILVLAFLIPRIGRLAMRIIK	47	PRIMARY	23	26
3	62	QLAFAGVGVYIAQIVAFFMLAVS	84	PRIMARY	23	27
4	86	MQAFGFSLAGAAIPATIASAAIG	108	SECONDARY	23	28
5	110	GAQSIVADFLAGFFILTEKQFGV	132	SECONDARY	23	29

(E) [Working Example 5]

[0107]

<Identification of Mutation Point of yggB Gene of 2A-1R Strain>

pL5K complemented the growth of the 2A-1R strain in the minimum medium, which suggested the possibility that the yggB gene of the 2A-1R strain has any mutations. Accordingly, a nucleotide sequence of the yggB gene of the 2A-1R strain was determined. As a result, it was found that in the 2A-1R strain, an IS was inserted into the C-terminal side region of the yggB gene (Figure 5). The nucleotide sequence of the mutant-type yggB gene of the 2A-1R strain is shown in SEQ ID NO: 7 and the corresponding amino acid sequence is shown in SEQ ID NO: 8. This suggested the possibility that an L-glutamic acid-producing ability of the 2A-1R strain was maintained by the mutation in the yggB gene. In this regard, this mutation was present not only in the 2A-1R strain, but also in the 2A-1 strain. This mutation is presumed to have occurred as a suppressor mutation to stably excrete L-glutamic acid out of the cell when the mutation was introduced into the odhA gene. The mutation in which an IS was inserted was named 2A-1-type mutation.

(F) [Working Example 6]

[0108]

<Construction of 2A-1-Type yggB Mutant Strain and Evaluation of L-glutamic Acid-Producing Ability>

(6-1) Introduction of 2A-1-Type Mutation into Wild-Type Strain and Evaluation Thereof (Single Cross-Over Recombinant Strain)

PCR was performed using the chromosomal DNA of the 2A-1 strain as a template and synthetic DNAs shown in SEQ ID NOS: 9 and 10 as primers to amplify

a fragment of yggB gene having the 2A-1-type mutation. The amplified product was digested with SacI and inserted into the SacI site of pBS3 of Working Example 1. A plasmid in which a yggB fragment having 2A-1-type mutation was cloned was named pBS3yggB2A. The pBS3yggB2A was introduced into C. glutamicum ATCC13869 by the electric pulse method and was spread over a CM-Dex agar medium containing 25 μg/ml kanamycin. The strains that appeared after culturing at 31.5°C were evaluated by PCR to confirm that they are single cross-over recombinants in which pBS3yggB2A was incorporated into the chromosome by homologous recombination. The obtained single cross-over recombinant strain was named 13869-2A. In this strain, the wild-type yggB gene and the mutant-type yggB gene were both expressed.

[0109]

The L-glutamic acid-producing ability of the obtained yggB mutation-introduced strain 13869-2A was confirmed by the method stated in Working Example 2. The results are shown in Table 4 (OD620 indicates an amount of the bacterial cells diluted to 101 times; Glu (g/L) indicates an amount of accumulated L-glutamic acid). It could be confirmed that 13869-2A strain clearly had an L-glutamic acid-producing ability even under the condition that ATCC13869 strain did not produce L-glutamic acid. From these results, it has been shown that the mutation in the yggB gene can induce L-glutamic acid production.

[0110]

[Table 4]

<Table 4 Amount of L-glutamic Acid Produced by Mutant-Type yggB Gene-Introduced Strain>

	OD620(x101)	Glu(g/L)
ATCC13869	0.625	0.3
2A-1R	0.334	15.5
13869-2A	0.582	3.6
Blank	0.002	0.6

[0111]

(6-2) Introduction of 2A-1-Type Mutation into Wild-Type Strain, and Evaluation Thereof (Double Cross-Over Recombinant Strain)

In order to construct a strain having only the mutant-type gene, the 13869-2A strain was cultured on a CM-Dex liquid medium for one night and the obtained suspension was spread over an S10 agar medium (100 g/l sucrose, 10 g/l polypeptone, 10 g/l yeast extract, 1 g/l KH_2PO_4, 0.4 g/l $MgSO_4$-$7H_2O$, 0.01 g/l $FeSO_4$-$7H_2O$, 0.01

g/l $MnSO_4$-4-5H_2O, 3 g/l urea, 1.2 g/l soybean protein hydrolysate solution, 20 g/l agar, adjusted to pH 7.5 with NaOH: autoclaved at 120°C for 20 minutes) and cultured at 31.5°C. Among the colonies that appeared, the strain exhibiting sensitivity to kanamycin was purified on an s2B agar medium. Chromosomal DNAs were prepared from these strains. Then, PCR was performed using synthetic DNAs shown in SEQ ID NOS: 9 and 10 as primers to confirm the mutation. The strain in which an IS-like sequence was inserted into the yggB gene was named 13869-2A-7.

[0112]

The L-glutamic acid-producing ability of the obtained 13869-2A-7 strain was confirmed by the method stated in Working Example 2. The results are shown in Table 5 (OD620 indicates an amount of the bacterial cells diluted to 101 times; Glu (g/L) indicates an amount of accumulated L-glutamic acid). It was shown that the 13869-2A-7 strain has the L-glutamic acid-producing ability which was equivalent to or higher than that of the 2A-1R strain, from which it could be confirmed that L-glutamic acid production in the presence of an excess of biotin was caused by the mutation in the yggB gene.

[0113]

[Table 5]

<Table 5 Amount of L-glutamic Acid Produced by Mutant-Type yggB Chromosome-Introducing Strain>

	OD620(x101)	Glu(g/L)
ATCC13869	0.648	0.4
2A-1R	0.420	13.8
13869-2A-7	0.414	16.1
Blank	0.002	0.7

(G) [Working Example 7]

[0114]

<Construction of A1-Type YggB Mutant Strain, and Evaluation Thereof>

The above L-glutamic acid-producing odhA mutant strains were analyzed. As a result, five types of mutations were identified on the yggB gene in addition to the above 2A-1 mutation. Hereinafter, these mutations are called A1-type mutation, 19-type mutation, L30-type mutation, 8-type mutation, and 66-type mutation, respectively. Each of the former three mutations; i.e., the A1-type mutation, 19-type mutation, and L30-type mutation, was introduced into the chromosome of ATCC13869 strain, and effects of these mutations were confirmed. In addition, the

8-type mutation was introduced into the chromosome of the ATCC14067 strain, and the effect of the mutation was confirmed. Further, the 66-type mutation was introduced into the chromosome of the C. melassecola ATCC17965 strain, and the effect of the mutation was confirmed.

[0115]

The A1-type mutation is a mutation in which TTCATTGTG is inserted next to G at position 1480 in SEQ ID NO: 5, and a mutation in which cysteine-serine-leucine residues are inserted between leucine residue at position 14 and tryptophan residue at position 15 in SEQ ID NO: 6. The nucleotide sequence of the mutant-type yggB gene into which this mutation is introduced is shown in SEQ ID NO: 19, and the amino acid sequence of the mutant-type YggB protein encoded by this mutant-type yggB gene is shown in SEQ ID NO: 20.

[0116]

The A1-type mutant gene can be obtained as follows. PCR is performed by using the synthetic DNAs shown in SEQ ID NOS: 30 and 31 as primers and the chromosomal DNA of ATCC13869 strain as a template to prepare an N-terminal side fragment. Similarly, PCR is performed by using the synthetic DNAs shown in SEQ ID NOS: 32 and 33 as primers to prepare a C-terminal side fragment. Subsequently, PCR can be performed by using an equimolar mixture of the N-terminal side fragment and the C-terminal side fragment as a template and the synthetic DNAs shown in SEQ ID NOS: 9 and 34 as primers to obtain a partial fragment of the A1-type mutant yggB gene. The obtained yggB gene fragment can be digested with SacI and inserted into the SacI site of pBS4S to construct a plasmid for introducing this type of mutation. pBS4 yggBA1 thus obtained is introduced into the chromosome of ATCC13869 strain similarly to the method in Working Example 6, and then removed. The sequence of the yggB gene of the obtained kanamycin sensitive strain can be determined and the strain having the A1-type substituted yggB gene is selected, to thereby construct a strain having the A1-type mutation. The A1-type mutant strain having these mutations was named ATCC13869-A1 strain.

[0117]

ATCC13869-A1 strain and the parent ATCC13869 strain were cultured similarly to the method as stated in Working Example 2. After completion of the culture, an amount of L-glutamic acid contained in the culture broth was measured by the known method.

The A1-type mutation-introduced ATCC13869-A1 strain on the chromosome greatly enhanced L-glutamic acid accumulation as compared to the parent

ATCC13869 strain.

[0118]

[Table 6]

<Table 6 Amount of L-glutamic Acid Produced by Mutant-Type yggB Gene (A1) Chromosome-Introduced Strain>

	OD620(x101)	Glu(g/L)
ATCC13869	0.650	0.5
ATCC13869-A1	0.548	8.6

(H) [Working Example 8]

[0119]

<Construction of 19-Type YggB Mutant Strain, and Evaluation Thereof>

The 19-type mutation is a mutation in which G at position 1734 is substituted with A in SEQ ID NO: 5, and a mutation in which alanine at position 100 is substituted with threonine in the amino acid sequence of SEQ ID NO: 6. The nucleotide sequence of the mutant-type YggB gene into which this mutation is introduced is shown in SEQ ID NO: 21, and the amino acid sequence of the mutant-type YggB protein encoded by this gene is shown in SEQ ID NO: 22. Similarly to the method in Working Example 7, the 19-type mutation-introduced yggB mutant strain is constructed. Specifically, PCR is performed by using the synthetic DNAs shown in SEQ ID NOS: 30 and 35 as a primer and the chromosomal DNA of ATCC13869 strain as a template to prepare an N-terminal side fragment. Similarly, PCR is performed by using the synthetic DNAs shown in SEQ ID NOS: 33 and 36 as primers to prepare a C-terminal side fragment. Subsequently, PCR can be performed by using an equimolar mixture of the N-terminal side fragment and the C-terminal side fragment as a template and the synthetic DNAs shown in SEQ ID NOS: 9 and 34 as primers to obtain a partial fragment of the 19-type YggB gene. The obtained mutant-type yggB gene fragment can be digested with SacI and inserted into the SacI site of pBS4S to construct a plasmid for introducing this type of mutation. pBS4 YggB19 thus obtained is introduced into the chromosome of ATCC13869 strain similarly to the method as stated in Working Example 6, and then removed. The sequence of the yggB gene of the obtained kanamycin sensitive strain can be determined and the strain which has been substituted with 19-type is selected, to thereby construct a strain having 19-type mutation. The 19-type mutant strain having these mutations was named ATCC13869-19 strain.

[0120]

ATCC13869-19 strain and the parent ATCC13869 strain were cultured similarly to the method in Working Example 2. After completion of the culture, the amount of L-glutamic acid contained in the culture broth was measured by the known method. The ATCC13869-19 strain in which the 19-type mutation was introduced on the chromosome greatly enhanced L-glutamic acid accumulation as compared to the parent ATCC13869 strain.

[0121]

[Table 7]

<Table 7 Amount of L-glutamic Acid Produced by Mutant-Type yggB Gene (19 Mutation) Chromosome-Introduced Strain>

	OD620(x101)	Glu(g/L)
ATCC13869	0.650	0.5
ATCC13869-19	0.614	0.7

(I) [Working Example 9]

[0122]

<Construction of L30-Type YggB Mutant Strain >

The L30-type mutation is a mutation in which C at position 1768 is substituted with T in SEQ ID NO: 5, and a mutation in which alanine at position 111 is substituted with valine in SEQ ID NO: 6. The nucleotide sequence of the mutant-type YggB gene in which this mutation is introduced is shown in SEQ ID NO: 23, and the amino acid sequence of the mutant-type YggB protein encoded by this gene is shown in SEQ ID NO: 24.

Similar to the method in Working Example 7, the L30-type mutation-introduced yggB mutant strain is constructed. Specifically, PCR is performed by using the synthetic DNAs shown in SEQ ID NOS: 30 and 37 as primers and the chromosomal DNA of ATCC13869 strain as a template to prepare an N-terminal side fragment. Similarly, PCR is performed by using the synthetic DNAs shown in SEQ ID NOS: 34 and 38 as primers to prepare a C-terminal side fragment. Subsequently, PCR was performed by using a mixture of an equal amount of the N-terminal side fragment and the C-terminal side fragment as a template and the synthetic DNAs shown in SEQ ID NOS: 9 and 34 as primers to obtain a partial fragment of the L30-type YggB gene. The obtained mutant-type YggB fragment is digested with SacI and inserted into the SacI site of pBS4S to construct a plasmid for introducing this

type of mutation. pBS4 YggB-L thus obtained was introduced into the chromosome of ATCC13869 strain similarly to the method as stated in Working Example 6, and then removed. The sequence of the yggB gene of the obtained kanamycin sensitive strain was determined and the strain which had been substituted with L30-type was selected, to thereby construct a strain having L30-type mutation. The L30-type mutant strain having these mutations was named ATCC13869-L strain.

[0123]

ATCC13869 strain and ATCC13869-L strain were cultured similarly to the method in Working Example 2. After completion of the culture, the amount of L-glutamic acid contained in the culture broth was measured by the known method. The results are shown in Table 8 (OD620 indicates an amount of the bacterial cells diluted to 101 times; Glu (g/L) indicates an amount of accumulated L-glutamic acid). ATCC13869-L30 into which L30 mutation was introduced greatly enhanced L-glutamic acid accumulation as compared to the parent ATCC13869 strain.

[0124]

[Table 8]

<Table 8 Amount of L-glutamic Acid Produced by Mutant-Type yggB Gene Chromosome-Introduced Strain (L-30 Mutation)>

	OD620(x101)	Glu(g/L)
ATCC13869	0.650	0.5
ATCC13869-L30	0.389	15.9

(J) [Working Example 10]

[0125]

<Culture of the Mutant-Type yggB Gene-Introduced Strains Under L-glutamic Acid-Producing Conditions, and Evaluation Thereof>

L-glutamic acid production of Coryneform bacterium can be induced by adding fatty acid-based surfactants such as Tween 40 or by limiting biotin. Accordingly, the ATCC13869 strain and the ATCC13869-19 strain were also cultured under Tween40-added condition and biotin-limited condition, respectively. For seed culture, each of the strains was inoculated into 20 ml of flask medium (80 g/l glucose, 30 g/l ammonium sulfate, 1 g/l KH_2PO_4, 0.4 g/l $MgSO_4$-$7H_2O$, 0.01 g/l $FeSO_4$-$7H_2O$, 0.01 g/l $MnSO_4$-4-$5H_2O$, 200 μg/l VB1, 60 μg/l Biotin, 0.48 g/l soybean hydrolysates (T-N), adjusted to pH 8.0 with KOH: autoclaved at 115°C for 10 minutes), and then 1 g of calcium carbonate which had been dry heat-sterilized beforehand was added thereto,

followed by a shaking culture at 31.5°C. The culture broth obtained after complete consumption of sugars was used as a seed culture broth. For Tween40-added culture, 2 ml of the seed culture broth was inoculated into 20 ml of the flask medium (80 g/l glucose, 30 g/l ammonium sulfate, 1 g/l KH_2PO_4, 0.4 g/l $MgSO_4$-$7H_2O$, 0.01 g/l $FeSO_4$-$7H_2O$, 0.01 g/l $MnSO_4$-4-$5H_2O$, 200 μg/l VB1, 60 μg/l Biotin, 0.48 g/l soybean hydrolysates (T-N), adjusted to pH 8.0 with KOH: autoclaved at 115°C for 10 minutes), and then 1 g of calcium carbonate which had been dry heat-sterilized beforehand was added thereto, followed by a shaking culture at 31.5°C. At the time when OD620 (× 101) = 0.2 was reached after the start of culture, Tween 40 was added so as to attain a final concentration of 5 g/L, and then the culture was continued. For biotin-limited culture, 1 ml of the seed culture broth was inoculated into 20 ml of the flask medium (80 g/l glucose, 30 g/l ammonium sulfate, 1 g/l KH_2PO_4, 0.4 g/l $MgSO_4$-$7H_2O$, 0.01 g/l $FeSO_4$-$7H_2O$, 0.01 g/l $MnSO_4$-4-$5H_2O$, 200 μg/l VB1, 0.48 g/l soybean hydrolysates (T-N), adjusted to pH 8.0 with KOH: autoclaved at 115°C for 10 minutes), and then 1 g of calcium carbonate which had been dry heat-sterilized beforehand was added thereto, followed by a shaking culture at 31.5°C. Under these culture conditions, a final concentration of biotin became about 2.9 μg/L.

After 40 hours from the start of culture, the concentrations of L-glutamic acid in the medium were measured for both of the Tween40-added culture and the biotin-limited culture. The results are shown in Table 9 (OD620 indicates an amount of the bacterial cells diluted to 101 times; Glu (g/L) indicates an amount of accumulated L-glutamic acid). It has been found that ATCC13869-19 strain increased L-glutamic acid accumulation even under L-glutamic acid-producing condition.

[0126]

[Table 9]

<Table 9 Amount of L-glutamic Acid Produced by Mutant-Type yggB Gene-Introduced Strain under the Glutamic Acid Production-Inducing Condition>

	OD620(x101)	Glu(g/L)
Tween40-added		
ATCC13869	0.538	25.6
ATCC13869-19	0.395	28.6
biotin-limited		
ATCC13869	0.462	36.0
ATCC13869-19	0.431	40.0

[0127]

The ATCC13869 strain, the ATCC13869-L30 strain, the ATCC13869-A1

strain, the ATCC13869-19 strain, and the strain in which yggB was amplified by plasmid were also cultured with Tween 40 added. Each of these strains were cultured on a CM-Dex plate medium overnight, and cells collected from 1/6 area of the plate were inoculated in 20 ml of a flask medium (80 g/l glucose, 30 g/l ammonium sulfate, 1 g/l KH_2PO_4, 0.4 g/l $MgSO_4$-$7H_2O$, 0.01 g/l $FeSO_4$-$7H_2O$, 0.01 g/l $MnSO_4$-4-$5H_2O$, 200 μg/l VB1, 60 μg/l Biotin, and 0.48 g/l soybean hydrolysates (T-N), adjusted to pH 8.0 with KOH: autoclaved at 115°C for 10 minutes), followed by addition of 1 g of calcium carbonate which had been dry heat-sterilized beforehand, and each of the strains was cultured with shaking at 31.5°C. After 5 hours from the start of culture, Tween 40 was added so as to attain a final concentration of 1 g/L. After 24 hours from the start of culture, an amount of bacterial cells and a yield of L-glutamic acid were each analyzed. As a result, as shown in Table 10, it is shown that all of the ATCC13869-19 strain, the ATCC13869-L30 strain, the ATCC13869-A1 strain, and the wild-type yggB gene-amplified strain (ATCC13869/pL5k-1) enhanced the yield of L-glutamic acid under L-glutamic acid-producing condition.

[0128]

[Table 10]

<Table 10 Amount of L-glutamic Acid Produced by Each Strain in Tween40-Added Culture>

	OD620 (x101)	Glu(g/L)
ATCC13869	0.887	12.8
ATCC13869/pVK9	0.748	12.4
ATCC13869/pL5k-1	0.711	19.2
ATCC13869-19	0.786	21.0
ATCC13869-A1	0.629	34.9
ATCC13869-L30	0.649	28.3
Blank	0.001	0.5

(K) [Working Example 11]

[0129]

<Construction of 8-Type YggB Mutant Strain>

The 8-type mutation is a mutation in which G at position 837 is substituted with an A in SEQ ID NO: 61, and a mutation in which alanine at position 111 is substituted with threonine in SEQ ID NO: 62. The nucleotide sequence of the mutant-type YggB gene into which this mutation is introduced is shown in SEQ ID NO: 63, and the amino acid sequence of the mutant-type YggB protein encoded by this gene is shown in SEQ ID NO: 64.

Similar to the method in Working Example 7, the 8-type mutation-introduced yggB mutant strain is constructed. Specifically, PCR is performed by using the synthetic DNAs shown in SEQ ID NOS: 30 and 65 as a primer and the chromosomal DNA of Brevibacterium flavumATCC14067 strain as a template to prepare an N-terminal side fragment. Similarly, PCR is performed by using the synthetic DNAs shown in SEQ ID NOS: 34 and 66 as primers to prepare a C-terminal side fragment. Subsequently, PCR is performed by using a mixture of an equal amount of the N-terminal side fragment and the C-terminal side fragment as a template and the synthetic DNAs shown in SEQ ID NOS: 9 and 34 as primers to obtain a partial fragment of the 8-type YggB gene. The obtained mutant-type YggB fragment was digested with SacI and inserted into the SacI site of pBS4S to construct a plasmid for introducing this type of mutation. pBS4 YggB8 thus obtained is introduced into the chromosome of ATCC14067 strain similarly to the method as stated in Working Example 6, and then removed. The sequence of the yggB gene of the obtained kanamycin sensitive strain is determined and the strain which has been substituted with 8-type is selected, to thereby construct a strain having 8-type mutation. The 8-type mutant strain having these mutations is named ATCC14067yggB8 strain.

(L) [Working Example 12]

[0130]

<Construction of 66-Type YggB Mutant Strain>

The 66-type mutation is a mutation in which C at position 1673 is substituted with T in SEQ ID NO: 67, and a mutation in which proline at position 424 is substituted with leucine in SEQ ID NO: 68. The nucleotide sequence of the mutant-type YggB gene in which this mutation is introduced is shown in SEQ ID NO: 69, and the amino acid sequence of the mutant-type YggB protein encoded by this gene is shown in SEQ ID NO: 70.

Similar to the method in Working Example 7, the 66-type mutation-introduced yggB mutant strain is constructed. Specifically, PCR is performed by using the synthetic DNAs shown in SEQ ID NOS: 30 and 71 as a primer and the chromosomal DNA of C.melassecolaATCC17965 strain as a template to prepare an N-terminal side fragment. Similarly, PCR is performed by using the synthetic DNAs shown in SEQ ID NOS: 34 and 72 as primers to prepare a C-terminal side fragment. Subsequently, PCR can be performed by using a mixture of an equal amount of the N-terminal side fragment and the C-terminal side fragment as a template and the synthetic DNAs shown in SEQ ID NOS: 9 and 10 as primers to obtain a partial fragment of the 66-type yggB gene. The obtained mutant-type YggB fragment was digested with SacI

and inserted into the SacI site of pBS4S to construct a plasmid for introducing this type of mutation. pBS4 YggB66 thus obtained is introduced into the chromosome of ATCC17965 strain similarly to the method stated in Working Example 6, and then removed. The sequence of the yggB gene of the obtained kanamycin sensitive strain can be determined and the strain which has been substituted with 66-type is selected, to thereby construct a strain having 66-type mutation. The 66-type mutant strain having these mutations is named yggB66 strain.

(M) [Working Example 13]

[0131]

<Screening of the Mutant-Type yggB Genes by In Vitro Mutation>

(13-1) Construction of yggB-Deleted Strain

Mutant-type yggB genes can be also obtained by introducing a mutation into yggB in vitro at random, and selecting a clone capable of producing L-glutamic acid without the addition of surfactants. In order to perform screening for mutant-type genes, a yggB gene-deleted strain was constructed first. PCR was performed by using the synthetic DNAs shown in SEQ ID NOS: 39 and 40 as primers and the chromosomal DNA of ATCC13869 strain as a template to prepare an N-terminal side fragment. Similarly, PCR was performed by using the synthetic DNAs shown in SEQ ID NOS: 41 and 42 as primers to prepare a C-terminal side fragment. SEQ ID NO: 40 and SEQ ID NO: 41 are complementary to each other. Subsequently, PCR was performed by using a mixture of an equal amount of the N-terminal side fragment and the C-terminal side fragment as a template and the synthetic DNAs shown in SEQ ID NOS: 39 and 42 as primers to obtain a fragment in which an ORF of the yggB gene was deleted.

[0132]

The obtained PCR fragment was digested with SacI and inserted into the SacI site of pBS4S to construct a plasmid for introducing deletion mutation. pBS4ΔYggB thus obtained is introduced into the chromosome of ATCC13869 strain by the method stated in Working Example 6, and then removed. PCR was performed by using the chromosomal DNA of the obtained kanamycin-sensitive strain as a template and the synthetic DNAs of SEQ ID NOS: 39 and 42 as primers to confirm that the yggB gene was deleted. The obtained yggB-deleted strain was named ATCC13869ΔyggB strain.

[0133]

(13-2) In Vitro Screening of Mutant-Type yggB Genes

On the other hand, mutagenesis treatment on the yggB gene was performed as follows. First, since the plasmid pL5k contains some regions other than the yggB

gene, pL5k was treated with XhoI and SalI, and then self-ligated to obtain the plasmid pL5kXS. A SalI recognition site is not present in the nucleotide sequence of SEQ ID NO: 5, but is present in the multi-cloning site of pBS3. About 10 μg of the obtained pL5kXS was dissolved in 500 mM phosphate buffer, 400 mM hydroxylamine, and 1 mM EDTA (pH 6.0), and then heated at 75°C for 30 to 90 minutes to introduce a mutation. The plasmid after mutation introduction was desalted using SUPREC-02 (manufactured by Takara Bio Inc.), and then introduced into ATCC13869ΔyggB strain by the method stated in Working Example 6. Thereafter, transformed cells were selected in the CM2B medium containing 25 μg/ml Km. On the other hand, pL5kXS before mutagenesis treatment was introduced into the ATCC13869ΔyggB strain as a control. The appeared transformants are inoculated into 2 ml of a liquid CM2BGU2 medium (CM2B medium stated in Working Example 3 further containing 10 g/L glucose and 15 g/L urea) and cultured at 31.5°C for 5 hours with shaking, followed by determining a concentration of glutamic acid.

The strain obtained by transforming the ATCC13869ΔyggB strain with the mutated pL5kXS was cultured in the CM2BGU2 medium in vitro. The evaluation results of the culture are shown in Table 11. There were three clones which cause accumulation of 1 g/L or more of L-glutamic acid among the transformants obtained from a plasmid mixture with a mutagenesis treatment time of 60 minutes and 90 minutes. The amount of L-glutamic acid contained in the starting medium is 0.16 g/L, and the amount of L-glutamic acid which had accumulated by the ATCC13869ΔyggB/pL5kXS (without mutagenesis treatment) strain was 0.31 g/L.

The strain obtained by transforming the ATCC13869ΔyggB strain with pL5kXS was cultured in the CM2BGU medium (the same composition as CM2BGU2 medium, except that urea concentration is 1.5g/L) in vitro. The evaluation results of the culture are shown in Table 12. One clone which causes accumulation of 1 g/L or more of L-glutamic acid was obtained among the transformants obtained from a plasmid mixture with a mutagenesis treatment time of 90 minutes.

[0134]

[Table 11]

<Table 11 Amount of L-glutamic Acid Produced by yggB Prasmid Subjected to Mutagenesis Treatment - 1>

Amount of Accumulated Glu (g/L)	Number of Clones		
	Mutagenesis Treatment Time		
	30min	60min	90min
Glu≤0.4	40	36	39
0.4<Glu≤0.6	8	11	6
0.6<Glu≤0.8	0	0	1
0.8<Glu≤1	0	0	0
1<Glu	0	1	2

[0135]

[Table 12]

<Table 12 Amount of L-glutamic Acid Produced by yggB Prasmid Subjected to Mutagenesis Treatment - 2>

Amount of Accumulated Glu (g/L)	Number of Clones	
	Mutagenesis Treatment Time	
	60min	90min
Glu≤0.7	45	41
0.7<Glu≤0.9	2	7
0.9<Glu	1	0

[0136]

A plasmid which gained a producing ability to accumulate 1 g/L or more of L-glutamic acid by mutagenesis treatment for 60 minutes as shown in Table 11 was named pL5kXSm-22. A plasmid which gained a producing ability to accumulate 0.9 g/L or more of L-glutamic acid by mutagenesis treatment for 90 minutes as shown in Table 12 was named pL5kXSm-27. ATCC13869ΔyggB/pL5kXS strain, ATCC13869ΔyggB/pL5kXSm-27 strain, and ATCC13869ΔyggB/pL5kXSm-22 strain were cultured under the conditions stated in Working Example 2, and amounts of bacterial cells and L-glutamic acid accumulation after 4 hours were analyzed. Table 13 shows the mean value of three independent experiments. It could be confirmed that the ATCC13869ΔyggB/pL5kXSm-27 strain and the ATCC13869ΔyggB/pL5kXSm-22 strain significantly enhanced L-glutamic acid accumulation. These results demonstrate that the mutant-type gene which is advantageous to L-glutamic acid can be constructed even by introducing mutation in

vitro at random. The sequence of the yggB gene which pL5kXSm-22 contains is shown in SEQ ID NO: 73 (22-Type Mutation).

The 22-type mutation is a mutation in which proline at position 437 is substituted with serine and a mutation in which C at position 2745 is substituted with T in SEQ ID NO: 5. Further, this mutation is accompanied by a mutation in which C at position 3060 is substituted with T. This mutant-type yggB gene is shown in SEQ ID NO: 73, and the amino acid sequence of the mutant-type YggB protein encoded by this mutant-type yggB gene is shown in SEQ ID NO: 74.

[0137]

[Table 13]

<Table 13 Amount of L-glutamic Acid Produced by yggB Prasmid Subjected to Mutagenesis Treatment - 3>

Strain	OD620(x101)	Glu(g/L)
ATCC13869⊿yggB/pL5kXS (Untreated)	0.253	0.58
ATCC13869⊿yggB/pL5kXSm22	0.232	1.10
ATCC13869⊿yggB/pL5kXSm27	0.245	0.82

[0138]

(13-3) Introduction of Mutant-Type Genes into Coryneform Bacteria, and Confirmation of L-glutamic Acid Production

The novel mutant-type yggB gene obtained in (13-2) is introduced into a coryneform bacterium. Method of introduction is as follows. The mutant-type yggB gene was introduced into pBS4S by the method stated in Example 6, and substituted with a wild-type yggB gene on the chromosome of ATCC13869. A strain in which this novel mutant-type yggB gene is introduced, and the parent ATCC13869 strain are cultured similarly to the method in Working Example 2. After completion of the culture, the amount of L-glutamic acid in the culture broth is measured by the known method to confirm that L-glutamic acid accumulation is enhanced due to the mutation-introduced strain. In this manner, the yggB gene mutant strain of which an L-glutamic acid-producing ability is enhanced can be obtained.

(N) [Working Example 14]

[0139]

<Sensitivity to Glutamic Acid Analogue of the Strains Having the Mutant-Type yggB Gene>

(14-1) Sensitivity to 4-Fluoroglutamic Acid on Solid Medium

It was predicted that strains which enhanced L-glutamic acid-producing ability due to yggB mutation would have decreased sensitivity to L-glutamic acid analogues. Accordingly, as an L-glutamic acid analogue due to yggB mutations, a change of sensitivity to 4-fluoroglutamic acid was investigated and considered. 4-fluoroglutamic acid which had been adjusted to pH 6.7 with NaOH and sterile-filtered was added to a minimum medium stated in Working Example 4 so that the final concentration of 4-fluoroglutamic acid was 7.5 mM. ATCC13869 strain, ATCC13869-L30 strain, and ATCC13869-A1 strain were spread over a CM-Dex medium and cultured overnight. Then, the bacterial cells were collected from the medium, washed with sterilized 0.85% NaCl solution, and diluted so as to attain the bacterium concentration shown in the top of Figure 6, and spotted onto the plate. The plate was cultured at 31.5°C. The time-course is shown in Figure 6. It was shown that the ATCC13869 strain grew the fastest of three strains under the condition where 4-fluoroglutamic acid was not added. In contrast, it was shown that under the condition where 4-fluoroglutamic acid was added, the growth of the ATCC13869 strain was suppressed, and the growth of the ATCC13869-L strain and the ATCC13869-A1 strain became better than that of the ATCC13869 strain.

[0140]

(14-2) Sensitivity to 4-Fluoroglutamic Acid on Liquid Medium

4-fluoroglutamic acid was adjusted to pH 6.7 with NaOH and was sterile-filtered. This 4-fluoroglutamic acid was added to a minimum medium having the same composition as stated in Working Example 4 (except for not containing agar) so as to attain a final concentration of 1.25 mM, 2.5 mM, 5 mM, 10 mM, and 20 mM, respectively. Each of ATCC13869 strain, ATCC13869ΔyggB strain, ATCC13869-L30 strain, and ATCC13869-A1 strain was spread over a CM-Dex medium and was cultured at 31.5°C overnight. Then, the bacterial cells were collected, washed with sterilized 0.85% NaCl solution, inoculated into the liquid medium, and cultured at 31.5°C with shaking. In a medium under the condition where 4-fluoroglutamic acid is not added in each sterilized strain, when the OD660 value of the medium reached 1.0, the culture was terminated, and the obtained culture broth was diluted appropriately and spread over a CM-Dex plate. The number of colonies which appeared on the next day was measured to be a viable cell number at the end of liquid culture. Figure 7 shows the change of the relative viable cell number at each concentration of 4-fluoroglutamic acid when the cell number of the culture under the condition where 4-fluoroglutamic acid was not added was set to 1. It has been found that the ATCC13869-A1 strain and the ATCC13869-L30 strain have decreased

sensitivity to 4-fluoroglutamic acid (i.e., enhanced resistance to 4-fluoroglutamic acid).

From these results, it has been shown that strains having a mutant-type yggB strain of the Present Invention can also be obtained by screening using sensitivity to L-glutamic acid structural analogues such as 4-fluoroglutamic acid.

(O) [Working Example 15]

[0141]

<Construction of the Mutant-Type yggB Gene-Introduced Strain in odhA Weakened Strain, and Evaluation Thereof>

[0142]

The yggB odhA-double mutant strain was constructed by using the ATCC13869-L30 strain which was constructed in Working Example 9, and the mutant-type odhA gene was evaluated.

First, a strain in which the mutation shown in Table 14 was introduced into the odhA gene of the ATCC13869-L strain is constructed. In Table 14, nucleotide sequences of the region corresponding to nucleotide numbers 2528 to 2562 of SEQ ID NO: 43 are shown. In Table 15, amino acid sequences of the region corresponding to amino acid numbers 696 to 707 of SEQ ID NO: 44 are shown.

L30sucA8 strain into which the odhA gene having the nucleotide sequence of SEQ ID NO: 45 was introduced can be obtained as follows. The odhA gene fragment is prepared by PCR using synthetic DNAs as shown in SEQ ID NOS: 53 and 54 as primers. The obtained odhA fragment is digested with BamHI and cloned to the BamHI site of plasmid pKF19m which is attached to Mutan-Super Express Km (manufactured by Takara Bio Inc.). Then, PCR is performed using a synthetic DNA of SEQ ID NO: 55 having a phosphorylated 5'-end and a selection primer attached to Mutan-Super Express Km, and the obtained PCR product is used to transform sup0-E. coli strain, for example MV 1184 strain, thereby to construct a plasmid containing the mutant odhA fragment. Then, this fragment can be cut at BamHI and inserted into the BamHI site of the pBS4S plasmid, to thereby construct a plasmid for introducing this mutation. The ATCC13869-L strain is transformed similarly to the method as stated in Working Example 1, to thereby obtain a strain in which the plasmid for introducing this mutation is inserted into the chromosome. Then, a strain which is resistant to sucrose and is sensitive to kanamycin is isolated from these strains in which the plasmid is inserted into the chromosome. L30sucA8 (odhA8) strain in which the odhA gene is deleted can be constructed by further confirming the sequence of the odhA gene and selecting the strain into which an objective frame-shift was

introduced.

[0143]

In addition, other odhA mutant strains can be obtained by the following methods using a yggB mutant strain.

L30sucA801 strain into which a mutant odhA gene having a nucleotide sequence of SEQ ID NO: 47 is introduced can be obtained according to the above method by using a synthetic DNA of SEQ ID NO: 56 having a phosphorylated 5'-end instead of a synthetic DNA of SEQ ID NO: 55 having a phosphorylated 5'-end.

L30sucA805 strain into which a mutant odhA gene having a nucleotide sequence of SEQ ID NO: 49 is introduced can be obtained according to the above method by using a synthetic DNA of SEQ ID NO: 57 having a phosphorylated 5'-end instead of a synthetic DNA of SEQ ID NO: 55 having a phosphorylated 5'-end.

L30sucA77 strain into which a mutant odhA gene having a nucleotide sequence of SEQ ID NO: 51 is introduced can be obtained according to the above method by using a synthetic DNA of SEQ ID NO: 58 having a phosphorylated 5'-end instead of a synthetic DNA of SEQ ID NO: 55 having a phosphorylated 5'-end.

The L30sucA8 strain does not have an α-KGDH activity, because the odhA gene of the L30sucA8 strain has a frame-shift mutation. On the other hand, in the L30sucA801 strain, the L30sucA805 strain, and the L30sucA77 strain, these strains have mutations in which there is deletion in odhA gene but frame-shift will not occur. These strains have the α-KGDH activity, but their activity was lower than that of a non-modified strain (data not shown).

[0144]

[Table 14]

<Table 14 Partial Sequences of Mutant-type odhA Gene>

Strain Name	odhA Sequence
ATCC13869-L	CTG GCT AAG CTG CGT GGC TAC GAC GTC GGA GGC AC
L30sucA8	CTG GCT AAG CTG CGT C GAC GTC GGA GGC AC
sucA801	CTG GCT AAG CTG CGT CTC GAC GTC GGA GGC AC
sucA805	CTG GCT AAA AGC TGC GTC GAC GTC GGA GGC AC
sucA77	CTG GCT ATA AGC TGC GTC GAC GTC GGA GGC AC

[0145]
[Table 15]

<Table 15 Partial Sequences of Mutant-type α-Ketoglutarate Dehydrogenase>

Strain Name	α-KGDH Sequence
wild	Leu Ala Lys Leu Arg Gly Tyr Asp Val Gly Gly Thr
⊿sucA(L30sucA8)	Leu Ala Lys Leu Arg
sucA801	Leu Ala Lys Leu Arg --- Leu Asp Val Gly Gly Thr
sucA805	Leu Ala Lys Ser Cys --- Val Asp Val Gly Gly Thr
sucA77	Leu Ala Ile Ser Cys --- Val Asp Val Gly Gly Thr

[0146]

<Culture of odhA Modified Strain, and Production of L-glutamic Acid by the odhA Modified Strain>

L-glutamic acid-producing ability of these odhA modified strains was evaluated by culturing these strains in a Sakaguchi flask. Each of the strains shown in Table 14 was cultured on a CM-Dex agar medium at 31.5°C for a whole day and night. Then, 1/6 plate bacterial cells of this culture were inoculated to 20 ml of a flask medium (60 g/l glucose, 22.5 g/l ammonium sulfate, 1 g/l KH_2PO_4, 0.4 g/l $MgSO_4$-$7H_2O$, 0.01 g/l $FeSO_4$-$7H_2O$, 0.01 g/l $MnSO_4$-4-$5H_2O$, 200 μg/l vitamin B1, 0.48 g/l soybean protein hydrolysate solution, and 300 μg/l biotin, adjusted to pH 8.0 with KOH). 1 g of calcium carbonate which had been dry heat-sterilized beforehand was added to this flask medium, and each of the strains was cultured with shaking at 115 rpm at 31.5°C. The amount of accumulated L-glutamic acid after 19 hours from the start of culture is shown in Table 16. odhA modified strains include a strain which exhibited L-glutamic acid accumulation equivalent to that of the ATCC13869-L strain. However, the sucA801 strain, the sucA805 strain, and the sucA77 strain exhibited higher L-glutamic acid accumulation than did the sucA8 strain. From these results, it has been found that the yield of L-glutamic acid is further enhanced by introducing mutations into the odhA gene in addition to the yggB gene.

(P) [Working Example 16]

[0148]

<Construction of odhA Weakened Strain from ATCC14067 Strain and ATCC14067yggB8 Strain>

odhA-deleted strain was constructed from yggB mutant strain having 8-type mutation, and the constructed strain was cultured as well as a strain having only odhA deletion, to compare the two strains. First, a plasmid for completely deleting odhA

was constructed. PCR was performed using chromosomal DNA of ATCC14067 strain as a template and synthetic DNAs shown in SEQ ID NOS: 77 and 78 to prepare an upstream side fragment of the sucA gene. Subsequently, PCR was performed using chromosomal DNA of ATCC14067 strain as a template and the synthetic DNAs shown in SEQ ID NOS: 79 and 80 to prepare a downstream side fragment of the sucA gene. Then, PCR was performed using a mixture of equimolar amounts of the upstream side fragment and the downstream side fragment as a template and synthetic DNAs of SEQ ID NOS: 81 and 82 to prepare a gene fragment in which odhA was deleted. The obtained PCR fragment was digested with BamHI and was cloned in the pBS4S constructed in Working Example 1. The plasmid thus obtained was named pBSΔsucA47.

The obtained pBSΔsucA47 was inserted into the chromosome of ATCC14067 strain or ATCC14067yggB8 strain similarly to the method stated in Working Example 6, and then was removed. Then, a chromosomal DNA was prepared from the obtained kanamycin-sensitive strain. Thereafter, PCR was performed by using this chromosomal DNA as a template and the synthetic DNAs of SEQ ID NOS: 77 and 80 to select a strain in which odhA region was deleted. odhA-deleted strains thus constructed were named ATCC14067ΔodhA strain and ATCC14067ΔodhA yggB8 strain, respectively.

The constructed ATCC14067ΔodhA strain and the ATCC14067ΔodhA yggB8 strain were cultured according to the method stated in Working Example 3. The results of this culture are shown in Table 17. It has been found that yggB8 mutation can enhance the yield of L-glutamic acid of the odhA mutant strain.

[0149]

[Table 17]

<Table 16 Amount of L-glutamic Acid Produced by odhA Modified Strain>

Strain Name	Glu (g/L)
ATCC13869-L	4.9
L30sucA8	19.8
L30sucA801	22.1
L30sucA805	23.8
L30sucA77	21.6

(Q) [Working Example 17]

[0150]

<Construction of symA-Deleted Strain from 2A-1R Strain>

The symA (suppressor of ygg mutation A) gene was deleted from the 2A-1R strain having a mutation of IS insertion into yggB, which was constructed in Working Example 3, thereby to construct a strain. The obtained strain was cultured, as was the 2A-1R strain, and the two strains were compared. The nucleic acid sequence of NCgl1867 gene from the ATCC13869 strain is shown in SEQ ID NO: 86, and the amino acid sequence from the ATCC13869 strain is shown in SEQ ID NO: 87. First, a plasmid for the purpose of completely deleting the symA gene was constructed. PCR was performed using chromosomal DNA of the ATCC13869 strain as a template and the synthetic DNAs shown in SEQ ID NOS: 88 and 89 to prepare an upstream side fragment of the symA gene. Subsequently, PCR was performed using chromosomal DNA of ATCC13869 strain as a template and the synthetic DNAs shown in SEQ ID NOS: 90 and 91 to prepare a downstream side fragment of the symA gene. Then, PCR was performed using a mixture of equimolar amounts of the upstream side fragment and the downstream side fragment as a template and synthetic DNAs of SEQ ID NOS: 88 and 91 to prepare a gene fragment in which the symA gene was deleted. The obtained PCR fragment was digested with BglII and was cloned in the pBS4S constructed in Working Example 1. The plasmid thus obtained was named pBSΔsymA.

The obtained pBSΔsymA was inserted into the chromosome of 2A-1R strain similarly to the method stated in Working Example 6, and then was removed. Then, a chromosomal DNA was prepared from the obtained kanamycin-sensitive strain. Thereafter, PCR was performed by using this chromosomal DNA as a template and the synthetic DNAs of SEQ ID NOS: 88 and 91 to select a strain in which symA region was deleted. The symA-deleted strain thus constructed was named 2A-1RΔsymA strain.

The constructed 2A-1RΔsymA strain and the parent 2A-1R strain were cultured according to the method stated in Working Example 3. The results of these culture are shown in Table 18. It has been found that the deletion of the symA gene can enhance L-glutamic acid producing ability of a strain having the mutant-type yggB gene.

[0151]

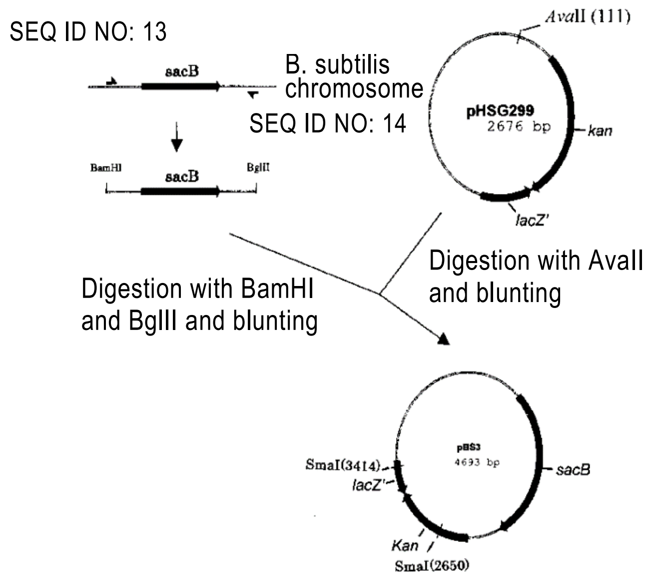
[Table 18]

<Table 18 Enhancement of L-glutamic Acid-Producing Ability by symA Gene Deletion>

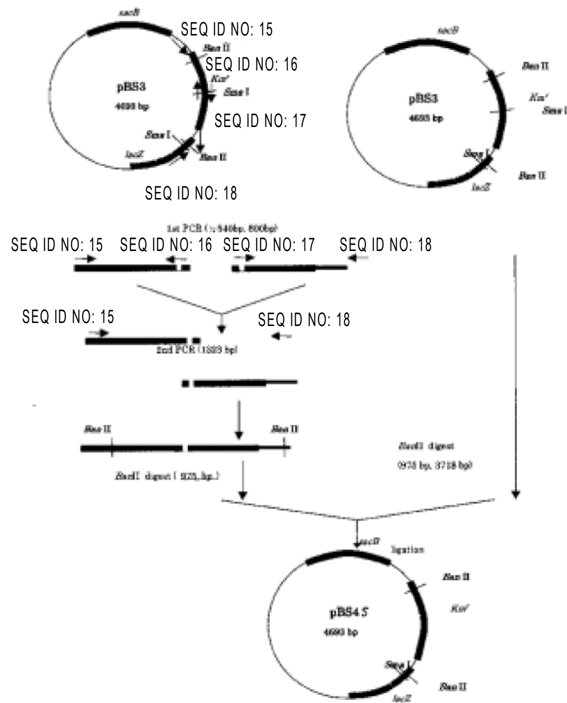
	OD620(×51)	Glu (g/L)
2A-1R	0.846	12.4
2A-1R Δ symA	0.709	15.8

H. Drawings

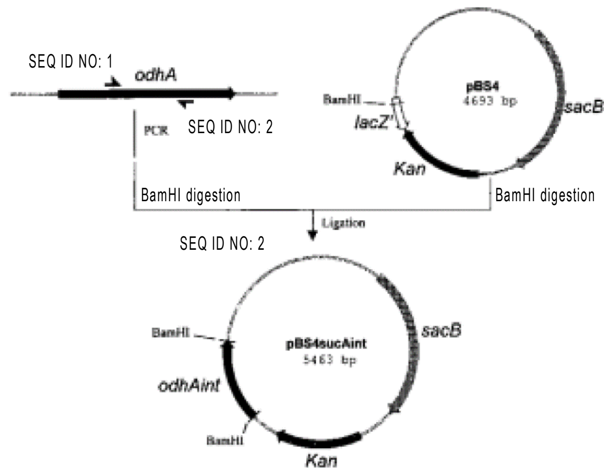
[Figure 1]



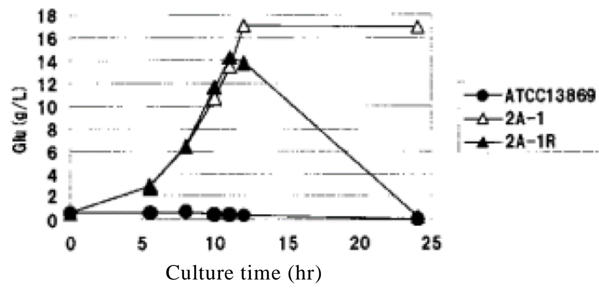
[Figure 2]



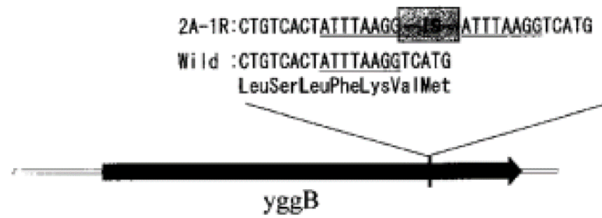
[Figure 3]



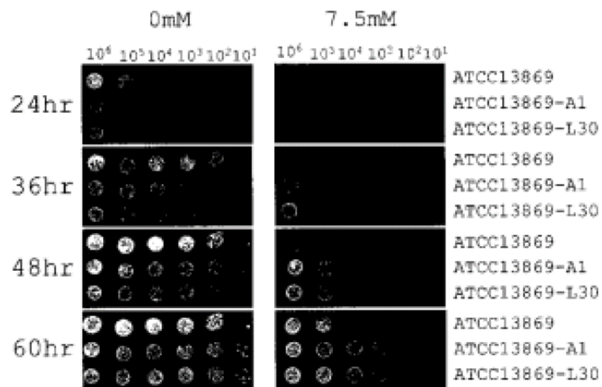
[Figure 4]



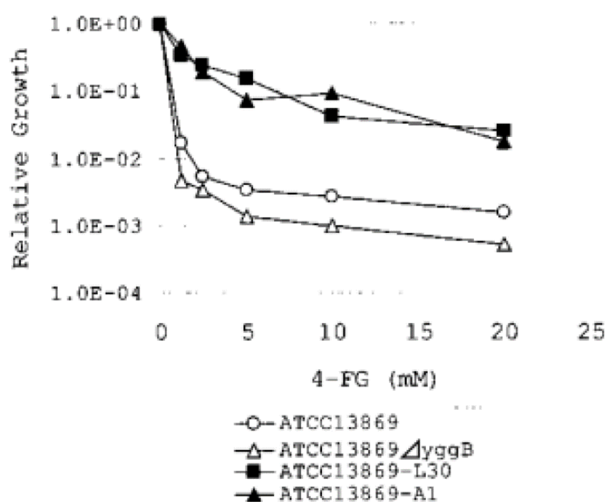
[Figure 5]



[Figure 6]



[Figure 7]



(2) Summary of the Present Invention

According to the above (1), the summary of the Present Invention is as follows.

A. Technical Field, Background Art

The Present Invention relates to the fermentation industry, and relates to a method for producing L-glutamic acid, which is widely used as seasoning ingredients, and further relates to a bacterium for using the method (paragraph [0001]).

L-glutamic acid has been conventionally produced on an industrial scale by a fermentation method using coryneform bacteria which have L-glutamic acid-producing ability, such as bacteria belonging to the genus Brevibacterium or the genus Corynebacterium (paragraph [0002]).

L-glutamic acid production by coryneform bacteria is typically performed under an inducing condition such as a biotin-limited condition, a surfactant-added condition, and a penicillin-added condition. Further, as strains which can produce L-glutamic acid under the non-inducing condition in the presence of sufficient biotin without applying these methods, a surfactant-temperature-sensitive strain, a penicillin-sensitive strain, a cerulenin-sensitive strain, a lysozyme-sensitive strain, etc. have been developed. However, it is very probable that these strains cause reduced adaptation to environmental changes in exchange for L-glutamic acid production. Therefore, considerable efforts have been required for the development of a strain capable of accumulating a significant amount of L-glutamic acid by using these methods (paragraphs [0003] and [0004]).

A strain which produces L-glutamic acid in the presence of sufficient biotin

can be achieved by deleting a gene encoding α-ketoglutarate dehydrogenase. However, the α-ketoglutarate dehydrogenase gene-deleted strain blocks the TCA cycle in the midway and thus grows slowly. Therefore, it has been difficult to surely obtain a sufficient amount of bacterial cells, which has been a problem (paragraph [0005]).

A yggB gene of coryneform bacteria is a homologue of a yggB gene of Escherichia coli, and has been analyzed as a kind of mechanosensitive channel. However, an effect of the yggB gene on L-glutamic acid production has not been known (paragraph [0006]).

B. Problem of the Present Invention

A problem of the Present Invention is to provide a novel art to enhance an L-glutamic acid-producing ability when L-glutamic acid is produced by using the coryneform bacterium (paragraph [0007]).

C. Means for Solving the Problem

It has been clarified that a yggB gene is involved in the production of L-glutamic acid by a coryneform bacterium. It has been found that an L-glutamic acid-producing ability can be greatly enhanced by modifying the coryneform bacterium by using the yggB gene and completed the Present Invention (paragraph [0008]).

The coryneform bacterium of the Present Invention has an L-glutamic acid-producing ability, and is modified by introducing a mutation into a yggB gene, and thereby the L-glutamic acid-producing ability of the coryneform bacterium is enhanced as compared to a non-modified strain (paragraph [0011]). However, the mutant-type yggB gene of the Present Invention is not particularly limited so far as the mutant-type yggB gene has a mutation to be able to enhance the L-glutamic acid producing ability of a coryneform bacterium in the presence of an excess of biotin (paragraph [0069]).

(A) One embodiment of a mutation which is introduced into a yggB gene in the Present Invention is a C-terminal side mutation; that is, a mutation is introduced into the C-terminal region of the yggB gene. This mutation is a mutation which is introduced into a portion of a nucleotide sequence of a region encoding a sequence of amino acid numbers 419 to 533 of SEQ ID NOS: 6, 68, 84, or 85, or a sequence of amino acid numbers 419 to 529 of SEQ ID NO: 62 (paragraph [0070]). Examples of C-terminal side mutation include [i] a mutation in which a transposable element is introduced (2A-1-type mutation) and [ii] a mutation in which proline is substituted with another amino acid (66-type mutation, 22-type mutation) ([Claim 1], paragraphs [0070] to [0072], [Working Example 2] to [Working Example 6], [Working Example

12], [Working Example 13], and [Working Example 17]).

(B) Another embodiment of a mutation which is introduced into a yggB gene in the Present Invention is a mutation which is introduced into a portion encoding five transmembrane regions (in the amino acid sequence of wild-type YggB protein of SEQ ID NOS: 6, 62, 68, 84, or 85, transmembrane regions are amino acid numbers 1 to 23 [the first transmembrane region], amino acid numbers 25 to 47 [the second transmembrane region], amino acid numbers 62 to 84 [the third transmembrane region], amino acid numbers 86 to 108 [the fourth transmembrane region], and amino acid numbers 110 to 132 [the fifth transmembrane region]) which is presumably contained in a YggB protein encoded by the yggB. Examples of these mutations include [i] a mutation in the first transmembrane region (A1-type mutation), [ii] a mutation in the fourth transmembrane region (19-type mutation), and [iii] a mutation in the fifth transmembrane region (L30-type mutation, 8-type mutation) ([Claim 1], paragraphs [0073] to [0076], [Working Example 7] to [Working Example 11], [Working Example 15], and [Working Example 16]).

D. Effect of the Present Invention

L-glutamic acid can be efficiently produced by using a coryneform bacterium which is modified by using a yggB gene of the Present Invention (paragraph [0010]).

2. Erroneous Findings and Determinations on the Violation of the Enablement Requirement and the Support Requirement on 19-Type Mutation (Reason 1 for Rescission)

(1) Common Technical Knowledge on Production of Glutamic Acid by Fermentation Methods Using Coryneform Bacteria as of the Priority Date and the Filing Date of the Present Case

A. Glutamic acid has been industrially produced by a fermentation method using coryneform bacteria since around the 1960's (Exhibits Otsu 2 and 24, the entire import of the oral argument).

B. The production of glutamic acid by the fermentation method is a process which comprises: adding various raw materials which serve as carbon sources and nitrogen sources, and other substances; culturing glutamic acid-producing coryneform bacteria to produce L-glutamic acid; and isolating the produced L-glutamic acid from the culture. In this regard, it has been general practice that the production of glutamic acid is performed under the inducing condition of limiting an amount of biotin in a medium, or adding a surfactant or penicillin until strains capable of producing glutamic acid even in the presence of sufficient biotin were developed (Exhibits Ko 1, 19 to 21, Exhibits Otsu 2 and 24, the entire import of the oral

argument).

(2) Compliance of Present Invention 11 with the Support Requirement on 19-type Mutation

A. Determinations on whether the statement of claims complies with the support requirement of the description shall be made as follows. First, comparison is made between the statement of the claims and the statement in the Detailed Description of the Invention. Then, consideration is made on whether or not the invention stated in the statement of the Claims is the invention stated in the Detailed Description of the Invention, and is within the scope where a person ordinarily skilled in the art can recognize that the statement in the Detailed Description of the Invention can solve the problem of the invention, and even if there are neither such statements nor such suggestions, consideration is made on whether or not the invention stated in the statement of the Claims is within the scope where a person ordinarily skilled in the art can recognize that the statement in the Detailed Description of the Invention can solve the problem of the invention in light of common technical knowledge at the time of filing the application. Based on the above comparison and consideration, the determination on the compliance with the support requirement shall be made.

B.(A) The problem of the Present Invention is to "provide a novel art to enhance an L-glutamic acid-producing ability of a coryneform bacterium when L-glutamic acid is produced by using the coryneform bacterium." Further, Present Invention 11 includes embodiments which attempt to enhance the producing ability not only in the inducing condition but also in the non-inducing condition. In this regard, it can be deemed that the present description discloses in Working Example 10 (paragraphs [0125] to [0128], [Table 9], and [Table 10]) that a producing ability of ATCC13869-19 strain in which 19-type mutation is introduced is enhanced as compared to that of the wild-type in the inducing condition. Therefore, it can be deemed that a person ordinarily skilled in the art can recognize that the 19-type mutation enhances the L-glutamic acid-producing ability in the inducing condition.

(B) Next, whether the 19-type mutation enhances the producing ability in the non-inducing condition is considered. The culture in Working Example 8 of the present description is performed similarly to the method in Working Example 2. It can be found that the medium used in Working Example 8 contains 300 μg/l biotin corresponding to “an excess of biotin” described in Claim 6 etc. and does not contain a surfactant and the like. Thus, Working Example 8 is an experiment performed in order to determine whether the 19-type mutation enhances the producing ability in the non-inducing condition (paragraphs [0120], [0097], and [0032] of the present

description).

In addition, [Table 7] of Working Example 8 shows that the ATCC13869-19 strain which is the 19-type mutant strain produced 0.2 g/L more L-glutamic acid than did the wild-type strain. Based on this result, the present description states in paragraph [0120] that "the ATCC13869-19 strain greatly enhanced L-glutamic acid accumulation as compared to the parent ATCC13869 strain." Thus, from these statements, it can be deemed that a person ordinarily skilled in the art recognizes that the 19-type mutation can solve the problem of the Present Invention even if under the non-inducing condition.

<Table 7 Amount of L-glutamic Acid Produced by Mutant-Type yggB Gene (19 Mutation) Chromosome-Introduced Strain>

	OD620(x101)	Glu(g/L)
ATCC13869	0.650	0.5
ATCC13869-19	0.614	0.7

C. With regard to Working Example 8, the Plaintiffs assert that: [i] in light of an amount value of glutamic acid produced by the wild-type strain and a blank value in Working Example 2, and a blank value in Working Example 3, and values in Working Examples 2, 3, 5, 7, and 9, the difference in glutamic acid production between the wild-type strain and the 19-type mutant strain is merely within the margin of error, and thus, a person ordinarily skilled in the art cannot recognize from Working Example 8 that a glutamic acid-producing ability is enhanced; [ii] without comparing between blank values and results of the parent strain and the mutant strain, the reliability of Working Example 8 cannot be evaluated; [iii] from the experiment results in Exhibits Ko 28 and 34 as well, it is supported that 19-type mutant strain does not produce glutamic acid under the non-inducing condition.

(A) Item [i] Mentioned Above

In the present description, all of Working Examples 3 and 6 to 9 were worked under the non-inducing condition, such as presence of 300 μg/l biotin in a medium, by the method stated in Working Example 2 or a method similar thereto (paragraphs [0097], [0100], [0109], [0112], [0117], [0120], and [0123] of the present description). With regard to blank values stated in [Table 1], [Table 2], [Table 4], and [Table 5] of Working Examples 2, 3, and 6, the present description does not explicitly explain these blank values. However, OD620 value which indicates an amount of bacterial

cells is 0.002, which is extremely low, in all of the Working Examples. Thus, in view of the above, as the Defendant asserts, it can be found that the blank value indicates a concentration of glutamic acid in the medium at the start of culture (initial medium) which was not inoculated with glutamic acid-producing bacterium. Further, according to Exhibit Ko 36 and Exhibit Otsu 6, it can be found that the amount value of glutamic acid produced by the wild-type strain (ATCC13869) under the non-inducing condition is a value after the glutamic acid had been degraded as the culture proceeded.

Working Examples 3 and 6 to 9 were worked by the method stated in Working Example 2 or the similar method thereto. However, considering that four blank values as mentioned above are different from each other ([Table 1] is 0.4 g/L, [Table 2] and [Table 4] are 0.6 g/L, and [Table 5] is 0.7 g/L), it can be found that the culture conditions such as the concentration of glutamic acid in the initial medium are different depending on each Working Example or depending on each culture. Taking into consideration the statement of paragraph [0097] of the present description and the statements of Exhibit Ko 36 and Exhibit Otsu 6, it can be found that the difference of the blank values in the Working Examples as disclosed above is due to soybean hydrolysates originated from natural products.

In the first place, the blank values and the amount value of glutamic acid produced by the wild-type strain in the present description are as disclosed in the above findings. Thus, based on these values, it cannot be deemed that the difference in glutamic acid production between the wild-type strain and the 19-type mutant strain, which are different from these values, in Working Example 8 is based on an error. In addition, as the above findings, the culture conditions differ depending on each Working Example or depending on each culture. Thus, even more, it cannot be deemed that the difference of 0.2 g/L in glutamic acid production between the wild-type strain and the 19-type mutant strain in Working Example 8 is based on an error on the basis of the values shown in Working Examples 2 and 3.

Furthermore, in view of other numerical values in Working Examples 2, 3, 5, 7, and 9 as well, it cannot be deemed that the difference of 0.2 g/L in glutamic acid production between the wild-type strain and the 19-type mutant strain in Working Example 8 is based on an error.

In view of the foregoing, it cannot be deemed that the above item [i] affects the determination in the above B.

(B) Item [ii] Mentioned Above

The problem of the Present Invention is to "provide a novel art to enhance an

L-glutamic acid-producing ability of a coryneform bacterium when L-glutamic acid is produced by using the coryneform bacterium." As used herein, the phrase "enhance producing ability" means that "the L-glutamic acid-producing ability is enhanced as compared to the case of a non-modified strain such as wild-type strain" ([Claim 1], [Claim 4], [Claim 5], and paragraphs [0015] and [0031] of the present description). Thus, since Working Example 8 shows that the 19-type mutant strain produced more glutamic acid than did the wild-type strain, even if the blank value is not stated, it cannot be deemed that the results of Working Example 8 are not reliable.

(C) Item [iii] Mentioned Above

a. Exhibit Ko 19 (Unexamined Patent Application Publication No. 1988-214189) discloses that "a method for producing an L-glutamic acid by performing glutamic acid fermentation using thus obtained multiple enriched strain of the present invention is almost the same as using the known and conventional glutamic acid-producing bacterium. ... As equipment for culture, test tubes, flasks, and jar fermenters can be used. It is obviously possible to use them for industrial scale production as well. ... The method for culture may be aerobic, and may be either a shaking culture or an aeration and agitation culture." (page 503, upper right column, line 4 from the bottom to lower right column, line 12). Exhibit Ko 20 (Unexamined Patent Application Publication No. 2004-313202) also discloses that "Fermentation may be performed ... under aerobic conditions such as a shaking culture or an aeration and agitation culture" (paragraph [0020]). Taking into consideration the combination of the disclosures in the literature on fermentation method published before the filing date of the present case (Exhibits Otsu 14 and 15) and the entire import of the oral argument with the above disclosures of Exhibits Ko 19 and 20, it can be found that various vessels and equipment can be used in performing the fermentation method, and that it had been the common technical knowledge as of the filing date of the present case that it is necessary to supply sufficient oxygen by an appropriate method according to the container to be used.

b. In the experiment in Exhibit Ko 28, while an Erlenmeyer flask was used, with regard to a shaking speed in this experiment, a shaking speed of a Sakaguchi flask was adopted on the basis of the statement of paragraph [0146] in the present description. In addition, it is not clear from Exhibit Ko 28 what kind of shaking method was adopted. Taking into consideration the common technical knowledge as of the filing date of the present case, even if the vessel identified in paragraph [0146] of the present description is a Sakaguchi flask, it is considered that a person skilled in the art would select an appropriate method of oxygen supply according to the vessel

and equipment to be used for the culture to perform the fermentation method. However, in the experiment in Exhibit Ko 28, the shaking speed disclosed as that of the Sakaguchi flask was mechanically applied to the Erlenmeyer flask for the culture. In addition, it is not clear what kind of the shaking method was adopted. In view of these considerations, it cannot be found that the experiment in Exhibit Ko 28 was carried out by an appropriate method. Therefore, it cannot be deemed that the experimental results in Exhibit Ko 28 affect the determination in the above B.

In this regard, the Plaintiffs further assert that the Sakaguchi flask is not known internationally, and that a person ordinarily skilled in the art who has read the present description understands that an ordinary flask such as an Erlenmeyer flask was used. However, as mentioned above, it can be found that a person ordinarily skilled in the art could appropriately set the aerobic condition necessary to produce glutamic acid by using various vessels and equipment as of the filing date of the present case. Therefore, even if there are no statements on a culture vessel anywhere other than in paragraph [0146], it cannot be deemed that a person ordinarily skilled in the art understands that an Erlenmeyer flask is used as the culture vessel. Not to mention, it cannot be deemed that a person ordinarily skilled in the art understands that the shaking speed stated in the same paragraph was applied to the Erlenmeyer flask.

With regard to the experiments in Exhibit Ko 34, it is stated that the culture was performed under "the ordinary aerobic culture condition of 31.5°C and 200 rpm utilizing a baffled flask which a person ordinarily skilled in the art uses." However, other culture conditions are not clear. In addition, it is not clear how an amount of produced glutamic acid was measured. Thus, Exhibit Ko 34 is not immediately reliable. Therefore, Exhibit Ko 34 does not affect the finding in the above B as well.

(3) Compliance with the Enablement Requirement

A. Present Invention 11 is an invention of a process. In this regard, if an invention is a process, working of the invention means an act of using the process (Article 2, paragraph (3), item (ii) of the Patent Act). Thus, whether or not a statement of an invention of a process complies with the enablement requirement should depend on whether or not the Detailed Description of the Invention in the description states the extent to which a person ordinarily skilled in the art can use the process without undue trial-and-error on the basis of the statement of the description and common technical knowledge at the time of filing the application.

B. In view of the statement of the present description in the above 1(1) and consideration in the above (1) and (2), with regard to the 19-type mutation, it can be deemed that a person ordinarily skilled in the art can work Present Invention 11

without undue trial-and-error on the basis of the disclosure of the present description and the common technical knowledge on the fermentation method as of the filing date of the present case.

(4) Summary

In view of the foregoing, with regard to the 19-type mutation, it cannot be deemed that Present Invention 11 violates the support requirement and the enablement requirement. Therefore, Reason 1 for Rescission asserted by the Plaintiffs is unfounded.

3. Erroneous Findings and Determinations on Lack of an Inventive Step (Reasons 2 and 3 for Rescission)

(1) Knowledge on Efflux of Glutamic Acid in Coryneform Bacteria as of the Priority Date of the Present Case

A.(A) The industrial production of glutamic acid by a fermentation method using coryneform bacteria was put to practical use from early on. However, a mechanism by which the bacteria cause efflux of glutamic acid out of the bacterial cell has not been elucidated (Exhibit Ko 12, Exhibits Otsu 1, 2, and 33).

(B) It was known before the priority date of the present case that in the fermentation method, as the fermentation proceeds and the efflux of glutamic acid proceeds, an osmotic pressure out of the bacterial cell becomes higher, and that the efflux of glutamic acid proceeds even after such a condition has been reached. This phenomenon cannot be explained only by the fact that efflux of glutamic acid occurs from the osmoregulated channel when hypoosmotic conditions have occurred outside the bacterial cell (Exhibits Otsu 28 and 39, the entire import of the oral argument).

(C) Before the priority date of the present case, Dr. Reinhard Krämer (hereinafter referred to as "Dr. Krämer") et al. had published two papers which are Exhibit Otsu 39 (Reinhard Krämer et al. "Carrier-mediated glutamate secretion by *Corynebacterium glutamicum* under biotin limitation" Biochimica et Biophysica Acta 1112 pp 115-123, 1992) and Exhibit Otsu 40 (Reinhard Krämer "Secretion of amino acids by bacteria: Physiology and mechanism" FEMS Microbiology Reviews 13 pp 75-94, 1994), which reported that a carrier for amino acids such as L-lysine, L-threonine, and L-isoleucine was found in Corynebacterium glutamicum, and which asserted that efflux of glutamic acid is also caused by the carrier (Exhibit Ko 22, Exhibits Otsu 1, 2, and 39 to 41).

One of the co-authors of Exhibit Ko 8 written in 1997 was Dr. Krämer. Exhibit Ko 8 cited the above Exhibits Otsu 39 and 40 and, as mentioned below, concluded that the efflux of glutamic acid observed in the experiment in Exhibit Ko 8

is due to a carrier (Exhibit Ko 8, Exhibits Otsu 39, 40, and 42).

B. In connection with the above A, the Plaintiffs assert that taking into consideration the evidences (Exhibits Ko 47 to 50), it was well known as of the priority date of the present case that in Corynebacterium glutamicum, the efflux of glutamic acid occurs from the osmoregulated channel in response to osmotic pressure.

However, Exhibit Ko 47 merely states that "Relevant to our studies are observations that under special conditions E. coli excretes trehalose (Styrvold and Strem 1991) and that Corynebacterium glutamicum excretes glutamate (Shiio et al. 1962)." Based on this statement only, it is insufficient to find that there was common technical knowledge as the Plaintiffs assert.

In addition, both Documents of Exhibits Ko 48 and 49 relate to Escherichia coli, from which it is not possible to find the existence of common technical knowledge on efflux of glutamic acid in coryneform bacterium including Corynebacterium glutamicum and the like.

Exhibit Ko 50 states that in relation to the Figure on page 5, efflux of compatible solutes at low osmotic pressure in Corynebacterium glutamicum occurs through at least three kinds of mechanoreceptive channels (osmoregulated channels). However, taking into consideration the statement of Exhibit Ko 8 mentioned below, the efflux through the osmoregulated channel does not occur with regard to all solutes equally, but occurs with regard to the specific solutes selectively. Thus, it cannot be deemed that whether glutamic acid is included in the above "compatible solute" to be excreted has been necessarily made clear from the above Figure alone. Therefore, from Exhibit Ko 50, it is not possible to find the existence of common technical knowledge asserted by the Plaintiffs.

In view of the foregoing, there are no evidences sufficient to find the above assertions made by the Plaintiffs.

(2) Erroneous Determinations on Invention of Exhibit Ko 8 (Reason 2 for Rescission)

In light of the factual background of the above (1), as the Plaintiffs assert in Exhibit Ko 8, it is considered below whether it is possible to find that efflux of glutamic acid occurred from the osmoregulated channel.

A. Matters Stated in Exhibit Ko 8 (Exhibit Ko 8, Exhibits Otsu 31 and 31-2)

(A) Abstract

a. Page 572, lines 1 to 6.

"Bacteria respond to hypoosmotic stress by releasing low-molecular-mass solutes in order to maintain constant turgor pressure. We have studied the function

of osmoregulated channel(s) in Corynebacterium glutamicum, which are responsible for efflux of various solutes upon sudden decrease in osmotic pressure. The channels preferentially mediated efflux of compatible solutes such as glycine betaine and proline. The release of molecules of similar size, e.g. glutamate or lysine, was restricted, ATP was completely retained even after severe osmotic shock."

b. Page 572, lines 13 to 14.

"These results indicate the presence of an osmoregulated channel in C. glutamicum similar to the mechanosensitive channel(s) of Escherichia coli."

(B) Results

a. Table 1 on page 575

Efflux solute	Analytical method	Solute retained after dilution to osmolality of					
		before	1860	1060	860	710	540
		µmol · mg dm⁻¹ (%)					
Gly betaine	sc. count	0.92	0.90 (98)	0.69	0.56	0.43	0.28 (30)
Proline	sc. count	0.94	0.91 (97)	0.63	0.50	0.38	0.27 (29)
Ectoine	NMR	0.52	0.51 (98)	0.50	0.46	0.44	0.29 (56)
Gly betaine[a]	NMR	0.35	–	–	0.15	0.11	0.01 (3)
+ ectoine[a]	NMR	0.45	–	–	0.34	0.32	0.23 (51)
Alanine[d]	HPLC	0.32	0.32 (100)	0.22	0.20	0.20	0.15 (47)
Glutamate[d]	enzyme	0.46	0.42 (91)	0.40	0.41	–	0.37 (80)
Lysine[d]	HPLC	0.20	0.18 (90)	0.15	0.16	0.15	0.15 (75)
ATP[b]	luciferase	0.007	0.007 (100)	0.007	0.007	0.007	0.007 (99)
Na^+ [c,d]	flame ph.	0.57	0.22 (39)	–	0.21	0.17	0.16 (28)
K^+ [c,d]	flame ph.	1.03	0.95 (92)	–	0.86	0.79	0.67 (65)

b. Page 575, right column, lines 19 to page 576, left column, line 1

"The data in Table 1 demonstrate that osmotically triggered solute efflux in C. glutamicum shows a clear preference for particular solutes. ... The data in Table 1 prove that glycine betaine is preferred over ectoine with respect to osmotically triggered efflux. ... The extent of alanine exit was similar to that of ectoine, whereas efflux of glutamate and lysine was significantly restricted".

c. Page 576, left column, lines 43 to 52.

"To make sure that all major efflux solutes have been taken into consideration when measuring efflux by chemical or radiochemical methods in the present study, we analyzed the external medium of cells by ^{1}HNMR after hypoosmotic shock ... . The obtained spectra proved that glycine betaine, proline, and ectoine were the major compounds released by the cell in the corresponding experiments. Besides traces of lactate, no other compounds could be detected in these experiments in significant amounts."

(C) Discussion

a. Page 578, left column, lines 50 to 60.

"Efflux of low-molecular-mass compounds can in principle be mediated by a membrane leak, by a proteinaceous channel, or by an efflux carrier. Examples for all three possibilities have been described. The possibility of a simple membrane leak was eliminated for C. glutamicum by the finding that the efflux showed a distinct preference for particular substrates. The involvement of a carrier was shown to be very unlikely by several experimental results. The efflux rate is extremely high; i.e., far above all carriers described so far in C. glutamicum, (the fastest one being the fully induced betaine uptake carrier (BetP) with 110 μmol-min^{-1}-g dm^{-1} (Farwick et al., 1995))."

b. Page 578, right column, lines 26 to 40

"An interesting property of this system is its preference for particular solutes. Under severe osmotic shock, more or less, all low-molecular-mass compounds are released in E. coli. This is not the case in C. glutamicum where predominantly efflux of compatible solutes is observed. Molecules related in size to glycine betaine and proline, e.g. glutamic acid, or even small inorganic ions (Na^+, K^+), obviously do not use this channel to the same extent as glycine betaine or proline. A molecule like ATP, which is also released in E. coli after severe hypoosmotic shock (Berrier et al., 1992), is fully retained in C. glutamicum. Consequently, the properties of this channel must be different from the E. coli system(s). As long as the molecular identity of the channel in C. glutamicum is not known, however, the observed sequence of permeability through the C. glutamicum channel can also be explained by a multiplicity of channels with different specificities."

c. Page 578, right column, lines 46 to 55

"The lack of sensitivity of this channel to Gd^{3+}, an unspecific channel blocker (Berrier et al., 1992; Schleyer et al., 1993; Hase et al., 1995), furthermore defines it as being different from the Gd^{3+}-sensitive MscL channel, described and cloned in E. coli (Sukharev et al., 1994). There are, however, other examples where this unspecific blocking agent did not work (Berrier et al., 1996). Thus, the channel in C. glutamicum may be similar to another type identified in E. coli by using electrophysiological techniques; i.e., the MscS channel (Martinac at al., 1987, 1990; Zoratti and Petronilli, 1988; Berrier et al., 1996)."

d. Page 579, left column, lines 39 to 46.

"Finally, it should be emphasized here that the described efflux channel is not related to the well-known glutamate efflux in C. glutamicum observed under particular metabolic conditions. Although glutamate efflux under conditions of

continuous production of this glutamic acid seems to respond to osmotic changes with respect to its activity (Lambert et al., 1995), it is mediated by an energy-dependent, specific carrier system, as has been shown before (Gutmann et al., 1992; Krämer, 1994)."

B. Consideration

Exhibit Ko 8 discloses in Table 1 that efflux of glutamic acid occurs from osmoregulated channel when hypoosmotic conditions have occurred, as mentioned above. Even taking as a premise the plaintiffs' assertion that the efflux amount should be considered on the basis of the "before" value, the efflux of glutamic acid rarely occurred until the osmotic pressure reached 540 mOsm, and then, efflux occurred merely in 20% at 540 mOsm, which is the second smallest value after ATP among the 11 solutes studied. Based on the results of Table 1 as above, Dr. Krämer and other authors of Exhibit Ko 8 state that with regard to solutes such as glycine betaine of which efflux occurs with a high percentage, such efflux occurred from the osmoregulated channel, whereas they drew a conclusion that efflux of glutamic acid was not due to the osmoregulated channel, but due to the carrier.

With regard to lysine, which was observed to be the second most restricted efflux after glutamic acid in Table 1, as found in the above (1)A(C), by the time of the priority date of the present case, Dr. Krämer and others had discovered a carrier for transport of lysine, and proposed that the efflux of glutamic acid, as well as that of lysine, etc., was due to a carrier. Dr. Krämer performed experiments himself and analyzed the results of Table 1 to draw the conclusion as mentioned above as one of co-writer of Exhibit Ko 8. Taking this into consideration, it is unlikely that a person skilled in the art who have read Exhibit Ko 8 would conceive of a different conclusion.

In view of the foregoing, it should not be deemed that a person ordinarily skilled in the art recognizes the osmoregulated channel stated in Exhibit Ko 8 in connection with efflux of glutamic acid on the basis of Table 1 as the Plaintiffs assert.

Further, in view of the disclosure of Exhibit Ko 8 as found in the above A, the trial decision of the present case did not err in finding the Invention of Exhibit Ko 8. Therefore, Reason 2 for Rescission asserted by the Plaintiffs is unfounded.

(3) Erroneous Determinations on Whether the Present Invention Would be Easily Conceivable (Reason 3 for Rescission)

It can be found that there are the common feature and the different feature between Present Invention 1 and the Invention of Exhibit Ko 8 as mentioned in the above No. 2, 3(1)B as found in the trial decision of the present case.

Whether the above different feature would be easily conceivable is considered

below. As considered in the above (1) and (2), it cannot be found that it was well known to a person ordinarily skilled in the art as of the priority date of the present case that an osmoregulated channel is involved in efflux of glutamic acid in a coryneform bacterium such as Corynebacterium glutamicum. In addition, it also cannot be found that Exhibit Ko 8 discloses that the osmoregulated channel is involved in the efflux of glutamic acid.

The Plaintiffs assert that the configuration of the 19-type mutation can be obtained by applying Exhibits Ko 10 and 13 to 15, the well-known art, and the common technical knowledge to the Invention of Exhibit Ko 8. However, the disclosures of Exhibits Ko 10 and 13 to 15 do not suggest that efflux of glutamic acid occurs from an osmoregulated channel in a coryneform bacterium. In addition, it cannot be found that there was any other well-known art or common technical knowledge that supports such suggestion.

Thus, it should be deemed that a person ordinarily skilled in the art would not be motivated to apply Exhibits Ko 10 and 13 to 15, the well-known art, and the common technical knowledge to Invention of Exhibit Ko 8, and thus the person ordinarily skilled in the art would not be motivated to focus on a yggB gene encoding an osmoregulated channel in a coryneform bacterium and to introduce a mutation so as to promote efflux of glutamic acid.

Other assertions made by the Plaintiffs on this point do not affect the above findings at all.

Therefore, the trial decision of the present case did not err in determining that the above different feature would not be easily conceivable.

(4) Whether Present Invention 4 Would be Easily Conceivable

Present Invention 4 is an invention of a coryneform bacterium, in which a mutant-type yggB gene is introduced into the coryneform bacterium, in which the mutation in the mutant-type yggB gene is "a mutation in which alanine at position 100 is substituted with threonine, and/or, alanine at position 111 is substituted with threonine or valine, in an amino acid sequence of SEQ ID NOS: 6, 62, 68, 84, or 85" which is a more specific concept of the mutation (ii) of Present Invention 1 which is "substitution, deletion, or insertion of one to five amino acids in a region selected from the group consisting of amino acid numbers 1 to 23, 86 to 108, and 110 to 132 of an amino acid sequence of SEQ ID NOS: 6, 62, 68, 84, or 85." Therefore, since it cannot be deemed that Present Invention 1 would be easily conceivable to a person ordinarily skilled in the art from Exhibits Ko 8, 10, and 13 to 15, the well-known art, and the common technical knowledge, it also cannot be deemed that Present Invention

4 would be easily conceivable.

(5) Whether Present Inventions 6, 7, and 9 to 11 Would be Easily Conceivable

Present Inventions 6, 7, and 9 to 11 are inventions to which Present Invention 1 is further limited. Therefore, as mentioned in the above (3), since it cannot be deemed that Present Invention 1 would be easily conceivable to a person ordinarily skilled in the art from Exhibits Ko 8, 10, and 13 to 15, the well-known art, and the common technical knowledge, it also cannot be deemed that all of Present Inventions 6, 7, and 9 to 11 would be easily conceivable.

With regard to Present Invention 11, the Plaintiffs assert that Present Invention 11 lacks an inventive step, since the 19-type mutation does not enhance a glutamic acid-producing ability under the non-inducing conditions. However, as stated in the above 2, the 19-type mutation produces more glutamic acid than does a non-modified strain even under non-inducing conditions. Therefore, this assertion is not acceptable.

(6) Whether Present Invention 12 Would be Easily Conceivable

As considered in the above (2), it cannot be found that Exhibit Ko 8 discloses that efflux of glutamic acid occurs from osmoregulated channel. Therefore, the trial decision of the present case did not err in finding the different feature between Present Invention 12 and the Invention of Exhibit Ko 8 as mentioned in the above No. 2, 3(1)D.

Further, as mentioned in the above (3), a person ordinarily skilled in the art would not be motivated to apply Exhibits Ko 10 and 13 to 15, the well-known art, and the common technical knowledge to the Invention of Exhibit Ko 8, and thus the person ordinarily skilled in the art would not be motivated to focus on a yggB gene which encodes an osmoregulated channel in a coryneform bacterium and to introduce a mutation so as to promote efflux of glutamic acid.

Therefore, it cannot be deemed that Present Invention 12 would be easily conceivable by applying Exhibits Ko 10 and 13 to 15, the well-known art, and the common technical knowledge to the Invention of Exhibit Ko 8.

(7) Summary

In view of the foregoing, without determining the remainder of the other issues, both of Reasons 2 and 3 for Rescission asserted by the plaintiffs are unfounded.

4. Erroneous Findings and Determinations on the Violations of the Enablement Requirement and the Support Requirement with Regard to Other than 19-Type Mutation (Reason 4 for Rescission)

(1) 2A-1-Type Mutation ((i) and (i') of Present Invention 1)

A. In light of consideration in the above 1 and 3, it can be deemed that a technical idea of the Present Invention is to introduce a mutation into a yggB gene to modify a YggB protein, to thereby produce a strain which promotes efflux of glutamic acid, based on the finding that the YggB protein, which is an osmoregulated channel encoded by the yggB gene, is involved in efflux of L-glutamic acid production in a coryneform bacterium.

Further, in Present Invention 1, as a mutation to be introduced into a yggB gene, from viewpoints of a gene region and a transmembrane, two kinds of mutations are distinguished and shown as follows: [i] a mutation which is introduced into a portion which encodes the C-terminal region of the yggB gene (2A-1-type mutation [(i) and (i') of the Present Invention 1], 66-type mutation and 22-type mutation [(i'') of the Present Invention 1]. C-terminal side mutation); and [ii] a mutation which is introduced into a portion which encodes the first, the fourth, and the fifth transmembrane regions of the yggB gene. ((ii) of Present Invention 1. Mutation of the transmembrane region).

B. Among the mutations to be introduced into the yggB gene, the present description states in paragraph [0070] that C-terminal side mutation is generally explained, and in paragraph [0071] that with regard to 2A-1-type mutation, a mutation in which the region positioned downstream from position 419 in the C-terminal region is substituted with five amino acids as well as a mutation in which C-terminal region of SEQ ID NOS: 6, 62, 68, 84, and 85 is deleted or substituted. The present description further states in Working Examples 5 and 6 that it was confirmed that a glutamic acid-producing ability was enhanced by the 2A-1-type mutation in which the region positioned downstream from position 419 in the C-terminal region is substituted with five amino acids (paragraphs [0107] to [0113]).

Based on the basic technical idea as mentioned in the above A, it can be found that a person ordinarily skilled in the art who has read the statement of present description recognizes that with regard to 2A-1-type mutation ((i) and (i') of Present Invention 1), a three-dimensional structure of the C-terminal region is modified by introducing the mutation such as deletion or substitution in the C-terminal region, to thereby promote efflux of glutamic acid and enhance a producing ability. In addition, it can be deemed that taking a hint from the present description, a person ordinarily skilled in the art can work the mutations (i) and (i') of Present Invention 1 according to the 2A-1-type mutation without undue trial-and-error.

C. The Plaintiffs assert that the 2A-1-type mutation (mutation of (i) and (i') of Present Invention 1) includes embodiments of mutations other than deletion or

substitution with five amino acids as stated in the present description, and that it is unpredictable whether such mutations can also achieve the same effect, and there are no Working Examples of such deletion.

However, as mentioned above, with regard to the 2A-1-type mutation, it can be deemed that from the statement of the present description, a person ordinarily skilled in the art can recognize the basic principle that a glutamic acid-producing ability can be enhanced by modifying the three-dimensional structure of the C-terminal region.

Further, as mentioned above, the present description discloses in Working Examples 5 and 6 that a producing ability was enhanced by the 2A-1-type mutation in which the region positioned downstream from position 419 in the C-terminal region is substituted with a short amino acid residue consisting of five amino acids. Thus, in view of the embodiment of the mutation, a person ordinarily skilled in the art can recognize that the producing ability was enhanced by modifying the three-dimensional structure of the C-terminal region, and even if the C-terminal region is deleted, the person ordinarily skilled in the art can recognize that the three-dimensional structure of the C-terminal region is modified and can predict that a glutamic acid-producing ability will be enhanced.

In addition to the above, in view of the fact that the region into which the mutation is introduced is subject to certain limitations as well, it cannot be deemed that embodiments other than those which are not disclosed in the present description lack the support requirement and the enablement requirement. Therefore, the Plaintiffs' assertion as mentioned above is not acceptable.

(2) 66-Type Mutation and 22-type Mutation (Mutation (i") of Present Invention 1)

A. The present description discloses in paragraph [0072] twelve substitutable prolines in the C-terminal side of the yggB gene, and that it is considered that the same prolines play an important role in maintaining a three-dimensional structure of the YggB protein. In addition, the present description states in Working Examples 12 and 13 that proline at position 424 was substituted with leucine (66-type mutation) and proline at position 437 was substituted with serine (22-type mutation), to thereby produce a mutant strain, and in Working Example 13 that the 22-type mutant strain actually enhanced an L-glutamic acid-producing ability (paragraphs [0130] to [0138]).

In addition to the foregoing statement of the present description, according to the evidences (Exhibits Otsu 19 to 22) and the entire import of the oral argument, it can be deemed that it is well known to a person ordinarily skilled in the art that proline is generally an amino acid having a special property which is involved in the

formation of a three-dimensional structure of a protein, which is consistent with the above statement in paragraph [0072] of the present description. Therefore, it can be deemed that a person ordinarily skilled in the art can recognize that, based on the basic technical idea of the Present Invention as mentioned in the above (1), the problem of the Present Invention can be solved by substituting proline in the C-terminal region with another amino acid, to thereby modify the three-dimensional structure of the C-terminal region and to promote efflux of glutamic acid from YggB protein. In addition, it can be found that from the present description and the common technical knowledge, a person ordinarily skilled in the art can work the mutation (i'') of Present Invention 1 without undue trial-and-error.

B. The Plaintiffs assert that it is necessary for a person ordinarily skilled in the art to confirm that each substitution of proline results in enhancement of an L-glutamic acid-producing ability one after another, since whether most prolines contribute to the three-dimensional structure formation has not been investigated.

However, as mentioned above, the basic principle was made clear. In addition, the number of substitutable prolines is limited. Therefore, it cannot be deemed that the points asserted by the Plaintiffs cause undue trial-and-error.

(3) Mutation in Transmembrane Region (Mutation (ii) of Present Invention 1)

A. Among the mutations to be introduced in the yggB gene, the mutation in the transmembrane regions in Present Invention 1 is a mutation in which one to five amino acids are substituted, deleted, or inserted in each region of amino acid numbers 1 to 23 (the first transmembrane region), amino acid numbers 86 to 108 (the fourth transmembrane region), and 110 to 132 (the fifth transmembrane region) of amino acid sequences.

In the present description, one or two Working Examples of mutations are stated corresponding to each transmembrane region (paragraphs [0074] to [0076]), as follows: as an example of the mutation in the first transmembrane region, the A1-type mutation (a mutation in which cysteine, serine, and leucine are inserted between leucine at position 14 and tryptophan at position 15 of SEQ ID NO: 6) is stated; as an example of the mutation in the fourth transmembrane region, the 19-type mutation (alanine at position 100 is substituted with threonine in the amino acid sequence of SEQ ID NO: 6) is stated; and as examples of the mutation in the fifth transmembrane region, the L30-type mutation (alanine at position 111 is substituted with valine in the amino acid sequence of SEQ ID NO: 6) and 8-type mutation (alanine at position 111 is substituted with threonine in the amino acid sequence of SEQ ID NO: 62) are stated.

In addition, the present description states, as Working Example 7, that the

same strain as that produced by the method for producing the A1-type mutant strain enhanced glutamic acid-producing ability (paragraphs [0114] to [0118]); as Working Example 8, that the same strain as that produced by the method for producing the 19-type mutant strain enhanced glutamic acid-producing ability (paragraphs [0119] to [0121]); as Working Example 9, that the same strain as that produced by the method for producing the L30-type mutant strain enhanced glutamic acid-producing ability (paragraphs [0122] to [0124]); and as Working Example 11, the method for producing the 8-type mutant strain (paragraphs [0129]).

As described in (1) above, the basic technical idea of the Present Invention is to modify a YggB protein forming an osmoregulated channel by introducing a mutation into a yggB gene, to thereby produce a strain which promotes efflux of glutamic acid, and (ii) of the Present Invention 1 is to modify transmembrane region, to thereby modify the three-dimensional structure of the transmembrane region. As mentioned above, the basic principles for solving the problem were made clear. In addition, one or two Working Examples in which a glutamic acid-producing ability was enhanced are stated for each of the three transmembrane regions. Further, the region into which the mutation is introduced is limited, and the number of amino acids which are substituted, deleted, or inserted is limited to one to five. Therefore, it can be found that a person ordinarily skilled in the art recognized that (ii) of Present Invention 1 can solve the problem of Present Invention 1. In addition, it can be found that, taking a hint from each statement of the present description, a person ordinarily skilled in the art can work the mutation (ii) of Present Invention 1 without undue trial-and-error.

B. The Plaintiffs assert that it requires undue trial-and-error to construct a lot of coryneform bacteria and to measure a producing ability thereof.

However, the basic principle was made clear, as mentioned in the above A. In addition, the region into which the mutation is introduced is limited, and the number of amino acids of the mutation such as substitution is also limited. Therefore, it cannot be deemed that the points asserted by the Plaintiffs cause undue trial-and-error.

(4) Amino Acid Sequence of SEQ ID NO: 85

A. In paragraph [0035] of the present description, with respect to the amino acid sequence of SEQ ID NO: 85, the locations which may be substituted or deleted are indicated by Xaa. According to the evidences (Exhibits Otsu 23 and 26), it is stated that amino acid substitutions do not occur during the evolution process at an active center of a protein or at sites of functional importance in the protein (functional

constraints on protein). On the other hand, it is stated that substitutions between amino acids having similar size and similar polarity are observed at sites other than the above sites, so as to conserve a three-dimensional structure of the protein (conservative substitution). It can be found that these statements were well known to a person ordinarily skilled in the art. In addition to this, the present description states in paragraphs [0034] and [0078] that "the above substitution is preferably a conservative substitution", and discloses the specific examples of the conservative substitution. Taking this into consideration as well, it can be deemed that a person ordinarily skilled in the art recognize that the location indicated by Xaa in SEQ ID NO: 85 is not functionally important for the protein and is a site where substitution and deletion are allowable, and that with regard to what substitutions are allowable, a person ordinarily skilled in the art recognize can recognize a certain range of substitutions, based on the significance of the conservative substitution as mentioned above.

Further, as a mutation to be introduced into a yggB gene, the present description states the C-terminal side mutation and the transmembrane region mutation of the amino acid sequence of SEQ ID NO: 85 as well as other sequences as mutation to be introduced to yggB gene, and further states other Working Examples with regard to other sequences, as mentioned in the above (1) to (3). Taking this into consideration as well, it can be found that with regard to the amino acid sequence of SEQ ID NO: 85 of Present Invention 1, even if there are no Working Examples in the present description, a person ordinarily skilled in the art recognizes that the location indicated by Xaa in SEQ ID NO: 85 is a site where substitution and deletion are allowable, and such a site is included in a certain range of substitutions, based on the significance of the conservative substitution, and a person ordinarily skilled in the art recognizes that when the mutations (i), (i'), (i''), and (ii) of Present Invention 1 are introduced, the problem of Present Invention 1 can be solved, and that the person ordinarily skilled in the art can work Present Invention 1 without undue trial-and-error.

B. The Plaintiffs assert that substitution, deletion, and insertion of proline, which is involved in the formation of a three-dimensional structure of a protein, causes significant change of the three-dimensional structure of the protein, and as a result, the protein might no longer function as an osmoregulated channel. In addition, the Plaintiffs assert that the number of possible variations of SEQ ID NO: 85 is enormous.

However, it can be found that based on the knowledge of conservative substitution in the above A, a person ordinarily skilled in the art recognizes that

substitution without limitation is not possible, and that substitutions which cause a loss of function as an osmoregulated channel are not included in the scope of Present Invention 1. Further, with regard to the number of possible variations of SEQ ID NO: 85, it can be found that such variations are limited to a certain range. Therefore, the Plaintiffs' assertion is not acceptable and does not affect the finding in the above A.

(5) Summary

In view of the foregoing, it cannot be deemed that Present Invention violates the support requirement and the enablement requirement, based on the grounds asserted by the Plaintiffs in the lawsuit of the present case. Therefore, Reason 4 for Rescission asserted by the Plaintiffs is unfounded.

5. Erroneous Findings and Determinations on the Clarity Requirement Violation (Reason 5 for Rescission)

(1) Statement of Present Inventions 6 and 12

A. Whether or not an invention for which a patent is sought is clear should be determined from the viewpoint of whether or not the claims are so unclear as to cause unforeseen disadvantages to third parties, taking into consideration the statement of the claims as well as the statement of the description and the drawings, and based on common technical knowledge of a person ordinarily skilled in the art at the time of filing the application.

The present description states in paragraph [0032] that the "'an excess of biotin-containing condition' means, for example, a condition containing biotin of 30 μg/L or more, preferably 40 μg/L or more, and more preferably 50 μg/L or more, in the medium."

In addition, as found in the above 2(1), glutamic acid has been industrially produced by the fermentation method using coryneform bacteria since around the 1960's. It can be found that until strains capable of producing glutamic acid even in the presence of sufficient biotin were developed, the production of glutamic acid under biotin-limiting condition was widely performed as one of the inducing conditions. Thus, taking into consideration the combination of the present description in the above 1(1) with this finding, it can be found that Present Inventions 6 and 12 are inventions which can enhance a producing ability even under non-inducing conditions.

Therefore, it can be found that a person ordinarily skilled in the art can understand that the phrase "excess of biotin" in Present Inventions 6 and 12 refers to an amount of biotin which does not satisfy the inducing conditions as stated in

paragraph [0032] of the present description.

In view of the foregoing, it cannot be deemed that Present Inventions 6 and 12 lacks the clarity requirement.

B. The Plaintiffs assert that the matters stated in paragraph [0032] of the present description are not stated in [Claim 6] and therefore Present Invention 6 is not clear. However, as mentioned in the above A, when determining whether or not the statement of the claims is unclear, there is no case where it is not allowable to take the statements of the description or common technical knowledge into consideration. Therefore, the above assertion is not acceptable.

(2) Statement of Present Invention 7

A. With regard to "L-glutamic acid analogue" as stated in Present Invention 7, in paragraph [0068] of the present description, thirteen specific substances which correspond to "L-glutamic acid analogue" are given. In addition, the present description states Working Examples with regard to a strain having enhanced resistance to 4-fluoroglutamic acid which is one of the listed substances in paragraphs [0139] and [0140]. Thus, it can be deemed that a person ordinarily skilled in the art can understand what kind of substances are included in the "L-glutamic acid analogue." Therefore, it cannot be deemed that Present Invention 7 violates the clarity requirement.

B. The Plaintiffs assert that in paragraphs [0068], [0139], and [0140] of the present description, only a portion of substances included in the "L-glutamic acid analogue" is listed, and therefore Present Invention 7 is not clear. However, whether or not an invention violates the clarity requirement should be determined from the viewpoint of whether or not the claims are so unclear as to cause unforeseen disadvantages to third parties. In the present case, since a lot of specific substances are listed as in the above A, it can be deemed that a person ordinarily skilled in the art can recognize what kind of substances are included in "L-glutamic acid analogue".

Therefore, the above Plaintiffs' assertion is not acceptable.

(3) Statement of Present Invention 8

A. The present description states in paragraph [0082] that "Examples of the gene which suppresses a function of a mutant-type yggB gene include a symA gene (supresser of yggB mutation) regarding the symA gene in Claim 8. The symA gene is encoded in nucleotide numbers 2051306 to 2051845 of the genome sequence registered as Genbank Accession No. NC_003450 of Corynebacterium glutamicum ATCC13032, and is registered as NCgl 1867 (NP_601149. hypothetical prot...[gi:19553147]). The symA gene of C. glutamicum ATCC13869 is shown in

nucleotide numbers 585 to 1121 of SEQ ID NO: 86", and further states a concrete method for producing symA-deleted strain in paragraph [0150]. It can be found that from these statements, a person ordinarily skilled in the art can clearly understands the contents stated in Claim 8. Therefore, it cannot be deemed that Present Invention 8 violates the clarity requirement.

B. The Plaintiffs assert that since Claim 8 states "(i) a DNA containing nucleotide sequence of nucleotide numbers 585 to 1121 of SEQ ID NO: 86", it is unclear what sequence is contained in the DNA other than the nucleotide sequence of nucleotide numbers 585 to 1121 of SEQ ID NO: 86, and therefore, the statement of Present Invention 8 is unclear. However, the meaning of "symA gene" is clear as mentioned above. Therefore, it cannot be deemed that Present Invention 8 violates the clarity requirement.

(4) Statement of Present Invention 9

A. With regard to the phrase "decrease an activity of α-ketoglutarate dehydrogenase" in Claim 9, the present description states in paragraph [0083] that "The phrase 'decrease an activity of α-ketoglutarate dehydrogenase' means that the α-KGDH activity is decreased as compared to that of a wild-type strain or a non-modified strain such as a parent strain." Thus, it can be deemed that a person ordinarily skilled in the art can understand the meaning.

Therefore, it cannot be deemed that Present Invention 9 violates the clarity requirement.

B. The Plaintiffs assert that if the comparison for "decrease" is to be made with a non-modified strain, the claim should be clearly defined as such. However, as mentioned in the above (1)A, when determining whether or not the statement of the claims is unclear, there is no case where it is not allowable to take the statements of the description into consideration. Therefore, the above assertion is not acceptable.

(5) Statement of Present Inventions 10 and 11

Both of Present Inventions 8 and 9 are clear as mentioned in the above (3) and (4). Therefore, it cannot be deemed that Present Inventions 10 and 11 which depend from Present Inventions 8 and 9 violate the clarity requirement.

(6) Statement of a Portion of Present Invention 6 which Depends from Claims 4 and 5

Claim 4 defines the 19-type mutation, the L30-type mutation, and the 8-type mutation stated in paragraphs [0075] and [0076] of the present description. Thus, a person ordinarily skilled in the art can understand that a portion which depends from Claim 4 in Present Invention 6 corresponds to mutant-type yggB genes (e) to (j), and

that this portion has no relationship with (a) to (d) and (k) to (n).

Similarly, Claim 5 defines the A1-type mutation stated in paragraph [0074] of the present description. Thus, a person ordinarily skilled in the art can understand that a portion which depends from Claim 5 in Present Invention 6 corresponds to mutant-type yggB genes (c) to (d), and that this portion has no relationship with (a), (b), and (e) to (n).

In view of the foregoing, it cannot be deemed that the statement of the portion of Present Invention 6 which depends from Claims 4 and 5 as well as Present Inventions 7 to 11 which depend from Claim 6 violate the clarity requirement.

(7) Summary

As mentioned above, it cannot be deemed that any of Present Inventions 6 to 12 violate the clarity requirement. Therefore, Reason 5 for Rescission asserted by the Plaintiffs is unfounded.

No. 5 Conclusion

For the foregoing reasons, the Plaintiffs' claims in both cases are groundless, and thus shall be dismissed. Therefore, the judgment is rendered as mentioned in the main text.

Intellectual Property High Court, Second Division

Presiding Judge MORI Yoshiyuki

Judge MANABE Mihoko

Judge KUMAGAI Daisuke