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(54) **METHOD FOR PRODUCING L-GLUTAMIC ACID BY FERMENTATION ACCOMPANIED BY PRECIPITATION**

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**Related U.S. Patent Documents**

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(52) **U.S. Cl.** ..... **435/110; 435/106**

(58) **Field of Classification Search** ..... None  
 See application file for complete search history.

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(57) **ABSTRACT**

A microorganism is provided which can metabolize a carbon source at a specific pH in a liquid medium containing L-glutamic acid at a saturation concentration and the carbon source, and which has ability to accumulate L-glutamic acid in an amount exceeding the amount corresponding to the saturation concentration in the liquid medium at the pH. Also provided is a method for producing L-glutamic acid by fermentation, which comprises culturing the microorganism in a liquid medium of which pH is adjusted to a pH at which L-glutamic acid is precipitated, to produce and accumulate L-glutamic acid and precipitate L-glutamic acid in the medium.

**7 Claims, 8 Drawing Sheets**

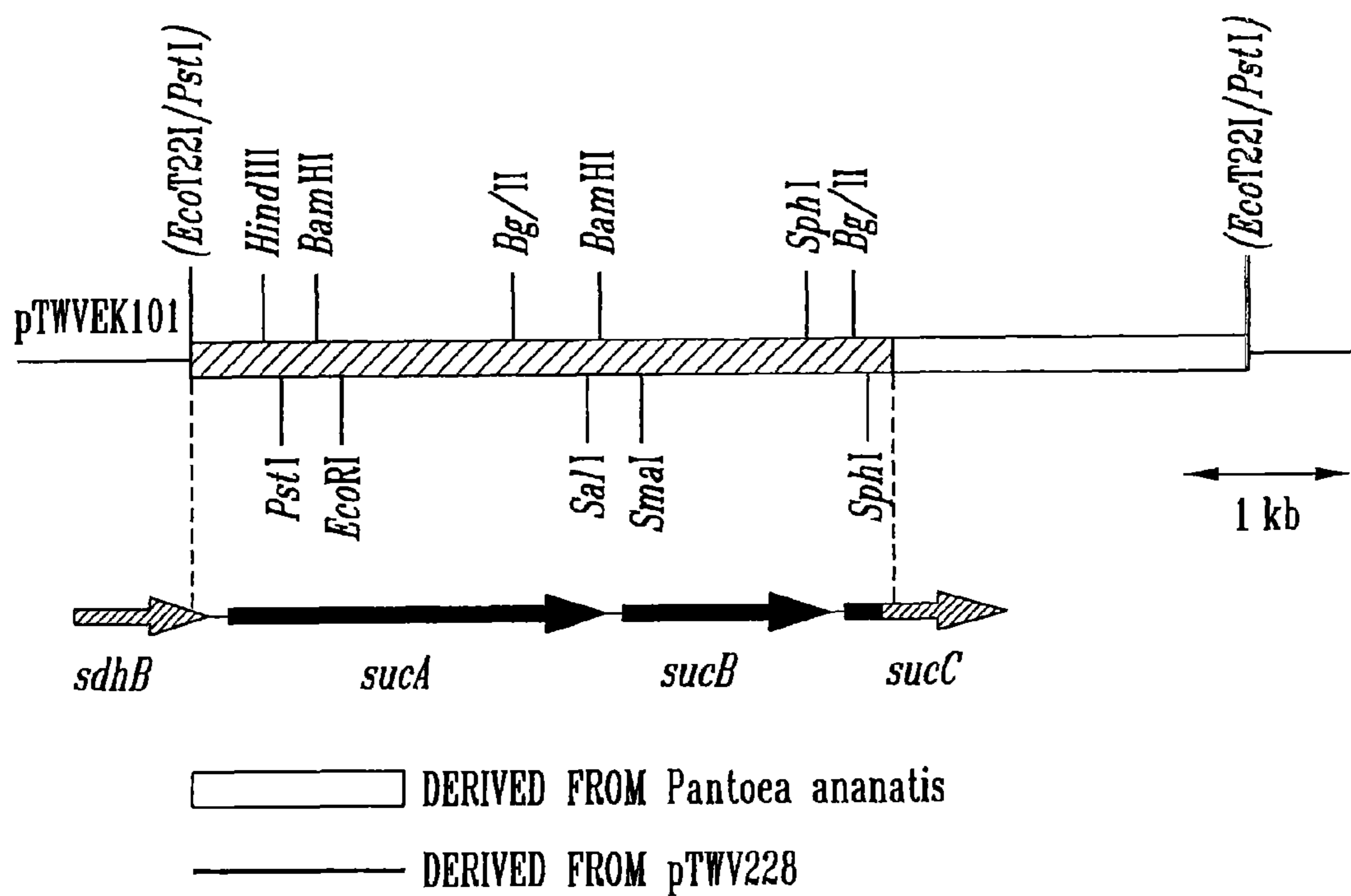
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*FIG. 1*  
AMENDED





481' KHPTPRKIYADRLEGEVASQEDATEMVNLYRDALDAGECVVPEWRPMSLHSFTWSPYLN  
 \*\*\*\*\* \*\* \* \*\* \*\*\*\*\* \*\* \* \*\* \*\*\*\*\*

479' KHPTPRKIYADKLEQEKVATLEDATEMVNLYRDALDAGDCVVAEWRPMMHSFTWSPYLN

541' HEWDEPYPAQVDMKRLKELALRISQVPEQIEVQSRVAKIYNDRKLMAGEKAFDWGGAEN  
 \*\*\*\*\* \*\* \* \*\* \*\*\*\*\* \*\* \* \*\* \*\*\*\*\*

539' HEWDEEYPNKVEMKRLQELAKRISTVPEAVEMQSRVAKIYGDRQAMAAGEKLDWGGAEN

601' LAYATLVDEGIPVRLSGEDSGRGTFFHRHAVVHNGANGSTYTPLHHIHNSQGEFKVWDSV  
 \*\*\*\*\* \*\* \* \*\* \*\*\*\*\* \*\* \* \*\* \*\*\*\*\*

599' LAYATLVDEGIPVRLSGEDSGRGTFFHRHAVIHNGSNGSTYTPLQHIHNGQGAFRVWDSV

661' LSEEAVLAFEYGYATAEPRVLT IWEAQFGDFANGAQVVIDQFISSGEQKWGRMCGLVMLL  
 \*\*\*\*\* \*\* \* \*\* \*\*\*\*\* \*\* \* \*\* \*\*\*\*\*

659' LSEEAVLAFEYGYATAEPRTL IWEAQFGDFANGAQVVIDQFISSGEQKWGRMCGLVMLL

721' PHGYEGQGPEHSSARLERYLQLCAEQNMQVCVPSTPAQVYHMLRRQALRGMRRPLVVMSP  
 \*\*\*\*\* \*\* \* \*\* \*\*\*\*\* \*\* \* \*\* \*\*\*\*\*

719' PHGYEGQGPEHSSARLERYLQLCAEQNMQVCVPSTPAQVYHMLRRQALRGMRRPLVVMSP

781' KSLLRHPLAISSLELANGSFQPAIGEIDDLDPQGVKRVVLCSGKVYYDLLEQRRKDEKT  
 \*\*\*\*\* \*\* \* \*\* \*\*\*\*\* \*\* \* \*\* \*\*\*\*\*

779' KSLLRHPLAVSSLEELANGTFLPAIGEIDELDPKGVKRVVMCSGKVYYDLLEQRRKNNQH

841' DVAIVRIEQLYPPHQAVQEALKAYSHVQDFVWCQEEPLNQGAWYCSQHFRDVVPPFGAT  
 \*\*\*\*\* \*\* \* \*\* \*\*\*\*\* \*\* \* \*\* \*\*\*\*\*

839' DVAIVRIEQLYPPHKAMQEVLLQQAHVKDFVWCQEEPLNQGAWYCSQHFRÉVÍPFGAS

901' LRYAGRPASASPAVGYSVHQQQQDLVNDALNVN  
 \*\*\*\*\* \*\* \* \*\* \*\*\*\*\*

899' LRYAGRPASASPAVGYSVHQQQQDLVNDALNVÉ

FIG. 2B



[88.2% / 407 aa]

1' MSSVDILVPDLPE SVADATVATWHKKPGDAVSRDEVIVEIETDKVVLEVPASADGVLEAV  
 \*\*\*\*\*  
 1' MSSVDILVPDLPE SVADATVATWHKKPGDAVVRDEVLVEIETDKVVLEVPASADGILDAV  
 61' LEDEGATVTSRQILGRLKEGNSAGKESSAKAESNDTTPAQRQTASLEEESDALSPAIRR  
 \*\*\*\*\*  
 61' LEDEGTTVTSRQILGRLREGNSAGKETS AKSEEKASTPAQRQQASLEEQNNDALSPAIRR  
 121' LIAEHNLDAAQIKGTGVGGRLTREDVEKHLANKPQAEKAAAPAAGAATAQQPVANRSEKR  
 \*\*\*\*\*  
 121' LLAEHNLDASA IKGTVGGRLTREDVEKHLAKAPAKE--SAPAAAAPAAQPALAAARSEKR  
 181' VPMTRLRKRVAERLLEAKNSTAMLTTFNEINMKPIMDLRKQYGD AFEKRGVRLGFMSFY  
 \*\*\*\*\*  
 179' VPMTRLRKRVAERLLEAKNSTAMLTTFNEVNMKPI MDLRKQYGEAF EKRHGIRLGFMSFY  
 241' IKAVVEALKRYPEVNASIDGEDVVYHNYFDVSI AVSTPRGLVTPVLRDVDALSMADIEKK  
 \*\*\*\*\*  
 239' VKAVVEALKRYPEVNASIDGDDVVYHNYFDVSM AVSTPRGLVTPVLRDVDTLGMADIEKK  
 301' IKELAVKGRDGKLTVDL TGGNFTITNGGVFGSLMSTPIINPPQSAILGMHA IKDRPMAV  
 \*\*\*\*\*  
 299' IKELAVKGRDGKLTVEDL TGGNFTITNGGVFGSLMSTPIINPPQSAILGMHA IKDRPMAV  
 361' NGQVVILPMMYLALS YDHRLIDGRESVGYLVAVKEMLEDPARLLLDV  
 \*\*\*\*\*  
 359' NGQVEILPMMYLALS YDHRLIDGRESVGFVTKELLEDPTRLLLDV

FIG. 3

[95.1% / 41 aa]

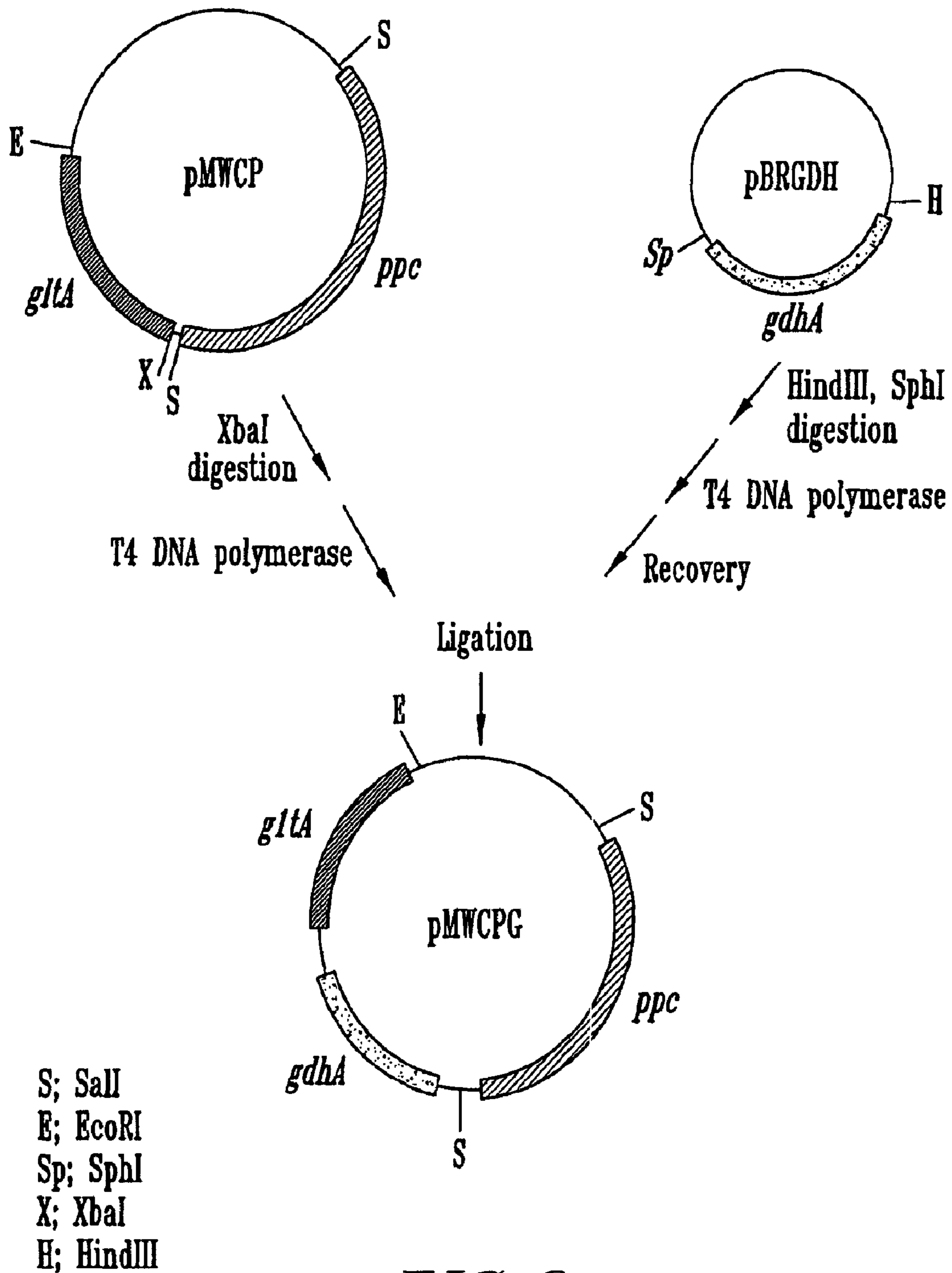
1' MNLHEYQAKQLFARYGMPAPTGYACTTPREAE EAASKIGAG  
 \*\*\*\*\*  
 1' MNLHEYQAKQLFARYGLPAPVGYACTTPREAE EAASKIGAGPWVVKCQVHAGGRGKAGGV

FIG. 4

[97.4% / 39 aa]

1' AFSVFRCHSIMNCVSVCPKGLNPTRAIGHIKSMLLQRSA  
 \*\*\*\*\*  
 181' FLIDSRDTETDSRLDGLSDAFSVFRCHSIMNCVSVCPKGLNPTRAIGHIKSMLLQRNA

FIG. 5



**FIG. 6**

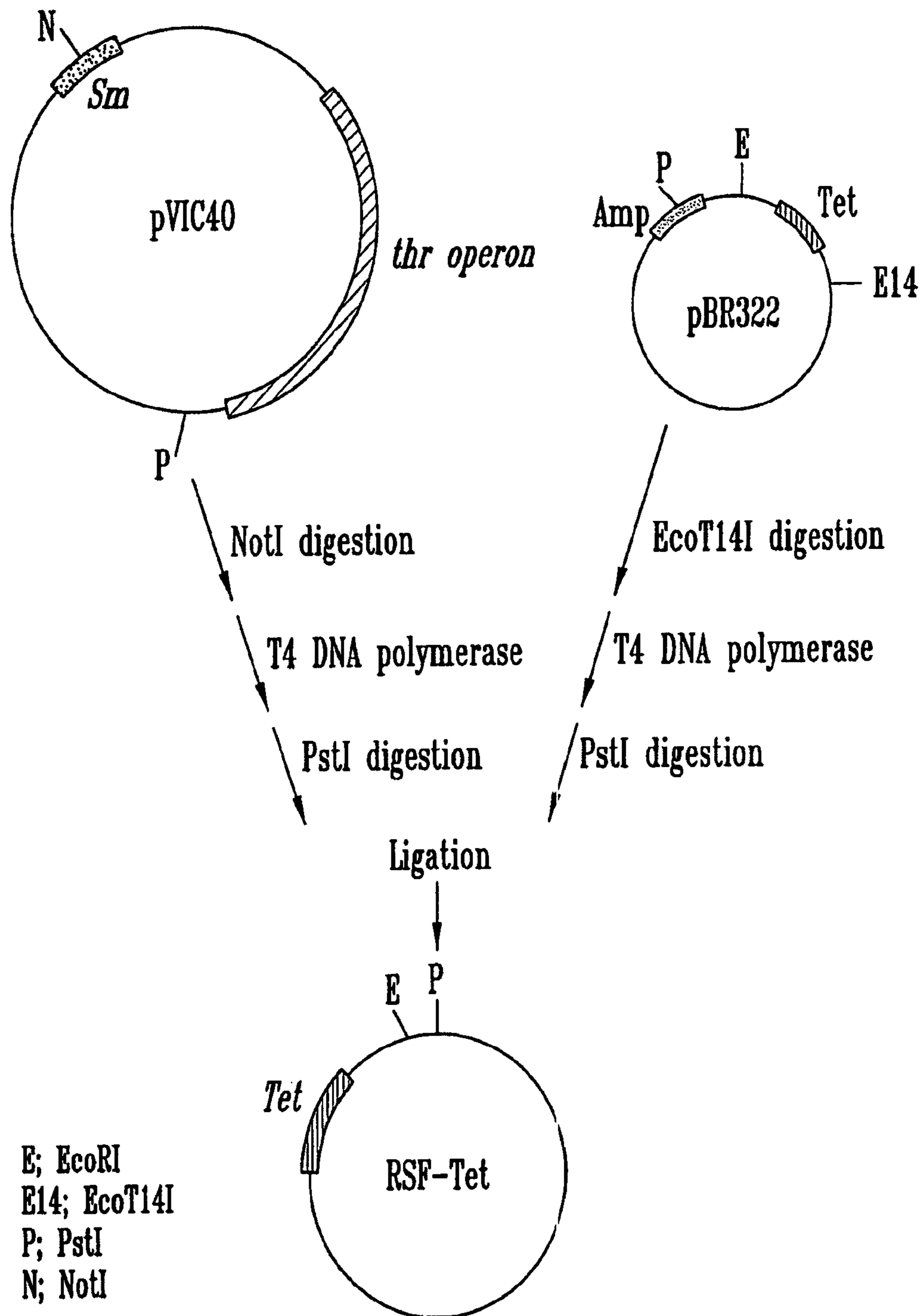
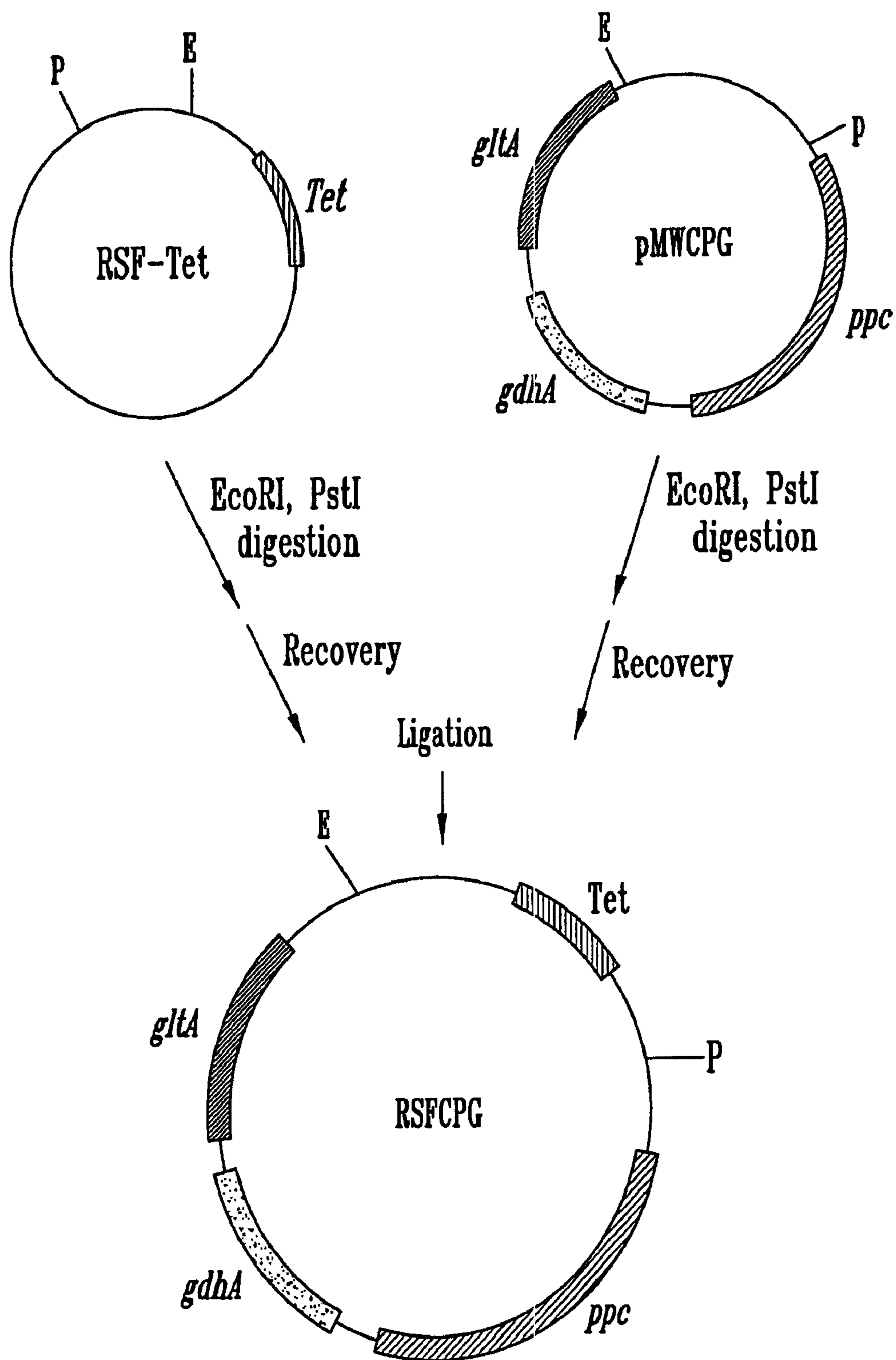


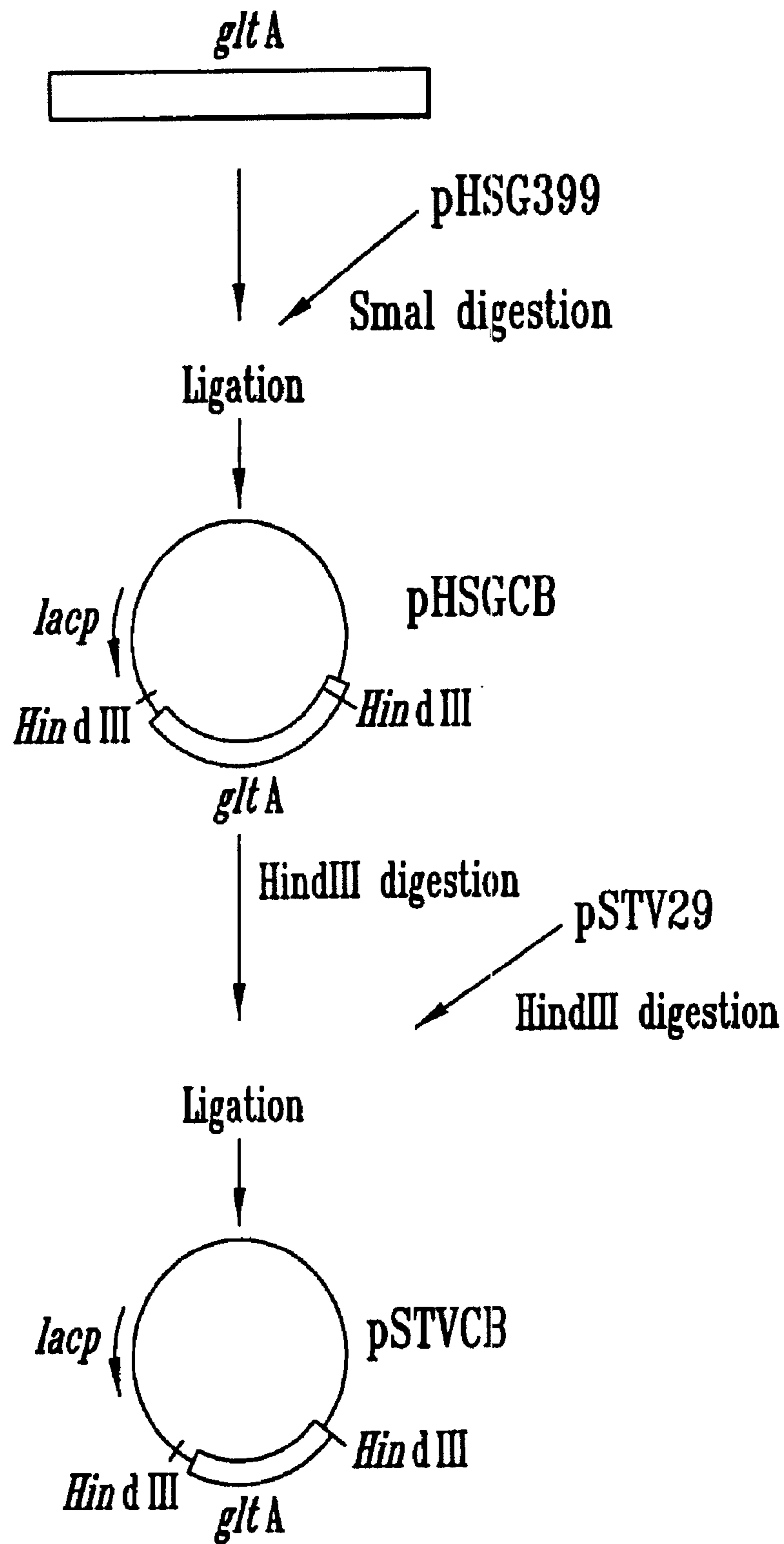
FIG. 7





E; EcoRI  
P; PstI

FIG. 8



**FIG. 9**



**METHOD FOR PRODUCING L-GLUTAMIC  
ACID BY FERMENTATION ACCOMPANIED  
BY PRECIPITATION**

**Matter enclosed in heavy brackets [ ] appears in the original patent but forms no part of this reissue specification; matter printed in italics indicates the additions made by reissue.**

CROSS-REFERENCE TO RELATED  
APPLICATIONS

The present application is a continuation of U.S. Ser. No. 09/641,892, filed on Aug. 18, 2000 (now U.S. Pat. No. 7,015,010), which claims priority to Japanese application No. JP 11-234806, filed on Aug. 20, 1999, and to Japanese application No. JP 2000-78771, filed on Mar. 21, 2000.

BACKGROUND OF THE INVENTION

The present invention relates to a method for producing L-glutamic acid by fermentation accompanied by precipitation. L-Glutamic acid is widely used as a material for seasonings and so forth.

L-Glutamic acid is mainly produced by fermentative methods using so-called coryneformbacteria producing L-glutamic acid, which belong to the genus *Brevibacterium*, *Corynebacterium* or *Microbacterium*, or mutant strains thereof (Amino Acid Fermentation, pp. 195-215, Gakkai Shuppan Center, 1986). Methods for producing L-glutamic acid by fermentation using other bacterial strains are known and include a method using a microorganism belonging to the genus *Bacillus*, *Streptomyces*, *Penicillium* or the like (U.S. Pat. No. 3,220,929), a method using a microorganism belonging to the genus *Pseudomonas*, *Arthrobacter*, *Serratia*, *Candida* or the like (U.S. Pat. No. 3,563,857), a method using a microorganism belonging to the genus *Bacillus*, *Pseudomonas*, *Serratia*, *Aerobacter aerogenes* (currently referred to as *Enterobacter aerogenes*) or the like (Japanese Patent Publication (Kokoku) No. 32-9393), a method using a mutant strain of *Escherichia coli* (Japanese Patent Application Laid-open (Kokai) No. 5-244970) and so forth. In addition, the inventors of the present invention have proposed a method for producing L-glutamic acid by using a microorganism belonging to the genus *Klebsiella*, *Erwinia* or *Pantoea* (Japanese Patent Application Laid-open No. 2000-106869).

Further, there have been disclosed various techniques for improving L-glutamic acid-producing ability by enhancing activities of L-glutamic acid biosynthetic enzymes through the use of recombinant DNA techniques. For example, it has been reported that the introduction of a gene coding for citrate synthase derived from *Escherichia coli* or *Corynebacterium glutamicum* was effective for the enhancement of L-glutamic acid-producing ability in *Corynebacterium* or *Brevibacterium* bacteria (Japanese Patent Publication No. 7-121228). In addition, Japanese Patent Application Laid-open No. 61-268185 discloses a cell harboring recombinant DNA containing a glutamate dehydrogenase gene derived from *Corynebacterium* bacteria. Further, Japanese Patent Application Laid-open No. 63-214189 discloses a technique for improving L-glutamic acid-producing ability by amplifying a glutamate dehydrogenase gene, an isocitrate dehydrogenase gene, an aconitate hydratase gene and a citrate synthase gene.

Although L-glutamic acid productivity has been considerably increased by breeding of the aforementioned microorganisms or improvement of production methods, develop-

ment of methods for more efficiently producing L-glutamic acid at a lower cost are still required to respond to the increasing future demand for L-glutamic acid.

A method wherein fermentation is performed with crystallizing L-amino acid accumulated in culture is known (Japanese Patent Application Laid-open No. 62-288). In this method, the L-amino acid concentration in the culture is maintained below a certain level by precipitating the accumulated L-amino acid in the culture. Specifically, L-tryptophan, L-tyrosine or L-leucine is precipitated during fermentation by adjusting the temperature and the pH of the culture or by adding a surface active agent to the medium.

While a fermentative method with precipitating L-amino acid is known as described above, amino acids suitable for this method are those of relatively low water solubility. No example exists for applying the method to highly water-soluble amino acids such as L-glutamic acid. In addition, the medium must have low pH to precipitate L-glutamic acid. However, L-glutamic acid-producing bacteria such as those mentioned above cannot grow under acidic conditions, and therefore L-glutamic acid fermentation is performed under neutral conditions (U.S. Pat. Nos. 3,220,929 and 3,032,474; Chao K. C. & Foster J. W., *J. Bacteriol.*, 77, pp. 715-725 (1959)). Thus, production of L-glutamic acid by fermentation accompanied by precipitation is not known.

Furthermore, it is known that growth of most acidophile bacteria is inhibited by organic acids such as acetic acid, lactic acid and succinic acid (Yasuro Oshima Ed., "Extreme Environment Microorganism Handbook", p. 231, Science Forum; Borichewski R. M., *J. Bacteriol.*, 93, pp. 597-599 (1967) etc.). Therefore, it is considered that many microorganisms are susceptible to L-glutamic acid, which is also an organic acid, under acidic conditions. There exists no report of microorganisms having L-glutamic acid-producing ability under acidic conditions has been attempted.

SUMMARY OF THE INVENTION

Based on the foregoing, an object of the present invention is to search and breed a microorganism that produces L-glutamic acid under low pH conditions and to provide a method for producing L-glutamic acid using an obtained microorganism by fermentation with precipitating L-glutamic acid.

The inventors of the present invention considered during the study for improvement of L-glutamic acid productivity by fermentation that inhibition of the production by L-glutamic acid accumulated in a medium at a high concentration was one of obstructions to the improvement of productivity. For example, cells have an excretory system and an uptake system for L-glutamic acid. However, if L-glutamic acid once excreted into the medium is incorporated into cells again, not only the production efficiency falls, but also the L-glutamic acid biosynthetic reactions are inhibited as a result. In order to avoid the inhibition of production by such accumulation of L-glutamic acid at high concentration, the inventors of the present invention screened microorganisms that can proliferate under acidic conditions and in the presence of a high concentration of L-glutamic acid. As a result, they successfully isolated microorganisms having such properties from a soil, and thus accomplished the present invention.

Thus, the present invention provides the following:  
(1) A microorganism which can metabolize a carbon source at a specific pH in a liquid medium containing L-glutamic acid at a saturation concentration and the carbon source, and has



ability to accumulate L-glutamic acid in an amount exceeding the amount corresponding to the saturation concentration in the liquid medium at the pH.

(2) The microorganism according to (1), which can grow in the liquid medium.

(3) The microorganism according to (1) or (2), wherein the pH is not more than 5.0.

(4) The microorganism according to any one of (1) to (3), which has at least one of the following characteristics:

(a) the microorganism is enhanced in activity of an enzyme that catalyzes a reaction for biosynthesis of L-glutamic acid; and

(b) the microorganism is decreased in or deficient in activity of an enzyme that catalyzes a reaction branching from a biosynthetic pathway of L-glutamic acid and producing a compound other than L-glutamic acid.

(5) The microorganism according to (4), wherein the enzyme that catalyzes the reaction for biosynthesis of L-glutamic acid is at least one selected from citrate synthase, phosphoenolpyruvate carboxylase and glutamate dehydrogenase.

(6) The microorganism according to (4) or (5), wherein the enzyme that catalyzes the reaction branching from the biosynthetic pathway of L-glutamic acid and producing a compound other than L-glutamic acid is  $\alpha$ -ketoglutarate dehydrogenase.

(7) The microorganism according to any one of (1) to (6), wherein the microorganism belongs to the genus *[Enterobacter] Pantoea*.

(8) The microorganism according to (7), which is *[Enterobacter agglomerans] Pantoea ananatis*.

(9) The microorganism according to (8), which has a mutation that causes less extracellular secretion of a viscous material compared with a wild strain when cultured in a medium containing a saccharide.

(10) A method for producing L-glutamic acid by fermentation, which comprises culturing a microorganism as defined in any one of (1) to (9) in a liquid medium of which pH is adjusted to a pH at which L-glutamic acid is precipitated, to produce and accumulate L-glutamic acid and precipitate L-glutamic acid in the medium.

(11) A method for screening a microorganism suitable for producing L-glutamic acid by fermentation with precipitating L-glutamic acid in a liquid medium, which comprises inoculating a sample containing microorganisms into an acidic medium containing L-glutamic acid at a saturation concentration and a carbon source, and selecting a strain that can metabolize the carbon source.

(12) The method according to (11), wherein a strain that can grow in the medium is selected as the strain that can metabolize the carbon source.

(13) The method according to (11) or (12), wherein a pH of the medium is not more than 5.0.

According to the method of the present invention, L-glutamic acid can be produced by fermentation with precipitating L-glutamic acid. As a result, L-glutamic acid in the medium is maintained below a certain concentration, and L-glutamic acid can be produced without suffering from the product inhibition by L-glutamic acid at a high concentration.

#### BRIEF EXPLANATION OF THE DRAWINGS

FIG. 1 shows a restriction map of a DNA fragment derived from *[Enterobacter agglomerans] Pantoea ananatis* pTWVEK101.

FIG. 2A and FIG. 2B show a comparison of the amino acid sequence deduced from the nucleotide sequence of the sucA gene derived from *[Enterobacter agglomerans] Pantoea*

*ananatis* and that derived from *Escherichia coli*. Upper sequence: *[Enterobacter agglomerans] Pantoea ananatis* (SEQ ID NO: 3), lower sequence: *Escherichia coli* (SEQ ID NO: 8) (the same shall apply hereafter).

5 FIG. 3 shows comparison of the amino acid sequence deduced from the nucleotide sequence of the sucB gene derived from *[Enterobacter agglomerans] Pantoea ananatis* (upper sequence—SEQ ID NO: 4) and that derived from *Escherichia coli* (lower sequence—SEQ ID NO: 9).

10 FIG. 4 shows comparison of the amino acid sequence deduced from the nucleotide sequence of the sucC gene derived from *[Enterobacter agglomerans] Pantoea ananatis* (upper sequence—SEQ ID NO: 10) and that derived from *Escherichia coli* (lower sequence—SEQ ID NO: 11).

15 FIG. 5 shows comparison of the amino acid sequence deduced from the nucleotide sequence of the sdhB gene derived from *[Enterobacter agglomerans] Pantoea ananatis* (upper sequence—SEQ ID NO: 2) and that derived from *Escherichia coli* (lower sequence—SEQ ID NO: 12).

20 FIG. 6 shows construction of plasmid pMWCPG having a *gltA* gene, a *ppc* gene and a *gdhA* gene.

FIG. 7 shows construction of plasmid RSF-Tet having the replication origin of the broad host spectrum plasmid RSF1010 and a tetracycline resistance gene.

25 FIG. 8 shows construction of plasmid RSFCPG having the replication origin of the broad host spectrum plasmid RSF1010 a tetracycline resistance gene, a *gltA* gene, a *ppc* gene and a *gdhA* gene.

30 FIG. 9 shows construction of plasmid pSTVCB having a *gltA* gene.

#### DETAILED DESCRIPTION OF THE INVENTION

Hereafter, the present invention will be explained in detail.

35 The microorganism of the present invention is a microorganism that (1) can metabolize a carbon source at a specific pH in a liquid medium containing L-glutamic acid at a saturation concentration and the carbon source and (2) has ability to accumulate L-glutamic acid in an amount exceeding the amount corresponding to the saturation concentration in the liquid medium at the pH.

The term "saturation concentration" means a concentration of L-glutamic acid dissolved in a liquid medium when the liquid medium is saturated with L-glutamic acid.

45 Hereafter, a method for screening a microorganism that can metabolize a carbon source in a liquid medium containing L-glutamic acid at a saturation concentration and the carbon source at a specific pH will be described. A sample containing microorganisms is inoculated into a liquid medium containing L-glutamic acid at a saturation concentration and a carbon source at a specific pH, and a strain that can metabolize the carbon source is selected. The specific pH is not particularly limited, but is usually not more than about 5.0, preferably not more than about 4.5, more preferably not more than about 4.3.

50 The microorganism of the present invention is used to produce L-glutamic acid by fermentation with precipitating L-glutamic acid. If the pH is too high, it becomes difficult to allow the microorganism to produce L-glutamic acid enough for precipitation. Therefore, pH is preferably in the aforementioned range.

60 If pH of an aqueous solution containing L-glutamic acid is lowered, the solubility of L-glutamic acid significantly falls around pKa of  $\gamma$ -carboxyl group (4.25, 25° C.). The solubility becomes the lowest at the isoelectric point (pH 3.2) and L-glutamic acid exceeding the amount corresponding to the saturation concentration is precipitated. While it depends on the medium composition, L-glutamic acid is usually dis-



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solved in an amount of 10 to 20 g/L at pH 3.2, 30 to 40 g/L at pH 4.0 and 50 to 60 g/L at pH 4.7, at about 30° C. Usually pH does not need to be made below 3.0, because the L-glutamic acid precipitating effect plateaus when pH goes below a certain value. However, pH may be below 3.0.

In addition, the expression that a microorganism "can metabolize the carbon source" means that it can proliferate or can consume the carbon source even though it cannot proliferate. Therefore, this phrase indicates that the microorganism catabolizes carbon sources such as saccharides or organic acids. Specifically, for example, if a microorganism proliferates when cultured in a liquid medium containing L-glutamic acid at a saturation concentration at pH 5.0 to 4.0, preferably pH 4.5 to 4.0, more preferably pH 4.3 to 4.0, still more preferably pH 4.0 at an appropriate temperature, for example, 28° C., 37° C. or 50° C. for 2 to 4 days, this microorganism can metabolize the carbon source in the medium.

Further, for example, even if a microorganism does not proliferate when it is cultured in a liquid medium containing L-glutamic acid at a saturation concentration at pH 5.0 to 4.0, preferably pH 4.5 to 4.0, more preferably pH 4.3 to 4.0, still more preferably pH 4.0 at an appropriate temperature, for example, 28° C., 37° C. or 50° C. for 2 to 4 days, the microorganism which consumes the carbon source in the medium is that can metabolize the carbon source in the medium.

The microorganism which can metabolize the carbon source includes a microorganism which can grow in the liquid medium.

The expression that a microorganism "can grow" means that it can proliferate or can produce L-glutamic acid even though it cannot proliferate. Specifically, for example, if a microorganism proliferates when cultured in a liquid medium containing L-glutamic acid at a saturation concentration at pH 5.0 to 4.0, preferably pH 4.5 to 4.0, more preferably pH 4.3 to 4.0, still more preferably pH 4.0 at an appropriate temperature, for example, 28° C., 37° C. or 50° C. for 2 to 4 days, this microorganism can grow in the medium.

Further, for example, even if a microorganism does not proliferate when it is cultured in a liquid synthetic medium containing L-glutamic acid at a saturation concentration at pH 5.0 to 4.0, preferably pH 4.5 to 4.0, more preferably pH 4.3 to 4.0, still more preferably pH 4.0 at an appropriate temperature, for example, 28° C., 37° C. or 50° C. for 2 to 4 days, the microorganism which increases the amount of L-glutamic acid in the medium is that can grow in the medium.

The selection described above may be repeated two or more times under the same conditions or with changing pH or the concentration of L-glutamic acid. An initial selection can be performed in a medium containing L-glutamic acid at a concentration lower than the saturation concentration, and thereafter a subsequent selection can be performed in a medium containing L-glutamic acid at a saturation concentration. Further, strains with favorable properties such as superior proliferation rate may be selected.

In addition to the property described above, the microorganism of the present invention has an ability to accumulate L-glutamic acid in an amount exceeding the amount corresponding to the saturation concentration of L-glutamic acid in a liquid medium. The pH of the aforementioned liquid medium is preferably the same as or close to that of the medium used for screening a microorganism having the aforementioned property (1). Usually, a microorganism becomes susceptible to L-glutamic acid at a high concentration as pH becomes lower. Therefore, it is preferred that pH is not low from the viewpoint of resistance to L-glutamic acid, but low pH is preferred from the viewpoint of production of

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L-glutamic acid with precipitating it. To satisfy these conditions, pH may be in the range of 3 to 5, preferably 4 to 5, more preferably 4.0 to 4.7, still more preferably 4.0 to 4.5, particularly preferably 4.0 to 4.3.

As the microorganism of the present invention or breeding materials therefor, there can be mentioned, for example, microorganisms belonging to the genus *Enterobacter*, *Klebsiella*, *Serratia*, *Pantoea*, *Erwinia*, *Escherichia*, *Corynebacterium*, *Alicyclobacillus*, *Bacillus*, *Saccharomyces* or the like. Among these, microorganisms belonging to the genus *Enterobacter* are preferred. Hereafter, the microorganism of the present invention will be explained mainly for microorganisms belonging to the genus *Enterobacter*, but the present invention can be applied to microorganism belonging to other genera and not limited to the genus *Enterobacter*.

As microorganisms belonging to the [*Enterobacter*] *Pantoea*, there can be specifically mentioned [*Enterobacter agglomerans*] *Pantoea ananatis*, preferably the [*Enterobacter agglomerans*] *Pantoea ananatis* AJ13355 strain. This strain was isolated from a soil in Iwata-shi, Shizuoka, Japan as a strain that can proliferate in a medium containing L-glutamic acid and a carbon source at low pH.

The physiological properties of AJ13355 are as follows:

- (1) Gram staining: negative
- (2) Behavior against oxygen: facultative anaerobic
- (3) Catalase: positive
- (4) Oxidase: negative
- (5) Nitrate-reducing ability: negative
- (6) Voges-Proskauer test: positive
- (7) Methyl Red test: negative
- (8) Urease: negative
- (9) Indole production: positive
- (10) Motility: motile
- (11) H<sub>2</sub>S production in TSI medium: weakly active
- (12) β-galactosidase: positive
- (13) Saccharide-assimilating property:
  - Arabinose: positive
  - Sucrose: positive
  - Lactose: positive
  - Xylose: positive
  - Sorbitol: positive
  - Inositol: positive
  - Trehalose: positive
  - Maltose: positive
  - Glucose: positive
  - Adonitol: negative
  - Raffinose: positive
  - Salicin: negative
  - Melibiose: positive
- (14) Glycerol-assimilating property: positive
- (15) Organic acid-assimilating property:
  - Citric acid: positive
  - Tartaric acid: negative
  - Gluconic acid: positive
  - Acetic acid: positive
  - Malonic acid: negative
- (16) Arginine dehydratase: negative
- (17) Ornithine decarboxylase: negative
- (18) Lysine decarboxylase: negative
- (19) Phenylalanine deaminase: negative
- (20) Pigment formation: yellow
- (21) Gelatin liquefaction ability: positive
- (22) Growth pH: growth is possible at pH 4.0, good growth at pH 4.5 to 7
- (23) Growth temperature: good growth at 25° C., good growth at 30° C., good growth at 37° C., growth is possible at 42° C., growth is not possible at 45° C.



Based on these bacteriological properties, AJ13355 was determined as [Enterobacter agglomerans] *Pantoea ananatis*.

The [Enterobacter agglomerans] *Pantoea ananatis* AJ13355 was deposited at the National Institute of Bio-  
 science and Human-Technology, Agency of Industrial Sci-  
 ence and Technology, Ministry of International Trade and  
 Industry (postal code: 305-8566, 1-3, Higashi 1-chome,  
 Tsukuba-shi, Ibaraki, Japan) on Feb. 19, 1998 and received an  
 accession number of FERM P-16644. It was then transferred  
 to an international deposition under the provisions of Budap-  
 est Treaty on Jan. 11, 1999 and received an accession number  
 of FERM BP-6614.

The microorganism of the present invention may be a  
 microorganism originally having L-glutamic acid-producing  
 ability or one having L-glutamic acid-producing ability  
 imparted or enhanced by breeding through use of mutation  
 treatment, recombinant DNA techniques or the like.

L-Glutamic acid-producing ability can be imparted or  
 enhanced by, for example, increasing activity of an enzyme  
 that catalyzes a reaction for biosynthesis of L-glutamic acid.  
 L-Glutamic acid-producing ability can also be enhanced by  
 decreasing activity of an enzyme that catalyzes a reaction  
 branching from the biosynthetic pathway of L-glutamic acid  
 and producing a compound other than L-glutamic acid, or  
 making the activity deficient.

Enzymes that catalyze are action for biosynthesis of  
 L-glutamic acid, include: glutamate dehydrogenase (hereaf-  
 ter, also referred to as "GDH"), glutamine synthetase,  
 glutamate synthase, isocitrate dehydrogenase, aconitate  
 hydratase, citrate synthase (hereafter, also referred to as  
 "CS"), phosphoenolpyruvate carboxylase (hereafter, also  
 referred to as "PEPC"), pyruvate dehydrogenase, pyruvate  
 kinase, enolase, phosphoglyceromutase, phosphoglycerate  
 kinase, glyceraldehyde-3-phosphate dehydrogenase, triose-  
 phosphate isomerase, fructose biphosphate aldolase, phos-  
 phofructokinase, glucose phosphate isomerase and so forth.  
 Among these enzymes, one, two or three of CS, PEPC and  
 GDH are preferred. Further, it is preferred that the activities of  
 all the three enzymes, CS, PEPC and GDH, are enhanced in  
 the microorganism of the present invention. In particular, CS  
 of *Brevibacterium lactofermentum* is preferred, because it  
 does not suffer from inhibition by  $\alpha$ -ketoglutaric acid,  
 L-glutamic acid and NADH.

In order to enhance the activity of CS, PEPC or GDH, for  
 example, a gene coding for CS, PEPC or GDH may be cloned  
 on an appropriate plasmid and a host microorganism may be  
 transformed with the obtained plasmid. The copy number of  
 the gene coding for CS, PEPC or GDH (hereafter, abbreviated  
 as "gltA gene", "ppc gene" and "gdhA gene", respectively) in  
 the transformed strain cell increases, resulting in the increase  
 of the activity of CS, PEPC or GDH.

The cloned gltA gene, ppc gene and gdhA gene are intro-  
 duced into the aforementioned starting parent strain solely or  
 in combination of arbitrary two or three kinds of them. When  
 two or three kinds of the genes are introduced, two or three  
 kinds of the genes may be cloned on one kind of plasmid and  
 introduced into the host, or separately cloned on two or three  
 kinds of plasmids that can coexist and introduced into the  
 host.

Two or more kinds of genes coding for enzymes of the  
 same kind, but derived from different microorganisms may be  
 introduced into the same host.

The plasmids described above are not particularly limited  
 so long as they are autonomously replicable in cells of a  
 microorganism belonging to, for example, the genus Enter-  
 obacter or the like, but, for example, there can be mentioned  
 pUC19, pUC18, pBR322, pHSG299, pHSG298, pHSG399,

pHSG398, RSF1010, pMW119, pMW118, pMW219,  
 pMW218, pACYC177, pACYC184 and so forth. Besides  
 these, vectors of phage DNA can also be used.

Transformation can be performed by, for example, the  
 method of D. M. Morrison (Methods in Enzymology, 68, 326  
 (1979)), the method wherein permeability of DNA is  
 increased by treating recipient bacterium cells with calcium  
 chloride (Mandel M. and Higa A., J. Mol. Biol., 53, 159  
 (1970)), the electroporation (Miller J. H., "A Short Course in  
 Bacterial Genetics", Cold Spring Harbor Laboratory Press,  
 U.S.A. 1992) or the like.

The activity of CS, PEPC or GDH can also be increased by  
 allowing multiple copies of a gltA gene, a ppc gene or a gdhA  
 gene to be present on chromosomal DNA of the aforemen-  
 tioned starting parent strain to be a host. In order to introduce  
 multiple copies of the gltA gene, the ppc gene or the gdhA  
 gene on chromosomal DNA of a microorganism belonging to  
 the genus Enterobacter or the like, a sequence of which mul-  
 tiple copies are present on the chromosomal DNA, such as  
 repetitive DNA and inverted repeats present at termini of a  
 transposable element, can be used. Alternatively, multiple  
 copies of the genes can be introduced on to chromosomal  
 DNA by utilizing transfer of a transposon containing the gltA  
 gene, the ppc gene or the gdhA gene. As a result, the copy  
 number of the gltA gene, the ppc gene or the gdhA gene in a  
 transformed strain cell is increased, and thus the activity of  
 CS, PEPC or GDH is increased.

As organisms to be a source of the gltA gene, the ppc gene  
 or the gdhA gene of which copy number is increased, any  
 organism can be used so long as it has activity of CS, PEPC or  
 GDH. Inter alia, bacteria, which are prokaryotes, for  
 example, those belonging to the genus Enterobacter, Kleb-  
 siella, Erwinia, Pantoea, Serratia, Escherichia, Corynebacte-  
 rium, Brevibacterium and Bacillus are preferred. As specific  
 examples, there can be mentioned Escherichia coli, Brevibacte-  
 rium lactofermentum and so forth. The gltA gene, the ppc  
 gene and the gdhA gene can be obtained from chromosomal  
 DNA of the microorganisms described above.

The gltA gene, the ppc gene and the gdhA gene can be  
 obtained by using a mutant strain which is deficient in the  
 activity of CS, PEPC or GDH to isolate a DNA fragment  
 which complements the auxotrophy from chromosomal DNA  
 of the aforementioned microorganisms. Since the nucleotide  
 sequences of these genes of Escherichia and Corynebacte-  
 rium bacteria have already been elucidated (Biochemistry,  
 22, pp. 5243-5249 (1983); J. Biochem., 95, pp. 909-916  
 (1984); Gene, 27, pp. 193-199 (1984); Microbiology, 140, pp.  
 1817-1828 (1994); Mol. Gen. Genet., 218, pp. 330-339  
 (1989); Molecular Microbiology, 6, pp. 317-326 (1992)),  
 they can also be obtained by PCR utilizing primers synthe-  
 sized based on each nucleotide sequence and chromosomal  
 DNA as a template.

The activity of CS, PEPC or GDH can also be increased by  
 enhancing the expression of the gltA gene, the ppc gene or the  
 gdhA gene besides the aforementioned amplification of the  
 genes. For example, the expression can be enhanced by  
 replacing a promoter for the gltA gene, the ppc gene or the  
 gdhA gene with other stronger promoters. For example,  
 strong promoters are known to include: lac promoter, trp  
 promoter, trc promoter, tac promoter,  $P_R$  promoter and  $P_L$   
 promoter of the lamda phage and so forth. The gltA gene, the  
 ppc gene and the gdhA gene of which promoter is replaced are  
 cloned on a plasmid and introduced into the host microorgan-  
 ism, or introduced onto the chromosomal DNA of the host  
 microorganism by using repetitive DNA, inverted repeats,  
 transposon or the like.



The activity of CS, PEPC or GDH can also be enhanced by replacing the promoter of the *gltA* gene, the *ppc* gene or the *gdhA* gene on the chromosome with other stronger promoters (see WO 87/03006 and Japanese Patent Application Laid-open No. 61-268183), or inserting a strong promoter in the upstream of the coding sequence of each gene (see Gene, 29, pp. 231-241 (1984)). Specifically, homologous recombination can be performed between DNA containing the *gltA* gene, the *ppc* gene or the *gdhA* gene of which promoter is replaced with a stronger one or a part thereof and the corresponding gene on the chromosome.

Examples of the enzyme which catalyze a reaction branching from the biosynthetic pathway of the L-glutamic acid and producing a compound other than L-glutamic acid include  $\alpha$ -ketoglutarate dehydrogenase (hereafter, also referred to as " $\alpha$ KGDH"), isocitrate lyase, phosphate acetyltransferase, acetate kinase, acetohydroxy acid synthase, acetolactate synthase, formate acetyltransferase, lactate dehydrogenase, glutamate decarboxylase, 1-pyrroline dehydrogenase and so forth. Among these enzymes,  $\alpha$ KGDH is preferred.

In order to obtain a decrease or deficiency of the activity of the aforementioned enzyme in a microorganism belonging to the genus *Enterobacter* or the like, mutation causing decrease or deficiency of the intracellular activity of the enzyme can be introduced into the gene of the aforementioned enzyme by a usual mutagenesis or genetic engineering method.

Examples of the mutagenesis method include, for example, methods utilizing irradiation with X-ray or ultraviolet ray, methods utilizing treatment with a mutagenic agent such as N-methyl-N'-nitro-N-nitrosoguanidine, and so forth. The site where the mutation is introduced to the gene may be in a coding region coding for an enzyme protein, or a region for regulating expression such as a promoter.

Examples of the genetic engineering methods include, for example, methods utilizing gene recombination, transduction, cell fusion and so forth. For example, a drug resistance gene is inserted into a cloned target gene to prepare a gene that has lost its function (defective gene). Subsequently, this defective gene is introduced into a cell of a host microorganism, and the target gene on the chromosome is replaced with the aforementioned defective gene by utilizing homologous recombination (gene disruption).

A decrease or deficiency of intracellular activity of the target enzyme and the degree of decrease of the activity can be determined by measuring the enzyme activity of a cell extract or a purified fraction thereof obtained from a candidate strain and comparing with that of a wild strain. For example, the  $\alpha$ KGDH activity can be measured by the method of Reed et al. (Reed L. J. and Mukherjee B. B., *Methods in Enzymology*, 13, pp. 55-61 (1969)).

Depending on the target enzyme, the target mutant strain can be selected based on the phenotype of the mutant strain. For example, a mutant strain which is deficient in the  $\alpha$ KGDH activity or decreases in the  $\alpha$ KGDH activity cannot proliferate or shows a markedly reduced proliferation rate in a minimal medium containing glucose or a minimal medium containing acetic acid or L-glutamic acid as an exclusive carbon source under aerobic conditions. However, normal proliferation is enabled even under the same condition by adding succinic acid or lysine, methionine and diaminopimelic acid to a minimal medium containing glucose. By utilizing these phenomena as indicators, mutant strains with decreased  $\alpha$ KGDH activity or deficient in the activity can be selected.

A method for preparing the  $\alpha$ KGDH gene deficient strain of *Brevibacterium lactofermentum* by utilizing homologous

recombination is described in detail in WO 95/34672. Similar methods can be applied to the other microorganisms.

Further, techniques such as cloning of genes and cleavage and ligation of DNA, transformation and so forth are described in detail in *Molecular Cloning*, 2nd Edition, Cold Spring Harbor Press, 1989 and so forth.

As a specific example of a mutant strain deficient in  $\alpha$ KGDH activity or with decreased  $\alpha$ KGDH activity obtained as described above, there can be mentioned [*Enterobacter agglomerans*] *Pantoea ananatis* AJ13356. [*Enterobacter agglomerans*] *Pantoea ananatis* AJ13356 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (postal code: 305-8566, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan) on Feb. 19, 1998 and received an accession number of FERM P-16645. It was then transferred to an international deposition under the provisions of Budapest Treaty on Jan. 11, 1999 and received an accession number of FERM BP-6615. The [*Enterobacter agglomerans*] *Pantoea ananatis* AJ13356 is deficient in  $\alpha$ KGDH activity as a result of disruption of the  $\alpha$ KGDH-E1 subunit gene (*sucA*).

When [*Enterobacter agglomerans*] *Pantoea ananatis*, an example of the microorganism used in the present invention, is cultured in a medium containing a saccharide, a viscous material is extracellularly secreted, resulting in low operation efficiency. Therefore, when [*Enterobacter agglomerans*] *Pantoea ananatis* having such a property of secreting the viscous material is used, it is preferable to use a mutant strain that secretes less the viscous material compared with a wild strain. Examples of mutagenesis methods include, for example, methods utilizing irradiation with X ray or ultraviolet ray, method utilizing treatment with a mutagenic agent such as N-methyl-N'-nitro-N-nitrosoguanidine and so forth. A mutant strain with decreased secretion of the viscous material can be selected by inoculating mutagenized bacterial cells in a medium containing a saccharide, for example, LB medium plate containing 5 g/L of glucose, culturing them with tilting the plate about 45 degrees and selecting a colony which does not show flowing down of liquid.

In the present invention, impartation or enhancement of L-glutamic acid-producing ability and impartation of other favorable properties such as mutation for less viscous material secretion described above can be carried out in an arbitrary order.

By culturing the microorganism of the present invention in a liquid medium of which pH is adjusted to a pH at which L-glutamic acid is precipitated, L-glutamic acid can be produced and accumulated with precipitating it in the medium. L-Glutamic acid can also be precipitated by starting the culture at a neutral pH and then ending it at a pH at which L-glutamic acid is precipitated.

The pH at which L-glutamic acid is precipitated means one at which L-glutamic acid is precipitated when the microorganism produces and accumulates L-glutamic acid.

As the aforementioned medium, a usual nutrient medium containing a carbon source, a nitrogen source, mineral salts and organic trace nutrients such as amino acids and vitamins as required can be used so long as pH is adjusted to a pH at which L-glutamic acid is precipitated. Either a synthetic medium or a natural medium can be used. The carbon source and the nitrogen source used in the medium can be any ones so long as they can be used by the cultured strain.

As the carbon source, saccharides such as glucose, glycerol, fructose, sucrose, maltose, mannose, galactose, starch hydrolysate and molasses are used. In addition, organic acids



such as acetic acid and citric acid may be used each alone or in combination with another carbon source.

As the nitrogen source, ammonia, ammonium salts such as ammonium sulfate, ammonium carbonate, ammonium chloride, ammonium phosphate and ammonium acetate, nitrates and so forth are used.

As the organic trace nutrients, amino acids, vitamins, fatty acids, nucleic acids, those containing these substances such as peptone, casamino acid, yeast extract and soybean protein decomposition products are used. When an auxotrophic mutant strain that requires an amino acid and so forth for metabolization or growth is used, the required nutrient must be supplemented.

As mineral salts, phosphates, magnesium salts, calcium salts, iron salts, manganese salts and so forth are used.

As for the culture method, aeration culture is usually performed with controlling the fermentation temperature to be 20 to 42° C. and pH to be 3 to 5, preferably 4 to 5, more preferably 4 to 4.7, particularly preferably 4 to 4.5. Thus, after about 10 hours to 4 days of culture, a substantial amount of L-glutamic acid is accumulated in the culture. Accumulated L-glutamic acid exceeding the amount corresponding to the saturation concentration is precipitated in the medium.

After completion of the culture, L-glutamic acid precipitated in the culture can be collected by centrifugation, filtration or the like. L-Glutamic acid dissolved in the medium can be collected according to known methods. For example, the L-glutamic acid can be isolated by concentrating the culture broth to crystallize it or isolated by ion exchange chromatography or the like. L-Glutamic acid precipitated in the culture broth may be isolated together with L-glutamic acid that have been dissolved in the medium after it is crystallized.

According to the method of the present invention, L-glutamic acid exceeding the amount corresponding to the saturation concentration is precipitated, and the concentration of L-glutamic acid dissolved in the medium is maintained at a constant level. Therefore, influence of L-glutamic acid at a high concentration on microorganisms can be reduced. Accordingly, it becomes possible to breed a microorganism having further improved L-glutamic acid-producing ability. Further, since L-glutamic acid is precipitated as crystals, acidification of the culture broth by accumulation of L-glutamic acid is suppressed, and therefore the amount of alkali used for maintaining pH of the culture can significantly be reduced.

## EXAMPLES

Hereafter, the present invention will be more specifically explained with reference to the following examples.

### <1> Screening of Microorganism Having L-Glutamic Acid Resistance in Acidic Environment

Screening of a microorganism having L-glutamic acid resistance in an acidic environment was performed as follows. Each of about 500 samples obtained from nature including soil, fruits, plant bodies, river water in an amount of 1 g was suspended in 5 mL of sterilized water, of which 200 µL, was coated on 20 mL of solid medium of which pH was adjusted to 4.0 with HCl. The composition of the medium was as follows: 3 g/L of glucose, 1 g/L of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.2 g/L of MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g/L of KH<sub>2</sub>PO<sub>4</sub>, 0.2 g/L of NaCl, 0.1 g/L of CaCl<sub>2</sub>·7H<sub>2</sub>O, 0.01 g/L of FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g/L of MnSO<sub>4</sub>·4H<sub>2</sub>O, 0.72 mg/L of ZnSO<sub>4</sub>·2H<sub>2</sub>O, 0.64 mg/L of CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.72 mg/L of CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.4 mg/L of boric acid, 1.2 mg/L of Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 50 µg/L of biotin, 50 µg/L of calcium pantothenate, 50 µg/L of folic acid, 50 µg/L of inositol, 50 µg/L of niacin, 50 µg/L of p-aminobenzoic acid,

50 µg/L of pyridoxine hydrochloride, 50 µg/L of riboflavin, 50 µg/L of thiamine hydrochloride, 50 mg/L of cycloheximide, 20 g/L of agar.

The media plated on which the above samples were plated were incubated at 28° C., 37° C. or 50° C. for 2 to 4 days and 378 strains each forming a colony were obtained.

Subsequently, each of the strains obtained as described above was inoculated in a test tube of 16.5 cm in length and 14 mm in diameter containing 3 mL of liquid medium (adjusted to pH 4.0 with HCl) containing a saturation concentration of L-glutamic acid and cultured at 28° C., 37° C. or 50° C. for 24 hours to 3 days with shaking. Then, the grown strains were selected. The composition of the aforementioned medium was follows: 40 g/L of glucose, 20 g/L of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g/L of MgSO<sub>4</sub>·7H<sub>2</sub>O, 2 g/L of KH<sub>2</sub>PO<sub>4</sub>, 0.5 g/L of NaCl, 0.25 g/L of CaCl<sub>2</sub>·7H<sub>2</sub>O, 0.02 g/L of FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.02 g/L of MnSO<sub>4</sub>·4H<sub>2</sub>O, 0.72 mg/L of ZnSO<sub>4</sub>·2H<sub>2</sub>O, 0.64 mg/L of CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.72 mg/L of CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.4 mg/L of boric acid, 1.2 mg/L of Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 2 g/L of yeast extract.

Thus, 78 strains of microorganisms having L-glutamic acid resistance in an acidic environment were successfully obtained.

### <2> Selection of Strains with Superior Growth Rate in Acidic Environment from Microorganisms Having L-glutamic Acid Resistance

The various microorganisms having L-glutamic acid resistance in an acidic environment obtained as described above were each inoculated into a test tube of 16.5 cm in length and 14 mm in diameter containing 3 mL of medium (adjusted to pH 4.0 with HCl) obtained by adding 20 g/L of glutamic acid and 2 g/L of glucose to M9 medium (Sambrook, J., Fritsh, E. F. and Maniatis, T., "Molecular Cloning", Cold Spring Harbor Laboratory Press, 1989), and the turbidity of the medium was measured in the time course to select strains with a favorable growth rate. As a result, as a strain showing favorable growth, the AJ13355 strain was obtained from a soil in Iwata-shi, Shizuoka, Japan. This strain was determined as **[Enterobacter agglomerans] Pantoea ananatis** based on its bacteriological properties described above.

### <3> Acquisition of Strain with Less Viscous Material Secretion from **[Enterobacter agglomerans] Pantoea ananatis** AJ13355 Strain

Since the **[Enterobacter agglomerans] Pantoea ananatis** AJ13355 strain extracellularly secretes a viscous material when cultured in a medium containing a saccharide, operation efficiency is not favorable. Therefore, a strain with less viscous material secretion was obtained by the ultraviolet irradiation method (Miller, J. H. et al., "A Short Course in Bacterial Genetics; Laboratory Manual", p. 150, Cold Spring Harbor Laboratory Press, 1992).

The **[Enterobacter agglomerans] Pantoea ananatis** AJ13355 strain was irradiated with ultraviolet ray for 2 minutes at the position 60 cm away from a 60-W ultraviolet lamp and cultured in LB medium overnight to fix mutation. The mutagenized strain was diluted and inoculated in LB medium containing 5 g/L of glucose and 20 g/L of agar so that about 100 colonies per plate would emerge and cultured at 30° C. overnight with tilting the plate about 45 degrees, and then 20 colonies showing no flowing down of the viscous material were selected.

As a strain satisfying conditions that no revertant emerged even after 5 times of subculture in LB medium containing 5 g/L of glucose and 20 g/L of agar, and that there should be observed growth equivalent to the parent strain in LB medium, LB medium containing 5 g/L of glucose and M9 medium (Sambrook, J. et al., Molecular Cloning, 2nd Edition, Cold Spring Harbor Press, 1989) to which 20 g/L of



L-glutamic acid and 2 g/L of glucose were added and of which pH was adjusted to 4.5 with HCl, SC17 strain was selected from the strains selected above.

<4> Construction of Glutamic Acid-Producing Bacterium from [Enterobacter agglomerans] *Pantoea ananatis* SC17 Strain

(1) Preparation of  $\alpha$ KGDH Deficient Strain from [Enterobacter agglomerans] *Pantoea ananatis* SC17 Strain

A strain deficient in  $\alpha$ KGDH and with enhanced L-glutamic acid biosynthetic system was prepared from the [Enterobacter agglomerans] *Pantoea ananatis* SC17 strain.

(i) Cloning of  $\alpha$ KGDH Gene (Hereafter, Referred to as "sucAB") of [Enterobacter agglomerans] *Pantoea ananatis* AJ13355 Strain

The sucAB gene of the [Enterobacter agglomerans] *Pantoea ananatis* AJ13355 strain was cloned by selecting a DNA fragment complementing the acetic acid-unassimilating property of the  $\alpha$ KGDH-E1 subunit gene (hereafter, referred to as "sucA") deficient strain of *Escherichia coli* from chromosomal DNA of the [Enterobacter agglomerans] *Pantoea ananatis* AJ13355 strain.

The chromosomal DNA of the [Enterobacter agglomerans] *Pantoea ananatis* AJ13355 strain was isolated by a method usually employed when chromosomal DNA is extracted from *Escherichia coli* (Text for Bioengineering Experiments, Edited by the Society for Bioscience and Bioengineering, Japan, pp. 97-98, Baifukan, 1992). The pTWV228 (resistant to ampicillin) used as a vector was commercially available one from Takara Shuzo Co., Ltd.

The chromosomal DNA of the AJ13355 strain digested with EcoT221 and pTWV228 digested with PstI were ligated by using T4 ligase and used to transform the sucA deficient *Escherichia coli* JRG465 strain (Herbert, J. et al., Mol. Gen. Genetics, 105, 182 (1969)). A strain growing in an acetate minimal medium was selected from the transformant strains obtained above, and a plasmid was extracted from it and designated as pTWVEK101. The *Escherichia coli* JRG465 strain harboring pTWVEK101 recovered auxotrophy for succinic acid or L-lysine and L-methionine besides the acetic acid-assimilating property. This suggests that pTWVEK101 contains the sucA gene of [Enterobacter agglomerans] *Pantoea ananatis*.

FIG. 1 shows the restriction map of a DNA fragment derived from [Enterobacter agglomerans] *Pantoea ananatis* in pTWVEK101. The determined nucleotide sequence of the hatched portion in FIG. 1 is shown as SEQ ID NO: 1. In this sequence, nucleotide sequences considered to be two full length ORFs and two nucleotide sequences considered to be partial sequences of the ORFs were found. SEQ ID NOS: 2 to 5 show amino acid sequences that can be encoded by these ORFs or partial sequences in an order from the 5' end. As a result of homology search for these, it was revealed that the portion of which nucleotide sequences were determined contained a 3'-end partial sequence of the succinate dehydrogenase iron-sulfur protein gene (sdhB), full length sucA and  $\alpha$ KGDH-E2 subunit gene (sucB), and 5'-end partial sequence of the succinyl CoA synthetase  $\beta$  subunit gene (sucC). The results of comparison of the amino acid sequences deduced from these nucleotide sequences with those derived from *Escherichia coli* (Eur. J. Biochem., 141, pp. 351-359 (1984); Eur. J. Biochem., 141, pp. 361-374 (1984); Biochemistry, 24, pp. 6245-6252 (1985)) are shown in FIGS. 2 to 5. Thus, the amino acid sequences each showed very high homology. In addition, it was found that a cluster of sdhB-sucA-sucB-sucC was constituted on the chromosome of [Enterobacter agglomerans] *Pantoea ananatis* as in *Escherichia coli* is (Eur. J.

Biochem., 141, pp. 351-359 (1984); Eur. J. Biochem., 141, pp. 361-374 (1984); Biochemistry, 24, pp. 6245-6252 (1985)).

(ii) Acquisition of  $\alpha$ KGDH Deficient Strain Derived from [Enterobacter agglomerans] *Pantoea ananatis* SC17 Strain

The homologous recombination was performed by using the sucAB gene of [Enterobacter agglomerans] *Pantoea ananatis* obtained as described above to obtain an  $\alpha$ KGDH deficient strain of [Enterobacter agglomerans] *Pantoea ananatis*.

After pTWVEK101 was digested with SphI to excise a fragment containing sucA, the fragment was blunt-ended with Klenow fragment (Takara Shuzo Co., Ltd.) and ligated with pBR322 digested with EcoRI and blunt-ended with Klenow fragment, by using T4 DNA ligase (Takara Shuzo Co., Ltd.) The obtained plasmid was digested at the restriction enzyme BglII recognition site positioned substantially at the center of sucA by using this enzyme, blunt-ended with Klenow fragment, and then ligated again by using T4 DNA ligase. It was considered that the sucA gene did not function because a frame shift mutation was introduced into sucA of the plasmid newly constructed through the above procedure.

The plasmid constructed as described above was digested with a restriction enzyme ApaLI, and subjected to agarose gel electrophoresis to recover a DNA fragment containing sucA into which the frame shift mutation was introduced and a tetracycline resistance gene derived from pBR322. The recovered DNA fragment was ligated again by using T4 DNA ligase to construct a plasmid for disrupting the  $\alpha$ KGDH gene.

The plasmid for disrupting the  $\alpha$ KGDH gene obtained as described above was used to transform the [Enterobacter agglomerans] *Pantoea ananatis* SC17 strain by electroporation (Miller, J. H., "A Short Course in Bacterial Genetics; Handbook", p. 279, Cold Spring Harbor Laboratory Press, U.S.A., 1992), and a strain where in sucA on the chromosome was replaced with a mutant type one by homologous recombination of the plasmid was obtained by using the tetracycline resistance as an indicator. The obtained strain was designated as SC17sucA strain.

In order to confirm that the SC17sucA strain was deficient in the  $\alpha$ KGDH activity, the enzyme activity was measured by the method of Reed et al. (Reed, L. J. and Mukherjee, B. B., Methods in Enzymology, 13, pp. 55-61, (1969)) by using cells of the strain cultured in LB medium until the logarithmic growth phase. As a result,  $\alpha$ KGDH activity of 0.073 ( $\Delta$ ABS/min/mg protein) was detected from the SC17 strain, whereas no  $\alpha$ KGDH activity was detected from the SC17sucA strain, and thus it was confirmed that the sucA was deficient as purposed.

(2) Enhancement of L-Glutamic Acid Biosynthetic System of [Enterobacter agglomerans] *Pantoea ananatis* SC17sucA Strain

Subsequently, a citrate synthase gene, a phosphoenolpyruvate carboxylase gene and a glutamate dehydrogenase gene derived from *Escherichia coli* were introduced into the SC17sucA strain.

(i) Preparation of Plasmid Having gltA Gene, ppc Gene and gdhA Gene Derived from *Escherichia coli*

The procedures of preparing a plasmid having a gltA gene, a ppc gene and a gdhA gene will be explained by referring to FIGS. 6 and 7.

A plasmid having a gdhA gene derived from *Escherichia coli*, pBRGDH (Japanese Patent Application Laid-open No. 7-203980), was digested with HindIII and SphI, the both ends were blunt-ended by the T4 DNA polymerase treatment, and then the DNA fragment having the gdhA gene was purified and recovered. Separately, a plasmid having a gltA gene and



a *ppc* gene derived from *Escherichia coli*, pMWCP (WO 97/08294), was digested with *Xba*I, and then the both ends were blunt-ended by using T4 DNA polymerase. This was mixed with the above purified DNA fragment having the *gdhA* gene and ligated by using T4 ligase to obtain a plasmid pMWCPG, which corresponded to pMWCP further containing the *gdhA* gene (FIG. 6).

At the same time, the plasmid pVIC40 (Japanese Patent Application Laid-open No. 8-047397) having the replication origin of the broad host spectrum plasmid RSF1010 was digested with *Not*I, treated with T4 DNA polymerase and digested with *Pst*I. pBR322 was digested with *Eco*T14I, treated with T4 DNA polymerase and digested with *Pst*I. The both products were mixed and ligated by using T4 ligase to obtain a plasmid RSF-Tet having the replication origin of RSF1010 and a tetracycline resistance gene (FIG. 7).

Subsequently, pMWCPG was digested with *Eco*RI and *Pst*I, and a DNA fragment having the *gltA* gene, the *ppc* gene and the *gdhA* gene was purified and recovered. RSF-Tet was similarly digested with *Eco*RI and *Pst*I, and a DNA fragment having the replication origin of RSF1010 was purified and recovered. The both products were mixed and ligated by using T4 ligase to obtain a plasmid RSFCPG, which corresponded to RSF-Tet containing the *gltA* gene, the *ppc* gene and the *gdhA* gene (FIG. 8). It was confirmed that the obtained plasmid RSFCPG expressed the *gltA* gene, the *ppc* gene and the *gdhA* gene, by the complementation of the auxotrophy of the *gltA*, *ppc* or *gdhA* gene deficient strain derived from *Escherichia coli* and measurement of each enzyme activity.

#### (ii) Preparation of Plasmid Having *gltA* Gene Derived from *Brevibacterium lactofermentum*

A plasmid having the *gltA* gene derived from *Brevibacterium lactofermentum* was constructed as follows. PCR was performed by using the primer DNAs having the nucleotide sequences represented by SEQ ID NOS: 6 and 7, which were prepared based on the nucleotide sequence of the *Corynebacterium glutamicum* *gltA* gene (Microbiology, 140, pp. 1817-1828 (1994)), and chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 as a template to obtain a *gltA* gene fragment of about 3 kb. This fragment inserted into a plasmid pHSG399 (purchased from Takara Shuzo Co., Ltd.) digested with *Sma*I to obtain a plasmid pHSGCB (FIG. 9). Subsequently, pHSGCB was digested with *Hind*III, and the excised *gltA* gene fragment of about 3 kb was inserted into a plasmid pSTV29 (purchased from Takara Shuzo Co., Ltd.) digested with *Hind*III to obtain a plasmid pSTVCB (FIG. 9). It was confirmed that the obtained plasmid pSTVCB expressed the *gltA* gene, by measuring the enzyme activity in the [Enterobacter agglomerans] *Pantoea ananatis* AJ13355 strain.

#### (iii) Introduction of RSFCPG and pSTVCB into SC17sucA Strain

The [Enterobacter agglomerans] *Pantoea ananatis* SC17sucA strain was transformed with RSFCPG by electroporation to obtain a transformant SC17sucA/RSFCPG strain having tetracycline resistance. Further, the SC17sucA/RSFCPG strain was transformed with pSTVCB by electroporation to obtain a transformant SC17sucA/RSFCPG+pSTVCB strain having chloramphenicol resistance.

#### <4> Acquisition of Strain with Improved Resistance to L-Glutamic Acid in Low pH Environment

A strain with improved resistance to L-glutamic acid at a high concentration in a low pH environment (hereafter, also referred to as "high-concentration Glu-resistant strain at low pH") was isolated from the [Enterobacter agglomerans] *Pantoea ananatis* SC17sucA/RSFCPG+pSTVCB strain.

The SC17sucA/RSFCPG+pSTVCB strain was cultured overnight at 30° C. in LBG medium (10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of NaCl, 5 g/L of glucose), and the

cells washed with saline was appropriately diluted and plated on an M9-E medium (4 g/L of glucose, 17 g/L of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 3 g/L of  $\text{KH}_2\text{PO}_4$ , 0.5 g/L of NaCl, 1 g/L of  $\text{NH}_4\text{Cl}$ , 10 mM of  $\text{MgSO}_4$ , 10  $\mu\text{M}$  of  $\text{CaCl}_2$ , 50 mg/L of L-lysine, 50 mg/L of L-methionine, 50 mg/L of DL-diaminopimelic acid, 25 mg/L of tetracycline, 25 mg/L of chloramphenicol, 30 g/L of L-glutamic acid, adjusted to pH 4.5 with aqueous ammonia) plate. The colony emerged after culture at 32° C. for 2 days was obtained as a high-concentration Glu-resistant strain at low pH.

For the obtained strain, growth level in M9-E liquid medium was measured and L-glutamic acid-producing ability was tested in a 50-ml volume large test tube containing 5 ml of L-glutamic acid production test medium (40 g/L of glucose, 20 g/L of  $(\text{NH}_4)_2\text{SO}_4$ , 0.5 g/L of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/L of  $\text{KH}_2\text{PO}_4$ , 0.5 g/L of NaCl, 0.25 g/L of  $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$ , 0.02 g/L of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02 g/L of  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.72 mg/L of  $\text{ZnSO}_4 \cdot 2\text{H}_2\text{O}$ , 0.64 mg/L of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.72 mg/L of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.4 mg/L of boric acid, 1.2 mg/L of  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 2 g/L of yeast extract, 200 mg/L of L-lysine hydrochloride, 200 mg/L of L-methionine, 200 mg/L of DL- $\alpha, \epsilon$ -diaminopimelic acid, 25 mg/L of tetracycline hydrochloride, 25 mg/L of chloramphenicol). A strain that exhibited the best growth level and the same L-glutamic acid producing ability as that of its parent strain, the SC17/RSFCPG+pSTVCB strain, was designated as [Enterobacter agglomerans] *Pantoea ananatis* AJ13601. The AJ13601 strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (postal code: 305-8566, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan) on Aug. 18, 1999 and received an accession number of FERM P-17516. It was then transferred to an international deposition under the provisions of Budapest Treaty on Jul. 6, 2000 and received an accession number of FERM BP-7207.

<5> Culture of [Enterobacter Agglomerans] *Pantoea ananatis* AJ13601 Strain for L-Glutamic Acid Production (1)  
The [Enterobacter agglomerans] *Pantoea ananatis* AJ13601 strain was inoculated into a 1-L jar fermenter containing 300 ml of medium containing 40 g/L of glucose, 20 g/L of  $(\text{NH}_4)_2\text{SO}_4$ , 0.5 g/L of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/L of  $\text{KH}_2\text{PO}_4$ , 0.5 g/L of NaCl, 0.25 g/L of  $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$ , 0.02 g/L of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02 g/L of  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.72 mg/L of  $\text{ZnSO}_4 \cdot 2\text{H}_2\text{O}$ , 0.64 mg/L of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.72 mg/L of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.4 mg/L of boric acid, 1.2 mg/L of  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 2 g/L of yeast extract, 200 mg/L of L-lysine hydrochloride, 200 mg/L of L-methionine, 200 mg/L of DL- $\alpha, \epsilon$ -diaminopimelic acid, 25 mg/L of tetracycline hydrochloride and 25 mg/L of chloramphenicol, and cultured at 34° C. and pH 6.0 for 14 hours. The culture pH was controlled by introducing ammonia gas into the medium.

The culture obtained as described above was centrifuged at 5000 rpm for 10 minutes, and the collected cells were inoculated into a 1-L jar fermenter containing 300 ml of medium containing 40 g/L of glucose, 5 g/L of  $(\text{NH}_4)_2\text{SO}_4$ , 1.5 g/L of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 6 g/L of  $\text{KH}_2\text{PO}_4$ , 1.5 g/L of NaCl, 0.75 g/L of  $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$ , 0.06 g/L of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.06 g/L of  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 2.16 mg/L of  $\text{ZnSO}_4 \cdot 2\text{H}_2\text{O}$ , 1.92 mg/L of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 2.16 mg/L of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 1.2 mg/L of boric acid, 3.6 mg/L of  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 6 g/L of yeast extract, 600 mg/L of L-lysine hydrochloride, 600 mg/L of L-methionine, 600 mg/L of DL- $\alpha, \epsilon$ -diaminopimelic acid, 25 mg/L of tetracycline hydrochloride and 25 mg/L of chloramphenicol and cultured at 34° C. and pH 4.5 to perform culture for L-glutamic acid production. The culture pH was controlled by introducing ammonia gas into the medium. As the initially added glucose was depleted, 600 g/L of glucose was continuously added.

As a result of the culture for L-glutamic acid production performed for 50 hours as described above, a substantial



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amount of L-glutamic acid crystals were precipitated in the jar fermenter. Table 1 shows the concentration of L-glutamic acid dissolved in the culture broth at that time and the L-glutamic acid concentration measured by dissolving the crystals in 2 M potassium hydroxide. L-Glutamic acid crystals were collected from the culture by decantation after the culture was left stood.

TABLE 1

Concentration of L-glutamic acid dissolved in culture broth	51 g/L
Amount of L-glutamic acid precipitated as crystals	67 g/L
Concentration of L-glutamic acid measured by dissolving crystals	118 g/L

<6> Culture of [Enterobacter Agglomerans] *Pantoea ananatis* AJ13601 Strain for L-Glutamic Acid Production (2)

The following experiment was performed in order to confirm that the [Enterobacter agglomerans] *Pantoea ananatis* AJ13601 strain still had L-glutamic acid-producing ability even under the condition that L-glutamic acid crystals were present.

The [Enterobacter agglomerans] *Pantoea ananatis* AJ13601 strain was inoculated into a 1-L jar fermenter containing 300 ml of medium containing 40 g/L of glucose, 20 g/L of  $(\text{NH}_4)_2\text{SO}_4$ , 0.5 g/L of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2 g/L of  $\text{KH}_2\text{PO}_4$ , 0.5 g/L of NaCl, 0.25 g/L of  $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$ , 0.02 g/L of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02 g/L of  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.72 mg/L of  $\text{ZnSO}_4 \cdot 2\text{H}_2\text{O}$ , 0.64 mg/L of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.72 mg/L of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.4 mg/L of boric acid, 1.2 mg/L of  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 2 g/L of yeast extract, 200 mg/L of L-lysine hydrochloride, 200 mg/L of L-methionine, 200 mg/L of DL- $\alpha, \epsilon$ -diaminopimelic acid, 25 mg/L of tetracycline hydrochloride and 25 mg/L of chloramphenicol, and cultured at 34° C. at pH 6.0 for 14 hours. The culture pH was controlled by bubbling the medium with ammonia gas. The culture obtained as described above was centrifuged at 5000 rpm for 10 minutes, and then the collected cells were cultured in a medium where L-glutamic acid was present as crystals. The used medium contained 40 g/L of glucose, 5 g/L of  $(\text{NH}_4)_2\text{SO}_4$ , 1.5 g/L of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 6 g/L of  $\text{KH}_2\text{PO}_4$ , 1.5 g/L of NaCl, 0.75 g/L of  $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$ , 0.06 g/L of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.06 g/L of  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 2.16 mg/L of  $\text{ZnSO}_4 \cdot 2\text{H}_2\text{O}$ , 1.92 mg/L of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 2.16 mg/L of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 1.2 mg/L of boric acid, 3.6 mg/L of  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 6 g/L of yeast extract, 600 mg/L of L-lysine hydrochloride, 600 mg/L of L-methionine, 600 mg/L of DL- $\alpha, \epsilon$ -diaminopimelic acid, 25 mg/L of tetracycline hydrochloride and 25 mg/L of chloramphenicol and L-glutamic acid crystals were added to 40 g/L. The cells were inoculated in a 1-L jar fermenter containing 300 ml of this medium and cultured at 34° C. and pH 4.3 to perform culture for L-glutamic acid production. The culture pH was controlled by introducing ammonia gas into the medium. As the initially added glucose was depleted, 600 g/L of glucose was continuously added. In this medium, only 39 g/L of the added L-glutamic acid was dissolved at pH 4.3 and the remaining 1 g/L was present as crystals.

As a result of the culture for L-glutamic acid production performed for 53 hours as described above, a substantial amount of L-glutamic acid crystals were precipitated in the jar fermenter. Table 2 shows the concentration of L-glutamic acid dissolved in the culture broth, the amount of L-glutamic acid present as crystals at that time and the L-glutamic acid concentration measured by dissolving the crystals in 2MKOH solution. L-Glutamic acid crystals were collected from the culture by decantation after the culture was left stood. The results showed that the [Enterobacter agglomerans] *Pantoea ananatis* AJ13101 strain accumulated

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L-glutamic acid and precipitated crystals thereof even under the condition that L-glutamic acid crystals were present.

TABLE 2

Concentration of L-glutamic acid dissolved in culture broth	39 g/L
Amount of L-glutamic acid precipitated as crystals	119 g/L
Concentration of L-glutamic acid measured by dissolving crystals	158 g/L
Amount of L-glutamic acid crystals newly produced by main culture	118 g/L

<7> Culture of [Enterobacter agglomerans] *Pantoea ananatis* AJ13601 Strain for L-Glutamic Acid Production (3)

The [Enterobacter agglomerans] *Pantoea ananatis* AJ13601 strain can grow not only at an acidic pH, but also at a neutral pH. Therefore, it was confirmed as follows that L-glutamic acid crystals could also be precipitated by starting the culture at a neutral pH and allowing production of L-glutamic acid during the culture so that pH of the culture should spontaneously be lowered.

Cells of one plate (8.5 cm in diameter) of the [Enterobacter agglomerans] *Pantoea ananatis* AJ13601 strain, cultured on LBG agar medium (10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of NaCl, 5 g/L of glucose, 15 g/L of agar) containing 25 mg/L of tetracycline hydrochloride and 25 mg/L of chloramphenicol at 30° C. for 14 hours, were inoculated into a 1-L jar fermenter containing 300 ml of medium containing 40 g/L of glucose, 5 g/L of  $(\text{NH}_4)_2\text{SO}_4$ , 1.5 g/L of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 6 g/L of  $\text{KH}_2\text{PO}_4$ , 1.5 g/L of NaCl, 0.75 g/L of  $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$ , 0.06 g/L of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.06 g/L of  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 2.16 mg/L of  $\text{ZnSO}_4 \cdot 2\text{H}_2\text{O}$ , 1.92 mg/L of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 2.16 mg/L of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 1.2 mg/L of boric acid, 3.6 mg/L of  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 6 g/L of yeast extract, 600 mg/L of L-lysine hydrochloride, 600 mg/L of L-methionine, 600 mg/L of DL- $\alpha, \epsilon$ -diaminopimelic acid, 25 mg/L of tetracycline hydrochloride and 25 mg/L of chloramphenicol and the culture was started at 34° C. and pH 7.0. The culture pH was controlled by introducing ammonia gas into the medium. As the initially added glucose was depleted, 600 g/L of glucose was continuously added.

As L-glutamic acid is accumulated, pH lowers spontaneously. The amount of the introduced ammonia gas was adjusted so that pH should be gradually lowered from 7.0 to 4.5 during the period between 15 hours and 24 hours after the start of the culture, and 24 hours after the start of the culture, pH became 4.5. Afterward, cultivation was continued for 12 hours.

As a result of the culture for L-glutamic acid production conducted for 36 hours as described above, a substantial amount of L-glutamic acid crystals were precipitated in the jar fermenter. Table 3 shows the concentration of L-glutamic acid dissolved in the culture broth, the amount of L-glutamic acid present as crystals at that time and the L-glutamic acid concentration measured by dissolving the crystals in 2MKOH solution. L-Glutamic acid crystals were collected from the culture by decantation after the culture was left stood.

TABLE 3

Concentration of L-glutamic acid dissolved in culture broth	45 g/L
Amount of L-glutamic acid precipitated as crystals	31 g/L
Concentration of L-glutamic acid measured by dissolving crystals	76 g/L



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          35

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cggcaacaaa tgaacctca aaaaagcata acattgctta agggatcaca atg cag      327
Met Gln
          40

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Thr Gly Val Lys Pro Glu Gln Phe His Ser Ala Thr Arg Glu Tyr Phe
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Pro Ala Thr Asn Ser Lys Gln Val Lys Val Leu Gln Leu Ile Asn Ala
          125             130             135

ttt cgt ttc cgc gga cat cag gaa gca aat ctc gat ccg ctt ggc ctg      663
Phe Arg Phe Arg Gly His Gln Glu Ala Asn Leu Asp Pro Leu Gly Leu
          140             145             150

tgg aaa cag gac cgc gtt gcc gat ctc gat cct gcc ttt cac gat ctg      711
Trp Lys Gln Asp Arg Val Ala Asp Leu Asp Pro Ala Phe His Asp Leu
          155             160             165

acc gac gcc gat ttt cag gaa agc ttt aac gta ggt tct ttt gcc att      759
Thr Asp Ala Asp Phe Gln Glu Ser Phe Asn Val Gly Ser Phe Ala Ile
          170             175             180             185

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ggc aaa gaa acc atg aag ctg gcc gat ctg ttc gac gcg ctg aag cag	807
Gly Lys Glu Thr Met Lys Leu Ala Asp Leu Phe Asp Ala Leu Lys Gln	
190 195 200	
acc tac tgt ggc tcg att ggt gca gag tat atg cac atc aat aac acc	855
Thr Tyr Cys Gly Ser Ile Gly Ala Glu Tyr Met His Ile Asn Asn Thr	
205 210 215	
gaa gag aaa cgc tgg atc cag cag cgt atc gaa tcc ggt gcg agc cag	903
Glu Glu Lys Arg Trp Ile Gln Gln Arg Ile Glu Ser Gly Ala Ser Gln	
220 225 230	
acg tca ttc agt ggc gaa gag aaa aaa ggt ttc ctg aaa gag ctg acc	951
Thr Ser Phe Ser Gly Glu Glu Lys Lys Gly Phe Leu Lys Glu Leu Thr	
235 240 245	
gcg gca gaa ggg ctg gaa aaa tat ctg gcc gcg aaa ttc ccg ggt gca	999
Ala Ala Glu Gly Leu Glu Lys Tyr Leu Gly Ala Lys Phe Pro Gly Ala	
250 255 260 265	
aaa cgt ttc tcg ctg gaa ggc ggt gat gcg ctg gtg ccg atg ctg cgc	1047
Lys Arg Phe Ser Leu Glu Gly Gly Asp Ala Leu Val Pro Met Leu Arg	
270 275 280	
gag atg att cgt cat gcg ggc aaa agc gcc aca cgt gaa gtg gta ctg	1095
Glu Met Ile Arg His Ala Gly Lys Ser Gly Thr Arg Glu Val Val Leu	
285 290 295	
ggg atg gcg cac cgt ggc cgt ctt aac gta ctg att aac gta ctg ggt	1143
Gly Met Ala His Arg Gly Arg Leu Asn Val Leu Ile Asn Val Leu Gly	
300 305 310	
aaa aag cca cag gat ctg ttc gac gaa ttc tcc ggt aaa cac aaa gag	1191
Lys Lys Pro Gln Asp Leu Phe Asp Glu Phe Ser Gly Lys His Lys Glu	
315 320 325	
cat ctg ggc acc ggt gat gtg aag tat cac atg gcc ttc tct tcg gat	1239
His Leu Gly Thr Gly Asp Val Lys Tyr His Met Gly Phe Ser Ser Asp	
330 335 340 345	
att gaa acc gaa ggt ggt ctg gtg cat ctg gcg ctg gcg ttt aac ccg	1287
Ile Glu Thr Glu Gly Gly Leu Val His Leu Ala Leu Ala Phe Asn Pro	
350 355 360	
tct cac ctg gaa att gtc agc ccg gtg gtc atg gga tcg gta cgt gca	1335
Ser His Leu Glu Ile Val Ser Pro Val Val Met Gly Ser Val Arg Ala	
365 370 375	
cgt ctc gat cgt ctg gcc gaa ccg gtc agc aat aaa gtg ttg cct atc	1383
Arg Leu Asp Arg Leu Ala Glu Pro Val Ser Asn Lys Val Leu Pro Ile	
380 385 390	
acc att cac ggt gat gcg gcg gtg att ggt cag gcc gtg gtt cag gaa	1431
Thr Ile His Gly Asp Ala Ala Val Ile Gly Gln Gly Val Val Gln Glu	
395 400 405	
acc ctg aac atg tct cag gcg cgc gcc tac gaa gtg gcc gcc acg gta	1479
Thr Leu Asn Met Ser Gln Ala Arg Gly Tyr Glu Val Gly Gly Thr Val	
410 415 420 425	
cgt atc gtc att aac aac cag gtt ggt ttt acc acc tcc aac ccg aaa	1527
Arg Ile Val Ile Asn Asn Gln Val Gly Phe Thr Thr Ser Asn Pro Lys	
430 435 440	
gat gcg cgt tca acc ccg tac tgt act gac atc gcc aag atg gtg ctg	1575
Asp Ala Arg Ser Thr Pro Tyr Cys Thr Asp Ile Gly Lys Met Val Leu	
445 450 455	
gca ccg att ttc cac gtc aat gct gac gat ccg gaa gcg gtg gcc ttt	1623
Ala Pro Ile Phe His Val Asn Ala Asp Asp Pro Glu Ala Val Ala Phe	
460 465 470	
gtt acc cgc ctg gcg ctg gac tat cgc aac acc ttc aaa cgc gat gtg	1671
Val Thr Arg Leu Ala Leu Asp Tyr Arg Asn Thr Phe Lys Arg Asp Val	
475 480 485	
ttt atc gat ctg gtg tgc tat cgc cgt cat ggt cac aac gag gcg gat	1719
Phe Ile Asp Leu Val Cys Tyr Arg Arg His Gly His Asn Glu Ala Asp	
490 495 500 505	



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gag cca agt gct acc cag ccg ttg atg tac cag aaa atc aaa aag cat	1767
Glu Pro Ser Ala Thr Gln Pro Leu Met Tyr Gln Lys Ile Lys Lys His	
510 515 520	
ccg acg ccg cgt aaa att tac gcc gat cgt ctg gaa ggc gaa ggt gtc	1815
Pro Thr Pro Arg Lys Ile Tyr Ala Asp Arg Leu Glu Gly Glu Gly Val	
525 530 535	
gcg tcg cag gaa gat gcc acc gag atg gtg aac ctg tac cgc gat gcg	1863
Ala Ser Gln Glu Asp Ala Thr Glu Met Val Asn Leu Tyr Arg Asp Ala	
540 545 550	
ctc gat gcg ggc gaa tgc gtg gtg ccg gaa tgg cgt ccg atg agc ctg	1911
Leu Asp Ala Gly Glu Cys Val Val Pro Glu Trp Arg Pro Met Ser Leu	
555 560 565	
cac tcc ttc acg tgg tcg cct tat ctg aac cac gaa tgg gat gag cct	1959
His Ser Phe Thr Trp Ser Pro Tyr Leu Asn His Glu Trp Asp Glu Pro	
570 575 580 585	
tat ccg gca cag gtt gac atg aaa cgc ctg aag gaa ctg gca ttg cgt	2007
Tyr Pro Ala Gln Val Asp Met Lys Arg Leu Lys Glu Leu Ala Leu Arg	
590 595 600	
atc agc cag gtc cct gag cag att gaa gtg cag tcg cgc gtg gcc aag	2055
Ile Ser Gln Val Pro Glu Gln Ile Glu Val Gln Ser Arg Val Ala Lys	
605 610 615	
atc tat aac gat cgc aag ctg atg gcc gaa ggc gag aaa gcg ttc gac	2103
Ile Tyr Asn Asp Arg Lys Leu Met Ala Glu Gly Glu Lys Ala Phe Asp	
620 625 630	
tgg ggc ggt gcc gag aat ctg gcg tac gcc acg ctg gtg gat gaa ggt	2151
Trp Gly Gly Ala Glu Asn Leu Ala Tyr Ala Thr Leu Val Asp Glu Gly	
635 640 645	
att ccg gtt cgc ctc tcg ggt gaa gac tcc ggt cgt gga acc ttc ttc	2199
Ile Pro Val Arg Leu Ser Gly Glu Asp Ser Gly Arg Gly Thr Phe Phe	
650 655 660 665	
cat cgc cac gcg gtc gtg cac aac cag gct aac ggt tca acc tat acg	2247
His Arg His Ala Val Val His Asn Gln Ala Asn Gly Ser Thr Tyr Thr	
670 675 680	
ccg ctg cac cat att cat aac agc cag gcc gag ttc aaa gtc tgg gat	2295
Pro Leu His His Ile His Asn Ser Gln Gly Glu Phe Lys Val Trp Asp	
685 690 695	
tcg gtg ctg tct gaa gaa gcg gtg ctg gcg ttt gaa tac ggt tac gcc	2343
Ser Val Leu Ser Glu Glu Ala Val Leu Ala Phe Glu Tyr Gly Tyr Ala	
700 705 710	
acg gct gag ccg cgc gtg ctg acc atc tgg gaa gcg cag ttt ggt gac	2391
Thr Ala Glu Pro Arg Val Leu Thr Ile Trp Glu Ala Gln Phe Gly Asp	
715 720 725	
ttt gcc aac ggt gct cag gtg gtg att gac cag ttc atc agc tct ggc	2439
Phe Ala Asn Gly Ala Gln Val Val Ile Asp Gln Phe Ile Ser Ser Gly	
730 735 740 745	
gaa cag aag tgg ggc cgt atg tgt gcc ctg gtg atg ttg ctg ccg cat	2487
Glu Gln Lys Trp Gly Arg Met Cys Gly Leu Val Met Leu Leu Pro His	
750 755 760	
ggc tac gaa ggt cag gga ccg gaa cac tcc tct gcc cgt ctg gaa cgc	2535
Gly Tyr Glu Gly Gln Gly Pro Glu His Ser Ser Ala Arg Leu Glu Arg	
765 770 775	
tat ctg caa ctt tgc gcc gag cag aac atg cag gtt tgc gtc ccg tcg	2583
Tyr Leu Gln Leu Cys Ala Glu Gln Asn Met Gln Val Cys Val Pro Ser	
780 785 790	
acg ccg gct cag gtg tat cac atg ctg cgc cgt cag gcg ctg cgc ggg	2631
Thr Pro Ala Gln Val Tyr His Met Leu Arg Arg Gln Ala Leu Arg Gly	
795 800 805	
atg cgc cgt ccg ctg gtg gtg atg tcg ccg aag tcg ctg tta cgc cat	2679
Met Arg Arg Pro Leu Val Val Met Ser Pro Lys Ser Leu Leu Arg His	
810 815 820 825	

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cca ctg gcg atc tcg tcg ctg gat gaa ctg gca aac ggc agt ttc cag	2727
Pro Leu Ala Ile Ser Ser Leu Asp Glu Leu Ala Asn Gly Ser Phe Gln	
830 835 840	
ccg gcc att ggt gag atc gac gat ctg gat ccg cag ggc gtg aaa cgc	2775
Pro Ala Ile Gly Glu Ile Asp Asp Leu Asp Pro Gln Gly Val Lys Arg	
845 850 855	
gtc gtg ctg tgc tcc ggt aag gtt tac tac gat ctg ctg gaa cag cgt	2823
Val Val Leu Cys Ser Gly Lys Val Tyr Tyr Asp Leu Leu Glu Gln Arg	
860 865 870	
cgt aaa gac gag aaa acc gat gtt gcc atc gtg cgc atc gaa cag ctt	2871
Arg Lys Asp Glu Lys Thr Asp Val Ala Ile Val Arg Ile Glu Gln Leu	
875 880 885	
tac ccg ttc ccg cat cag gcg gta cag gaa gca ttg aaa gcc tat tct	2919
Tyr Pro Phe Pro His Gln Ala Val Gln Glu Ala Leu Lys Ala Tyr Ser	
890 895 900 905	
cac gta cag gac ttt gtc tgg tgc cag gaa gag cct ctg aac cag ggc	2967
His Val Gln Asp Phe Val Trp Cys Gln Glu Glu Pro Leu Asn Gln Gly	
910 915 920	
gcc tgg tac tgt agc cag cat cat ttc cgt gat gtc gtg ccg ttt ggt	3015
Ala Trp Tyr Cys Ser Gln His His Phe Arg Asp Val Val Pro Phe Gly	
925 930 935	
gcc acc ctg cgt tat gca ggt cgc ccg gca tcg gct tct ccg gcc gtg	3063
Ala Thr Leu Arg Tyr Ala Gly Arg Pro Ala Ser Ala Ser Pro Ala Val	
940 945 950	
ggt tat atg tcc gta cac caa caa cag cag caa gac ctg gtt aat gac	3111
Gly Tyr Met Ser Val His Gln Gln Gln Gln Gln Leu Val Asn Asp	
955 960 965	
gca ctg aac gtc aat taa ttaaaaggaa agata atg agt agc gta gat att	3162
Ala Leu Asn Val Asn Met Ser Ser Val Asp Ile	
970 975 980	
ctc gtt ccc gac ctg cct gaa tcg gtt gca gat gcc aca gta gca acc	3210
Leu Val Pro Asp Leu Pro Glu Ser Val Ala Asp Ala Thr Val Ala Thr	
985 990 995	
tgg cac aag aaa cca ggc gat gca gtc agc cgc gat gaa gtc atc	3255
Trp His Lys Lys Pro Gly Asp Ala Val Ser Arg Asp Glu Val Ile	
1000 1005 1010	
gtc gaa att gaa act gac aaa gtc gtg ctg gaa gtg ccg gca tct	3300
Val Glu Ile Glu Thr Asp Lys Val Val Leu Glu Val Pro Ala Ser	
1015 1020 1025	
gcc gat ggc gtg ctg gaa gcc gtg ctg gaa gac gaa ggg gca acc	3345
Ala Asp Gly Val Leu Glu Ala Val Leu Glu Asp Glu Gly Ala Thr	
1030 1035 1040	
gtt acg tcc cgc cag atc ctg ggt cgc ctg aaa gaa ggc aac agt	3390
Val Thr Ser Arg Gln Ile Leu Gly Arg Leu Lys Glu Gly Asn Ser	
1045 1050 1055	
gcg ggt aaa gaa agc agt gcc aaa gcg gaa agc aat gac acc acg	3435
Ala Gly Lys Glu Ser Ser Ala Lys Ala Glu Ser Asn Asp Thr Thr	
1060 1065 1070	
cca gcc cag cgt cag aca gcg tcg ctt gaa gaa gag agc agc gat	3480
Pro Ala Gln Arg Gln Thr Ala Ser Leu Glu Glu Glu Ser Ser Asp	
1075 1080 1085	
gcg ctc agc ccg gcg atc cgt cgc ctg att gcg gag cat aat ctt	3525
Ala Leu Ser Pro Ala Ile Arg Arg Leu Ile Ala Glu His Asn Leu	
1090 1095 1100	
gac gct gcg cag atc aaa ggc acc ggc gta ggc gga cgt tta acg	3570
Asp Ala Ala Gln Ile Lys Gly Thr Gly Val Gly Gly Arg Leu Thr	
1105 1110 1115	
cgt gaa gac gtt gaa aaa cat ctg gcg aac aaa ccg cag gct gag	3615
Arg Glu Asp Val Glu Lys His Leu Ala Asn Lys Pro Gln Ala Glu	
1120 1125 1130	



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aaa gcc gcc gcg cca gcg gcg ggt gca gca acg gct cag cag cct	3660
Lys Ala Ala Ala Pro Ala Ala Gly Ala Ala Thr Ala Gln Gln Pro	
1135 1140 1145	
gtt gcc aac cgc agc gaa aaa cgt gtt ccg atg acg cgt tta cgt	3705
Val Ala Asn Arg Ser Glu Lys Arg Val Pro Met Thr Arg Leu Arg	
1150 1155 1160	
aag cgc gtc gcg gag cgt ctg ctg gaa gcc aag aac agc acc gcc	3750
Lys Arg Val Ala Glu Arg Leu Leu Glu Ala Lys Asn Ser Thr Ala	
1165 1170 1175	
atg ttg acg acc ttc aac gaa atc aac atg aag ccg att atg gat	3795
Met Leu Thr Thr Phe Asn Glu Ile Asn Met Lys Pro Ile Met Asp	
1180 1185 1190	
ctg cgt aag cag tac ggc gat gcg ttc gag aag cgt cac ggt gtg	3840
Leu Arg Lys Gln Tyr Gly Asp Ala Phe Glu Lys Arg His Gly Val	
1195 1200 1205	
cgt ctg ggc ttt atg tct ttc tac atc aag gcc gtg gtc gaa gcg	3885
Arg Leu Gly Phe Met Ser Phe Tyr Ile Lys Ala Val Val Glu Ala	
1210 1215 1220	
ctg aag cgt tat cca gaa gtc aac gcc tct atc gat ggc gaa gac	3930
Leu Lys Arg Tyr Pro Glu Val Asn Ala Ser Ile Asp Gly Glu Asp	
1225 1230 1235	
gtg gtg tac cac aac tat ttc gat gtg agt att gcc gtc tct acg	3975
Val Val Tyr His Asn Tyr Phe Asp Val Ser Ile Ala Val Ser Thr	
1240 1245 1250	
cca cgc gga ctg gtg acg cct gtc ctg cgt gac gtt gat gcg ctg	4020
Pro Arg Gly Leu Val Thr Pro Val Leu Arg Asp Val Asp Ala Leu	
1255 1260 1265	
agc atg gct gac atc gag aag aaa att aaa gaa ctg gca gtg aaa	4065
Ser Met Ala Asp Ile Glu Lys Lys Ile Lys Glu Leu Ala Val Lys	
1270 1275 1280	
ggc cgt gac ggc aag ctg acg gtt gac gat ctg acg ggc ggt aac	4110
Gly Arg Asp Gly Lys Leu Thr Val Asp Asp Leu Thr Gly Gly Asn	
1285 1290 1295	
ttt acc atc acc aac ggt ggt gtg ttc ggt tcg ctg atg tct acg	4155
Phe Thr Ile Thr Asn Gly Gly Val Phe Gly Ser Leu Met Ser Thr	
1300 1305 1310	
cca atc atc aac ccg cca cag agc gcg att ctg ggc atg cac gcc	4200
Pro Ile Ile Asn Pro Pro Gln Ser Ala Ile Leu Gly Met His Ala	
1315 1320 1325	
att aaa gat cgt cct atg gcg gtc aat ggt cag gtt gtg atc ctg	4245
Ile Lys Asp Arg Pro Met Ala Val Asn Gly Gln Val Val Ile Leu	
1330 1335 1340	
cca atg atg tac ctg gct ctc tcc tac gat cac cgt tta atc gat	4290
Pro Met Met Tyr Leu Ala Leu Ser Tyr Asp His Arg Leu Ile Asp	
1345 1350 1355	
ggc cgt gaa tct gtc ggc tat ctg gtc gcg gtg aaa gag atg ctg	4335
Gly Arg Glu Ser Val Gly Tyr Leu Val Ala Val Lys Glu Met Leu	
1360 1365 1370	
gaa gat ccg gcg cgt ctg ctg ctg gat gtc tga ttcactcactg	4378
Glu Asp Pro Ala Arg Leu Leu Leu Asp Val	
1375 1380	
ggcacgcggtt gcgtgcccaa tctcaatact cttttcagat ctgaatggat agaacatc	4436
atg aac tta cac gaa tac cag gct aaa cag ctg ttt gca cgg tat	4481
Met Asn Leu His Glu Tyr Gln Ala Lys Gln Leu Phe Ala Arg Tyr	
1385 1390 1395	
ggc atg cca gca ccg acc ggc tac gcc tgt act aca cca cgt gaa	4526
Gly Met Pro Ala Pro Thr Gly Tyr Ala Cys Thr Thr Pro Arg Glu	
1400 1405 1410	
gca gaa gaa gcg gca tcg aaa atc ggt gca	4556
Ala Glu Glu Ala Ala Ser Lys Ile Gly Ala	

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1415 1420

<210> SEQ ID NO 2  
 <211> LENGTH: 39  
 <212> TYPE: PRT  
 <213> ORGANISM: Enterobacter agglomerans

<400> SEQUENCE: 2

Ala Phe Ser Val Phe Arg Cys His Ser Ile Met Asn Cys Val Ser Val  
 1 5 10 15

Cys Pro Lys Gly Leu Asn Pro Thr Arg Ala Ile Gly His Ile Lys Ser  
 20 25 30

Met Leu Leu Gln Arg Ser Ala  
 35

<210> SEQ ID NO 3  
 <211> LENGTH: 935  
 <212> TYPE: PRT  
 <213> ORGANISM: Enterobacter agglomerans

<400> SEQUENCE: 3

Met Gln Asn Ser Ala Met Lys Pro Trp Leu Asp Ser Ser Trp Leu Ala  
 1 5 10 15

Gly Ala Asn Gln Ser Tyr Ile Glu Gln Leu Tyr Glu Asp Phe Leu Thr  
 20 25 30

Asp Pro Asp Ser Val Asp Ala Val Trp Arg Ser Met Phe Gln Gln Leu  
 35 40 45

Pro Gly Thr Gly Val Lys Pro Glu Gln Phe His Ser Ala Thr Arg Glu  
 50 55 60

Tyr Phe Arg Arg Leu Ala Lys Asp Ala Ser Arg Tyr Thr Ser Ser Val  
 65 70 75 80

Thr Asp Pro Ala Thr Asn Ser Lys Gln Val Lys Val Leu Gln Leu Ile  
 85 90 95

Asn Ala Phe Arg Phe Arg Gly His Gln Glu Ala Asn Leu Asp Pro Leu  
 100 105 110

Gly Leu Trp Lys Gln Asp Arg Val Ala Asp Leu Asp Pro Ala Phe His  
 115 120 125

Asp Leu Thr Asp Ala Asp Phe Gln Glu Ser Phe Asn Val Gly Ser Phe  
 130 135 140

Ala Ile Gly Lys Glu Thr Met Lys Leu Ala Asp Leu Phe Asp Ala Leu  
 145 150 155 160

Lys Gln Thr Tyr Cys Gly Ser Ile Gly Ala Glu Tyr Met His Ile Asn  
 165 170 175

Asn Thr Glu Glu Lys Arg Trp Ile Gln Gln Arg Ile Glu Ser Gly Ala  
 180 185 190

Ser Gln Thr Ser Phe Ser Gly Glu Glu Lys Lys Gly Phe Leu Lys Glu  
 195 200 205

Leu Thr Ala Ala Glu Gly Leu Glu Lys Tyr Leu Gly Ala Lys Phe Pro  
 210 215 220

Gly Ala Lys Arg Phe Ser Leu Glu Gly Gly Asp Ala Leu Val Pro Met  
 225 230 235 240

Leu Arg Glu Met Ile Arg His Ala Gly Lys Ser Gly Thr Arg Glu Val  
 245 250 255

Val Leu Gly Met Ala His Arg Gly Arg Leu Asn Val Leu Ile Asn Val  
 260 265 270

Leu Gly Lys Lys Pro Gln Asp Leu Phe Asp Glu Phe Ser Gly Lys His  
 275 280 285



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Lys Glu His Leu Gly Thr Gly Asp Val Lys Tyr His Met Gly Phe Ser  
 290 295 300

Ser Asp Ile Glu Thr Glu Gly Gly Leu Val His Leu Ala Leu Ala Phe  
 305 310 315 320

Asn Pro Ser His Leu Glu Ile Val Ser Pro Val Val Met Gly Ser Val  
 325 330 335

Arg Ala Arg Leu Asp Arg Leu Ala Glu Pro Val Ser Asn Lys Val Leu  
 340 345 350

Pro Ile Thr Ile His Gly Asp Ala Ala Val Ile Gly Gln Gly Val Val  
 355 360 365

Gln Glu Thr Leu Asn Met Ser Gln Ala Arg Gly Tyr Glu Val Gly Gly  
 370 375 380

Thr Val Arg Ile Val Ile Asn Asn Gln Val Gly Phe Thr Thr Ser Asn  
 385 390 395 400

Pro Lys Asp Ala Arg Ser Thr Pro Tyr Cys Thr Asp Ile Gly Lys Met  
 405 410 415

Val Leu Ala Pro Ile Phe His Val Asn Ala Asp Asp Pro Glu Ala Val  
 420 425 430

Ala Phe Val Thr Arg Leu Ala Leu Asp Tyr Arg Asn Thr Phe Lys Arg  
 435 440 445

Asp Val Phe Ile Asp Leu Val Cys Tyr Arg Arg His Gly His Asn Glu  
 450 455 460

Ala Asp Glu Pro Ser Ala Thr Gln Pro Leu Met Tyr Gln Lys Ile Lys  
 465 470 475 480

Lys His Pro Thr Pro Arg Lys Ile Tyr Ala Asp Arg Leu Glu Gly Glu  
 485 490 495

Gly Val Ala Ser Gln Glu Asp Ala Thr Glu Met Val Asn Leu Tyr Arg  
 500 505 510

Asp Ala Leu Asp Ala Gly Glu Cys Val Val Pro Glu Trp Arg Pro Met  
 515 520 525

Ser Leu His Ser Phe Thr Trp Ser Pro Tyr Leu Asn His Glu Trp Asp  
 530 535 540

Glu Pro Tyr Pro Ala Gln Val Asp Met Lys Arg Leu Lys Glu Leu Ala  
 545 550 555 560

Leu Arg Ile Ser Gln Val Pro Glu Gln Ile Glu Val Gln Ser Arg Val  
 565 570 575

Ala Lys Ile Tyr Asn Asp Arg Lys Leu Met Ala Glu Gly Glu Lys Ala  
 580 585 590

Phe Asp Trp Gly Gly Ala Glu Asn Leu Ala Tyr Ala Thr Leu Val Asp  
 595 600 605

Glu Gly Ile Pro Val Arg Leu Ser Gly Glu Asp Ser Gly Arg Gly Thr  
 610 615 620

Phe Phe His Arg His Ala Val Val His Asn Gln Ala Asn Gly Ser Thr  
 625 630 635 640

Tyr Thr Pro Leu His His Ile His Asn Ser Gln Gly Glu Phe Lys Val  
 645 650 655

Trp Asp Ser Val Leu Ser Glu Glu Ala Val Leu Ala Phe Glu Tyr Gly  
 660 665 670

Tyr Ala Thr Ala Glu Pro Arg Val Leu Thr Ile Trp Glu Ala Gln Phe  
 675 680 685

Gly Asp Phe Ala Asn Gly Ala Gln Val Val Ile Asp Gln Phe Ile Ser  
 690 695 700

Ser Gly Glu Gln Lys Trp Gly Arg Met Cys Gly Leu Val Met Leu Leu

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705	710	715	720
Pro His Gly Tyr	Glu Gly Gln Gly	Pro Glu His Ser Ser	Ala Arg Leu
	725	730	735
Glu Arg Tyr	Leu Gln Leu Cys Ala	Glu Gln Asn Met Gln	Val Cys Val
	740	745	750
Pro Ser Thr	Pro Ala Gln Val Tyr His	Met Leu Arg Arg	Gln Ala Leu
	755	760	765
Arg Gly Met	Arg Arg Pro Leu Val	Val Met Ser Pro Lys	Ser Leu Leu
	770	775	780
Arg His Pro	Leu Ala Ile Ser Ser	Leu Asp Glu Leu Ala	Asn Gly Ser
	785	790	795
Phe Gln Pro	Ala Ile Gly Glu Ile	Asp Asp Leu Asp Pro	Gln Gly Val
	805	810	815
Lys Arg Val	Val Leu Cys Ser Gly	Lys Val Tyr Tyr Asp	Leu Leu Glu
	820	825	830
Gln Arg Arg	Lys Asp Glu Lys Thr	Asp Val Ala Ile Val	Arg Ile Glu
	835	840	845
Gln Leu Tyr	Pro Phe Pro His Gln	Ala Val Gln Glu Ala	Leu Lys Ala
	850	855	860
Tyr Ser His	Val Gln Asp Phe Val	Trp Cys Gln Glu Glu	Pro Leu Asn
	865	870	875
Gln Gly Ala	Trp Tyr Cys Ser Gln	His His Phe Arg Asp	Val Val Pro
	885	890	895
Phe Gly Ala	Thr Leu Arg Tyr Ala	Gly Arg Pro Ala Ser	Ala Ser Pro
	900	905	910
Ala Val Gly	Tyr Met Ser Val His	Gln Gln Gln Gln Gln	Asp Leu Val
	915	920	925
Asn Asp Ala	Leu Asn Val Asn		
	930	935	

&lt;210&gt; SEQ ID NO 4

&lt;211&gt; LENGTH: 407

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Enterobacter agglomerans

&lt;400&gt; SEQUENCE: 4

Met Ser Ser Val	Asp Ile Leu Val	Pro Asp Leu Pro	Glu Ser Val Ala
1	5	10	15
Asp Ala Thr	Val Ala Thr Trp	His Lys Lys Pro	Gly Asp Ala Val Ser
	20	25	30
Arg Asp Glu	Val Ile Val Glu	Ile Glu Thr Asp	Lys Val Val Leu Glu
	35	40	45
Val Pro Ala	Ser Ala Asp Gly	Val Leu Glu Ala	Val Leu Glu Asp Glu
	50	55	60
Gly Ala Thr	Val Thr Ser Arg	Gln Ile Leu Gly	Arg Leu Lys Glu Gly
	65	70	75
Asn Ser Ala	Gly Lys Glu Ser	Ser Ala Lys Ala	Glu Ser Asn Asp Thr
	85	90	95
Thr Pro Ala	Gln Arg Gln Thr	Ala Ser Leu Glu	Glu Glu Ser Ser Asp
	100	105	110
Ala Leu Ser	Pro Ala Ile Arg	Arg Leu Ile Ala	Glu His Asn Leu Asp
	115	120	125
Ala Ala Gln	Ile Lys Gly Thr	Gly Val Gly Gly	Arg Leu Thr Arg Glu
	130	135	140
Asp Val Glu	Lys His Leu Ala	Asn Lys Pro Gln	Ala Glu Lys Ala Ala



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145	150	155	160
Ala Pro Ala Ala Gly Ala Ala Thr Ala Gln Gln Pro Val Ala Asn Arg	165	170	175
Ser Glu Lys Arg Val Pro Met Thr Arg Leu Arg Lys Arg Val Ala Glu	180	185	190
Arg Leu Leu Glu Ala Lys Asn Ser Thr Ala Met Leu Thr Thr Phe Asn	195	200	205
Glu Ile Asn Met Lys Pro Ile Met Asp Leu Arg Lys Gln Tyr Gly Asp	210	215	220
Ala Phe Glu Lys Arg His Gly Val Arg Leu Gly Phe Met Ser Phe Tyr	225	230	235
Ile Lys Ala Val Val Glu Ala Leu Lys Arg Tyr Pro Glu Val Asn Ala	245	250	255
Ser Ile Asp Gly Glu Asp Val Val Tyr His Asn Tyr Phe Asp Val Ser	260	265	270
Ile Ala Val Ser Thr Pro Arg Gly Leu Val Thr Pro Val Leu Arg Asp	275	280	285
Val Asp Ala Leu Ser Met Ala Asp Ile Glu Lys Lys Ile Lys Glu Leu	290	295	300
Ala Val Lys Gly Arg Asp Gly Lys Leu Thr Val Asp Asp Leu Thr Gly	305	310	315
Gly Asn Phe Thr Ile Thr Asn Gly Gly Val Phe Gly Ser Leu Met Ser	325	330	335
Thr Pro Ile Ile Asn Pro Pro Gln Ser Ala Ile Leu Gly Met His Ala	340	345	350
Ile Lys Asp Arg Pro Met Ala Val Asn Gly Gln Val Val Ile Leu Pro	355	360	365
Met Met Tyr Leu Ala Leu Ser Tyr Asp His Arg Leu Ile Asp Gly Arg	370	375	380
Glu Ser Val Gly Tyr Leu Val Ala Val Lys Glu Met Leu Glu Asp Pro	385	390	395
Ala Arg Leu Leu Leu Asp Val	405		

<210> SEQ ID NO 5  
 <211> LENGTH: 40  
 <212> TYPE: PRT  
 <213> ORGANISM: Enterobacter agglomerans

<400> SEQUENCE: 5

Met Asn Leu His Glu Tyr Gln Ala Lys Gln Leu Phe Ala Arg Tyr Gly	1	5	10	15
Met Pro Ala Pro Thr Gly Tyr Ala Cys Thr Thr Pro Arg Glu Ala Glu	20	25	30	
Glu Ala Ala Ser Lys Ile Gly Ala	35	40		

<210> SEQ ID NO 6  
 <211> LENGTH: 30  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <221> NAME/KEY: misc\_feature  
 <223> OTHER INFORMATION: Artificial Sequence: synthetic DNA

<400> SEQUENCE: 6

gtcgacaata gccygaatct gttctggtcg

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Ser His Leu Glu Ile Val Ser Pro Val Val Ile Gly Ser Val Arg Ala  
 325 330 335  
 Arg Leu Asp Arg Leu Asp Glu Pro Ser Ser Asn Lys Val Leu Pro Ile  
 340 345 350  
 Thr Ile His Gly Asp Ala Ala Val Thr Gly Gln Gly Val Val Gln Glu  
 355 360 365  
 Thr Leu Asn Met Ser Lys Ala Arg Gly Tyr Glu Val Gly Gly Thr Val  
 370 375 380  
 Arg Ile Val Ile Asn Asn Gln Val Gly Phe Thr Thr Ser Asn Pro Leu  
 385 390 395 400  
 Asp Ala Arg Ser Thr Pro Tyr Cys Thr Asp Ile Gly Lys Met Val Gln  
 405 410 415  
 Ala Pro Ile Phe His Val Asn Ala Asp Asp Pro Glu Ala Val Ala Phe  
 420 425 430  
 Val Thr Arg Leu Ala Leu Asp Phe Arg Asn Thr Phe Lys Arg Asp Val  
 435 440 445  
 Phe Ile Asp Leu Val Ser Tyr Arg Arg His Gly His Asn Asn Glu Ala  
 450 455 460  
 Asp Glu Pro Ser Ala Thr Gln Pro Leu Met Tyr Gln Lys Ile Lys Lys  
 465 470 475 480  
 His Pro Thr Pro Arg Lys Ile Tyr Ala Asp Lys Leu Glu Gln Glu Lys  
 485 490 495  
 Val Ala Thr Leu Glu Asp Ala Thr Glu Met Val Asn Leu Tyr Arg Asp  
 500 505 510  
 Ala Leu Asp Ala Gly Asp Cys Val Val Ala Glu Trp Arg Pro Met Asn  
 515 520 525  
 Met His Ser Phe Thr Trp Ser Pro Tyr Leu Asn His Glu Trp Asp Glu  
 530 535 540  
 Glu Tyr Pro Asn Lys Val Glu Met Lys Arg Leu Gln Glu Leu Ala Lys  
 545 550 555 560  
 Arg Ile Ser Thr Val Pro Glu Ala Val Glu Met Gln Ser Arg Val Ala  
 565 570 575  
 Lys Ile Tyr Gly Asp Arg Gln Ala Met Ala Ala Gly Glu Lys Leu Phe  
 580 585 590  
 Asp Trp Gly Gly Ala Glu Asn Leu Ala Tyr Ala Thr Leu Val Asp Glu  
 595 600 605  
 Gly Ile Pro Val Arg Leu Ser Gly Glu Asp Ser Gly Arg Gly Thr Phe  
 610 615 620  
 Phe His Arg His Ala Val Ile His Asn Gln Ser Asn Gly Ser Thr Tyr  
 625 630 635 640  
 Thr Pro Leu Gln His Ile His Asn Gly Gln Gly Ala Phe Arg Val Trp  
 645 650 655  
 Asp Ser Val Leu Ser Glu Glu Ala Val Leu Ala Phe Glu Tyr Gly Tyr  
 660 665 670  
 Ala Thr Ala Glu Pro Arg Thr Leu Thr Ile Trp Glu Ala Gln Phe Gly  
 675 680 685  
 Asp Phe Ala Asn Gly Ala Gln Val Val Ile Asp Gln Phe Ile Ser Ser  
 690 695 700  
 Gly Glu Gln Lys Trp Gly Arg Met Cys Gly Leu Val Met Leu Leu Pro  
 705 710 715 720  
 His Gly Tyr Glu Gly Gln Gly Pro Glu His Ser Ser Ala Arg Leu Glu  
 725 730 735  
 Arg Tyr Leu Gln Leu Cys Ala Glu Gln Asn Asn Gln Val Cys Val Pro

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740					745					750					
Ser	Thr	Pro	Ala	Gln	Val	Tyr	His	Met	Leu	Arg	Arg	Gln	Ala	Leu	Arg
		755					760					765			
Gly	Met	Arg	Arg	Pro	Leu	Val	Val	Met	Ser	Pro	Lys	Ser	Leu	Leu	Arg
	770					775					780				
His	Pro	Leu	Ala	Val	Ser	Ser	Leu	Glu	Glu	Leu	Ala	Asn	Gly	Thr	Phe
	785					790					795				800
Leu	Pro	Ala	Ile	Gly	Glu	Glu	Ile	Asp	Glu	Leu	Asp	Pro	Lys	Gly	Val
				805					810					815	
Lys	Arg	Val	Val	Met	Cys	Ser	Ser	Gly	Lys	Val	Tyr	Tyr	Asp	Leu	Leu
			820					825					830		
Glu	Gln	Arg	Arg	Lys	Asn	Asn	Gln	His	Asp	Val	Ala	Ile	Val	Arg	Ile
		835					840					845			
Glu	Gln	Leu	Tyr	Pro	Phe	Pro	His	Lys	Ala	Met	Gln	Glu	Val	Leu	Gln
		850				855					860				
Gln	Phe	Ala	His	Val	Lys	Asp	Phe	Val	Trp	Cys	Gln	Glu	Glu	Pro	Leu
	865					870					875				880
Asn	Gln	Gly	Ala	Trp	Tyr	Cys	Ser	Gln	His	His	Phe	Arg	Glu	Val	Ile
				885					890					895	
Pro	Phe	Gly	Ala	Ser	Leu	Arg	Tyr	Ala	Gly	Arg	Pro	Ala	Ser	Ala	Ser
			900					905					910		
Pro	Ala	Val	Gly	Tyr	Met	Ser	Val	His	Gln	Lys	Gln	Gln	Gln	Asp	Leu
		915					920					925			
Val	Asn	Asp	Ala	Leu	Asn	Val	Glu								
	930					935									

&lt;210&gt; SEQ ID NO 9

&lt;211&gt; LENGTH: 405

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Escherichia coli

&lt;400&gt; SEQUENCE: 9

Met	Ser	Ser	Val	Asp	Ile	Leu	Val	Pro	Asp	Leu	Pro	Glu	Ser	Val	Ala
1			5						10					15	
Asp	Ala	Thr	Val	Ala	Thr	Trp	His	Lys	Lys	Pro	Gly	Asp	Ala	Val	Val
			20					25					30		
Arg	Asp	Glu	Val	Leu	Val	Glu	Ile	Glu	Thr	Asp	Lys	Val	Val	Leu	Glu
		35					40					45			
Val	Pro	Ala	Ser	Ala	Asp	Gly	Ile	Leu	Asp	Ala	Val	Leu	Glu	Asp	Glu
		50				55					60				
Gly	Thr	Thr	Val	Thr	Ser	Arg	Gln	Ile	Leu	Gly	Arg	Leu	Arg	Glu	Gly
				70							75			80	
Asn	Ser	Ala	Gly	Lys	Glu	Thr	Ser	Ala	Lys	Ser	Glu	Glu	Lys	Ala	Ser
				85					90					95	
Thr	Pro	Ala	Gln	Arg	Gln	Gln	Ala	Ser	Leu	Glu	Glu	Gln	Asn	Asn	Asp
			100					105					110		
Ala	Leu	Ser	Pro	Ala	Ile	Arg	Arg	Leu	Leu	Ala	Glu	His	Asn	Leu	Asp
		115					120					125			
Ala	Ser	Ala	Ile	Lys	Gly	Thr	Gly	Val	Gly	Gly	Arg	Leu	Thr	Arg	Glu
		130				135					140				
Asp	Val	Glu	Lys	His	Leu	Ala	Lys	Ala	Pro	Ala	Lys	Glu	Ser	Ala	Pro
		145				150					155				160
Ala	Ala	Ala	Ala	Pro	Ala	Ala	Gln	Pro	Ala	Leu	Ala	Ala	Arg	Ser	Glu
				165					170					175	
Lys	Arg	Val	Pro	Met	Thr	Arg	Leu	Arg	Lys	Arg	Val	Ala	Glu	Arg	Leu



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180				185				190							
Leu	Glu	Ala	Lys	Asn	Ser	Thr	Ala	Met	Leu	Thr	Thr	Phe	Asn	Glu	Val
	195						200					205			
Asn	Met	Lys	Pro	Ile	Met	Asp	Leu	Arg	Lys	Gln	Tyr	Gly	Glu	Ala	Phe
	210					215					220				
Glu	Lys	Arg	His	Gly	Ile	Arg	Leu	Gly	Phe	Met	Ser	Phe	Tyr	Val	Lys
225					230					235					240
Ala	Val	Val	Glu	Ala	Leu	Lys	Arg	Tyr	Pro	Glu	Val	Asn	Ala	Ser	Ile
			245						250					255	
Asp	Gly	Asp	Asp	Val	Val	Tyr	His	Asn	Tyr	Phe	Asp	Val	Ser	Met	Ala
		260						265					270		
Val	Ser	Thr	Pro	Arg	Gly	Leu	Val	Thr	Pro	Val	Leu	Arg	Asp	Val	Asp
		275					280						285		
Thr	Leu	Gly	Met	Ala	Asp	Ile	Glu	Lys	Lys	Ile	Lys	Glu	Leu	Ala	Val
	290					295					300				
Lys	Gly	Arg	Asp	Gly	Lys	Leu	Thr	Val	Glu	Asp	Leu	Thr	Gly	Gly	Asn
305					310					315					320
Phe	Thr	Ile	Thr	Asn	Gly	Gly	Val	Phe	Gly	Ser	Leu	Met	Ser	Thr	Pro
			325						330					335	
Ile	Ile	Asn	Pro	Pro	Gln	Ser	Ala	Ile	Leu	Gly	Met	His	Ala	Ile	Lys
		340						345					350		
Asp	Arg	Pro	Met	Ala	Val	Asn	Gly	Gln	Val	Glu	Ile	Leu	Pro	Met	Met
	355						360						365		
Tyr	Leu	Ala	Leu	Ser	Tyr	Asp	His	Arg	Leu	Ile	Asp	Gly	Arg	Glu	Ser
	370					375					380				
Val	Gly	Phe	Leu	Val	Thr	Ile	Lys	Glu	Leu	Leu	Glu	Asp	Pro	Thr	Arg
385					390					395					400
Leu	Leu	Leu	Asp	Val											
			405												

<210> SEQ ID NO 10  
 <211> LENGTH: 41  
 <212> TYPE: PRT  
 <213> ORGANISM: Enterobacter agglomerans

<400> SEQUENCE: 10

Met	Asn	Leu	His	Glu	Tyr	Gln	Ala	Lys	Gln	Leu	Phe	Ala	Arg	Tyr	Gly
1			5						10					15	
Met	Pro	Ala	Pro	Thr	Gly	Tyr	Ala	Cys	Thr	Thr	Pro	Arg	Glu	Ala	Glu
		20						25					30		
Glu	Ala	Ala	Ser	Lys	Ile	Gly	Ala	Gly							
	35						40								

<210> SEQ ID NO 11  
 <211> LENGTH: 60  
 <212> TYPE: PRT  
 <213> ORGANISM: Escherichia coli

<400> SEQUENCE: 11

Met	Asn	Leu	His	Glu	Tyr	Gln	Ala	Lys	Gln	Leu	Phe	Ala	Arg	Tyr	Gly
1			5						10					15	
Leu	Pro	Ala	Pro	Val	Gly	Tyr	Ala	Cys	Thr	Thr	Pro	Arg	Glu	Ala	Glu
		20						25					30		
Glu	Ala	Ala	Ser	Lys	Ile	Gly	Ala	Gly	Pro	Trp	Val	Val	Lys	Cys	Gln
	35						40					45			
Val	His	Ala	Gly	Gly	Arg	Gly	Lys	Ala	Gly	Gly	Val				
	50					55					60				

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<210> SEQ ID NO 12  
 <211> LENGTH: 58  
 <212> TYPE: PRT  
 <213> ORGANISM: Escherichia coli

<400> SEQUENCE: 12

Phe Leu Ile Asp Ser Arg Asp Thr Glu Thr Asp Ser Arg Leu Asp Gly  
 1                   5                   10                   15

Leu Ser Asp Ala Phe Ser Val Phe Arg Cys His Ser Ile Met Asn Cys  
           20                   25                   30

Val Ser Val Cys Pro Lys Gly Leu Asn Pro Thr Arg Ala Ile Gly His  
           35                   40                   45

Ile Lys Ser Met Leu Leu Gln Arg Asn Ala  
       50                   55

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What is claimed is:

1. A method for producing L-glutamic acid by fermentation, which comprises culturing an isolated microorganism in a liquid medium of which pH is adjusted to the pH at which L-glutamic acid is precipitated, to produce and accumulate L-glutamic acid and precipitate L-glutamic acid in the medium,

wherein said microorganism can metabolize a carbon source at a specific pH in a liquid medium containing the carbon source and L-glutamic acid at a saturation concentration, and has the ability to accumulate L-glutamic acid in an amount exceeding the amount corresponding to the saturation concentration in the liquid medium at the pH,

wherein said microorganism is [Enterobacter agglomerans] *Pantoea ananatis*, and

wherein said microorganism has at least one of the following characteristics: (a) the microorganism has increased activity, as compared to a corresponding wild-type microorganism, of an enzyme that catalyzes a reaction for biosynthesis of L-glutamic acid; and (b) the microorganism has decreased activity, as compared to a corresponding wild-type microorganism, or deficient activ-

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ity of an enzyme that catalyzes a reaction of a pathway branching from a biosynthetic pathway of L-glutamic acid and producing a compound other than L-glutamic acid.

25 2. The method according to claim 1, wherein said microorganism can grow in the liquid medium.

3. The method according to claim 1, wherein the pH is not more than 5.0.

30 4. The method according to claim 1, wherein in said microorganism an activity of at least one enzyme selected from the group consisting of citrate synthase, phosphoenolpyruvate carboxylase and glutamate dehydrogenase, is increased.

35 5. The method according to claim 1, wherein in said microorganism the enzyme that catalyzes the reaction of the pathway branching from the biosynthetic pathway of L-glutamic acid and producing the compound other than L-glutamic acid is  $\alpha$ -ketoglutarate dehydrogenase.

40 6. The method according to claim 1, wherein said microorganism has a mutation that causes less extracellular secretion of a viscous material compared with a wild strain when cultured in a medium containing a saccharide.

7. The method according to claim 1, wherein said microorganism is *Pantoea ananatis* AJ13355 strain.

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