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Description

The invention relates to mutants and alleles of the prpD1 gene of coryneform bacteria coding for variants of 2-methylcitrate
5 dehydratase (EC No. 4.2.1.79) and methods for producing L-lysine using bacteria which comprise these alleles.

Prior art

10 amino acids are used in human medicine, in the pharmaceutical industry, in the food product industry and very especially in livestock nutrition.

It is known that amino acids are produced by fermentation of
15 strains of coryneform bacteria, especially *Corynebacterium glutamicum*. Because of the great importance, work on improving the production methods is continually being done. Improvements in the methods may be fermentation technology measures such as, for example, stirring and supplying oxygen, or relate to
20 the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by for example ion exchange chromatography or the intrinsic output properties of the microorganism itself.

25 The methods used for improving the output properties of these microorganisms are ones of mutagenesis, selection and choice of mutants. The strains obtained in this way are resistant to antimetabolites or are auxotrophic for metabolites of
30 regulatory importance, and produce the amino acids. A known antimetabolite is the lysine analogue S-(2-aminoethyl)-L-cysteine (AEC).

Methods of recombinant DNA technology have likewise been used
35 for some years for strain improvement of L-aminoacid-producing strains of *Corynebacterium* by amplifying individual amino acid biosynthesis genes and investigating the effect on amino acid production.

The chromosome of *Corynebacterium glutamicum* was completely sequenced some time ago (Kalinowski et al., Journal of Biotechnology 104, 5-25 (2003)). The chromosome of
5 *Corynebacterium efficiens* has likewise been sequenced (Nishio et al., Genome Res. 13 (7), 1572-1579 (2003)).

Corresponding sequence data can be taken from the public databases. Suitable databases are for example the database of
10 the European Molecular Biology Laboratories (EMBL, Heidelberg, Germany and Cambridge, UK), the database of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA), that of the Swiss Institute of Bioinformatics (Swissprot, Geneva, Switzerland), the Protein Information
15 Resource Database (PIR, Washington, DC, USA) and the DNA Data Bank of Japan (DDBJ, 1111 Yata, Mishima, 411-8540, Japan).

Summarizing descriptions of the genetics, the metabolism and the industrial importance of *Corynebacterium* are to be found
20 in the articles by Ikeda, by Pfefferle et al. and by Mueller and Huebner in the book "Microbial Production of L-Amino Acids" (Advances in Biochemical Engineering 79, (2003), Springer Verlag, Berlin, Germany, editor: T. Scheper), in the special edition "A New Era in *Corynebacterium glutamicum*
25 Biotechnology" of the Journal of Biotechnology (volume 104 (1-3), 2003, editors: A. Pühler and T. Tauch) and in the "Handbook of *Corynebacterium glutamicum*" (editors: L. Eggeling and M. Bott, CRC Press, Taylor & Francis Group, Boca Raton, FL, USA, 2005).

30 The nucleotide sequence of the *prpD1* gene coding for the 2-methylcitrate dehydratase of *Corynebacterium glutamicum* is generally available inter alia in the database of the National Center for Biotechnology Information (NCBI) of the National
35 Library of Medicine (Bethesda, MD, USA) under the access number AF434798. It can furthermore be found in the Patent Application EP 1 108 790 as sequence No. 770.

Claes et al. (Journal of Bacteriology 184(10), 2728-2739 (2002)) report on genetic, microbiological and biochemical investigations on the prpD1, prpB1 and prpC1 genes of *Corynebacterium glutamicum* ATCC 13032.

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For improved clarity, the nucleotide sequence of the prpD1 gene coding for the 2-methylcitrate dehydratase from *Corynebacterium glutamicum* ("wild-type gene") according to the data of the NCBI database is depicted in SEQ ID NO:1, and the amino acid sequence of the encoded 2-methylcitrate dehydratase resulting therefrom is depicted in SEQ ID NO:2 and 4. Nucleotide sequences located upstream and downstream are additionally indicated in SEQ ID NO:3.

10

15 Object of the invention

The inventors' object was to provide novel measures for the improved production of amino acids, especially L-lysine, L-tryptophan, L-valine, L-isoleucine and L-homoserine.

20

Description of the invention

The invention relates to generated and isolated mutants of coryneform bacteria which preferentially secrete amino acids and which comprise a gene or allele which codes for a polypeptide having 2-methylcitrate dehydratase activity, characterized in that the polypeptide comprises L-leucine at the position corresponding to position 272 of the amino acid sequence of SEQ ID NO: 2 or in that the polypeptide includes the amino acid sequence of SEQ ID NO: 2, with L-leucine being present at position 272.

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Among the coryneform bacteria, the genus *Corynebacterium* is preferred. In the genus *Corynebacterium*, the following species are preferred:

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Corynebacterium efficiens (type strain DSM44549),
Corynebacterium glutamicum (type strain ATCC13032),
Corynebacterium thermoaminogenes (for example the strain

FERM BP-1539), and

Corynebacterium ammoniagenes (type strain ATCC6871),
with very particular preference for the species
5 Corynebacterium glutamicum.

Some representatives of the species Corynebacterium glutamicum
are known in the state of the art also under other species
designations. These include for example:

10 Corynebacterium acetoacidophilum ATCC127270,
Corynebacterium lilium DSM20137,
Corynebacterium melassecola ATCC17965,
Brevibacterium flavum ATCC14067,
Brevibacterium lactofermentum ATCC127269,
15 Brevibacterium divaricatum ATCC14020, and
Microbacterium ammoniaphilum ATCC15354.

The term "Micrococcus glutamicus" for Corynebacterium
glutamicum has likewise been in use.

20

The strains of coryneform bacteria employed for the measures
of the invention preferably already have the ability to enrich
the desired amino acid in the cell and/or secrete it into the
nutrient medium surrounding it and accumulate it. The term
25 "produce" is also used for this hereinafter. In particular,
the strains of coryneform bacteria employed have the ability
to enrich or to accumulate $\geq$ (at least) 0.25 g/l, $\geq$ 0.5 g/l, $\geq$
1.0 g/l, $\geq$ 1.5 g/l, $\geq$ 2.0 g/l, $\geq$ 4 g/l or $\geq$ 10 g/l of the
desired amino acid in $\leq$ (at most) 120 hours, $\leq$ 96 hours, $\leq$
30 48 hours, $\leq$ 36 hours, $\leq$ 24 hours or $\leq$ 12 hours in the cell or
in the nutrient medium. In this connection, the strains may
have been produced by mutagenesis and selection, by
recombinant DNA techniques or by a combination of the two
methods.

35

Known representatives of L-lysine-producing or secreting
strains of coryneforme bacteria are for example:
Corynebacterium glutamicum DM58-1/pDM6 (= DSM4697) described

in EP 0 358 940,

Corynebacterium glutamicum MH20-22B (= DSM16835) described in Menkel et al. (Applied and Environmental Microbiology 55(3), 684-688 (1989)),

5 *Corynebacterium glutamicum* AHP-3 (= Ferm BP-7382) described in EP 1 108 790,

Corynebacterium glutamicum NRRL B-11474 described in US 4,275,157, and

10 *Corynebacterium thermoaminogenes* AJ12521 (= FERM BP-3304) described in US 5,250,423.

Known representatives of L-tryptophan-producing or secreting strains of coryneform bacteria are for example:

15 *Corynebacterium glutamicum* K76 (=Ferm BP-1847) described in US 5,563,052,

Corynebacterium glutamicum BPS13 (=Ferm BP-1777) described in US 5,605,818, and

Corynebacterium glutamicum Ferm BP-3055 described in US 5,235,940.

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Known representatives of L-valine-producing or secreting strains of coryneform bacteria are for example:

Brevibacterium lactofermentum FERM BP-1763 described in US 5,188,948,

25 *Brevibacterium lactofermentum* FERM BP-3007 described in US 5,521,074,

Corynebacterium glutamicum FERM BP-3006 described in US 5,521,074, and

30 *Corynebacterium glutamicum* FERM BP-1764 described in US 5,188,948.

Known representatives of L-isoleucine-producing or secreting strains of coryneform bacteria are for example:

Brevibacterium flavum FERM BP-760 described in US 4,656,135,

35 *Brevibacterium flavum* FERM BP-2215 described in US 5,294,547, and

Corynebacterium glutamicum FERM BP-758 described in US 4,656,135.

Known representatives of L-homoserine-producing or secreting strains of coryneform bacteria are for example:

Micrococcus glutamicus ATCC 14296 described in US 3,189,526

5 and

Micrococcus glutamicus ATCC 14297 described in US 3,189,526.

Data on the taxonomic classification of strains of this group of bacteria are to be found inter alia in Seiler (Journal of
10 General Microbiology 129, 1433-1477 (1983)), Kinoshita (1985, Glutamic Acid Bacteria, pp 115-142. In: Demain and Solomon (ed), Biology of Industrial Microorganisms. The Benjamin/Cummins Publishing Co., London, UK), Kämpfer and Kroppenstedt (Canadian Journal of Microbiology 42, 989-1005
15 (1996)), Liebl et al. (International Journal of Systematic Bacteriology 41, 255-260 (1991)) and in US-A-5,250,434.

Strains with the designation "ATCC" can be purchased from the American Type Culture Collection (Manassas, VA, USA). Strains
20 with the designation "DSM" can be purchased from the Deutsche Sammlung of Mikroorganismen and Zellkulturen (DSMZ, Brunswick, Germany). Strains with the designation "NRRL" can be purchased from the Agricultural Research Service Patent Culture Collection (ARS, Peoria, Illinois, US). Strains with the
25 designation "FERM" can be purchased from National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba Ibaraki, Japan).

The polypeptide encoded by the prpD1 gene and having
30 2-methylcitrate dehydratase activity (PrpD1) is allocated the EC number 4.2.1.79 in accordance with the nomenclature of the IUPAC (International Union of Pure and Applied Chemistry).

Chemically, a gene is a polynucleotide. Another term for this
35 is nucleic acid.

Proteinogenic amino acids mean the amino acids which occur in natural proteins, that is to say in proteins of microorganisms, plants, animals and humans. In connection with

the present invention, proteinogenic amino acids means the group of L-amino acids consisting of L-aspartic acid, L-asparagine, L-threonine, L-serine, L-glutamic acid, L-glutamine, glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-proline and L-arginine and, where appropriate, L-selenocysteine. The L-amino acids likewise include L-homoserine.

The mutants according to the invention preferably secrete the stated proteinogenic amino acids, especially L-lysine, L-tryptophan, L-valine, L-isoleucine or L-homoserine. The term amino acid also includes salts thereof such as, for example, lysine monohydrochloride or lysine sulphate in the case of the amino acid L-lysine.

The invention further relates to mutants of coryneform bacteria which comprise a prpD1 allele which codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity which includes the amino acid sequence of SEQ ID NO: 2, with L-leucine being present at position 272.

The invention further relates to mutants of coryneform bacteria which comprise a prpD1 allele which codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which comprises L-leucine at the position corresponding to position 272 of the amino acid sequence of SEQ ID NO:2, where the gene includes a nucleotide sequence which is identical to the nucleotide sequence of a polynucleotide which is obtainable by a polymerase chain reaction (PCR) using a primer pair whose nucleotide sequences in each case include at least 15 consecutive nucleotides which are selected from the nucleotide sequence between position 1 and 750 of SEQ ID NO:3 or SEQ ID NO:7 and from the complementary nucleotide sequence between position 2245 and 3000 of SEQ ID NO:3 or SEQ ID NO:7. An example of such a suitable primer pair is depicted in SEQ ID NO:9 and SEQ ID

NO:10. Preferred starting material ("template" DNA) is chromosomal DNA of coryneform bacteria which have been treated in particular with a mutagen. The chromosomal DNA is particularly preferably of the genus *Corynebacterium* and very particularly preferably that of the species *Corynebacterium glutamicum*.

The invention further relates to mutants of coryneform bacteria which comprise a *prpD1* allele which codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which includes an amino acid sequence with a length corresponding to 498 L-amino acids, with L-leucine being present at position 272.

The invention further relates to mutants of coryneform bacteria which comprise a *prpD1* allele which codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which comprises at position 252 to 291 of the amino acid sequence the amino acid sequence corresponding to position 252 to 291 of SEQ ID NO:6 or 8. The amino acid sequence of the encoded polypeptide preferably comprises an amino acid sequence corresponding to position 202 to 341 of SEQ ID NO:6 or 8 or position 152 to 391 of SEQ ID NO:6 or 8 or position 102 to 441 of SEQ ID NO:6 or 8 or position 52 to 491 of SEQ ID NO:6 or 8 or position 2 to 495 of SEQ ID NO:6 or 8 or position 2 to 496 of SEQ ID NO:6 or 8 or position 2 to 497 of SEQ ID NO:6 or 8. The length of the encoded polypeptide very particularly preferably includes 498 amino acids.

The invention further relates to mutants of coryneform bacteria which comprise a *prpD1* allele which codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which comprises L-leucine at position 272 or at the corresponding position of the amino acid sequence, and whose amino acid sequence is additionally at least 90%, preferably at least 92% or at least 94% or at least 96%, and very particularly preferably at least 97% or at least 98% or at least 99% identical to the amino acid sequence of SEQ ID NO:6

or SEQ ID NO:8.

The invention further relates to mutants of coryneform bacteria which comprise a prpD1 allele which codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which comprises L-leucine at position 272 or at the corresponding position of the amino acid sequence, and whose nucleotide sequence is additionally at least 90%, preferably at least 92% or at least 94% or at least 96%, and very particularly preferably at least 97% or at least 98% or at least 99% identical to the nucleotide sequence of SEQ ID NO:5.

It is known that conservative amino acid exchanges alter the enzymic activity only insubstantially. Accordingly, the prpD1 allele which is present in the mutants according to the invention and which codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity may, in addition to the amino acid sequence shown in SEQ ID NO:6 or SEQ ID NO:8, comprise one (1) or more conservative amino acid exchange(s). The polypeptide preferably comprises not more than two (2), not more than three (3), not more than four (4) or not more than five (5) conservative amino acid exchanges. The enzymatic activity is substantially unaffected by such conservative amino acid exchanges.

In the case of aromatic amino acids, mutual exchanges of phenylalanine, tryptophan and tyrosine are referred to as conservative exchanges. In the case of hydrophobic amino acids, mutual exchanges of leucine, isoleucine and valine are referred to as conservative exchanges. In the case of polar amino acids, mutual exchanges of glutamine and asparagine are referred to as conservative exchanges. In the case of basic amino acids, mutual exchanges of arginine, lysine and histidine are referred to as conservative exchanges. In the case of acidic amino acids, mutual exchanges of aspartic acid and glutamic acid are referred to as conservative exchanges. In the case of amino acids comprising hydroxyl groups, mutual exchanges of serine and threonine are referred to as

conservative exchanges.

When working on the present invention, it was found by comparing the amino acid sequence using the Clustal program
5 (Thompson et al., Nucleic Acids Research 22, 4637-4680 (1994)) that the amino acid sequences of the 2-methylcitrate dehydratase of different bacteria such as, for example, Mycobacterium tuberculosis, Mycobacterium bovis, Corynebacterium efficiens and Corynebacterium glutamicum
10 comprise a sequence motif consisting of the sequence Arg-Glu-Leu-Asp-Phe-His-Asp-Thr-Phe-Leu-Ala-Ala-Asp/Glu-Tyr-Ser-His-Pro, a sequence motif consisting of the sequence Gly-Ile-Cys-Leu-His-Glu-His-Lys-Ile-Asp-His-Val-Ala-His-Leu-Gly-Pro and also a sequence motif consisting of the sequence Pro-Ala-Pro-Ile-Trp-Glu-Gly-Glu-Asp-Gly-Val-Ile-Ala-Trp-Leu-Leu.
15 The designation "Asp/Glu" means that "Asp or Glu" may be present at the corresponding position.

Accordingly, preferred mutants of coryneform bacteria are
20 those comprising a prpD1 allele which codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which includes at least one amino acid sequence selected from the group of Arg-Glu-Leu-Asp-Phe-His-Asp-Thr-Phe-Leu-Ala-Ala-Asp/Glu-Tyr-Ser-His-Pro, Gly-Ile-Cys-Leu-His-Glu-His-Lys-Ile-Asp-His-Val-Ala-His-Leu-Gly-Pro and Pro-Ala-Pro-Ile-Trp-Glu-Gly-Glu-Asp-Gly-Val-Ile-Ala-Trp-Leu-Leu, and comprises at
25 position 272 or at the corresponding or comparable position of the amino acid sequence one of the proteinogenic amino acids except L-proline, preferably L-leucine.

30 The amino acid sequence motif Arg-Glu-Leu-Asp-Phe-His-Asp-Thr-Phe-Leu-Ala-Ala-Asp/Glu-Tyr-Ser-His-Pro is present for example in SEQ ID NO:2 and 4 or 6 and 8 from position 102 to 118. The amino acid sequence motif Gly-Ile-Cys-Leu-His-Glu-His-Lys-Ile-Asp-His-Val-Ala-His-Leu-Gly-Pro is present for example in SEQ
35 ID NO:2 and 4 or 6 and 8 from position 156 to 172. The amino acid sequence motif Pro-Ala-Pro-Ile-Trp-Glu-Gly-Glu-Asp-Gly-Val-Ile-Ala-Trp-Leu-Leu is present for example in SEQ ID NO: 2

and 4 or 6 and 8 from position 240 to 255.

The invention finally relates to mutants of coryneform bacteria which comprise a prpD1 allele which codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which includes the amino acid sequence of SEQ ID NO:6 or SEQ ID NO:8.

The invention finally relates also to mutants of coryneform bacteria which comprise a prpD1 allele corresponding to SEQ ID NO:5 or SEQ ID NO:7.

It is known that the host's own enzymes called aminopeptidases delete the terminal methionine during the protein synthesis.

The expression "a position corresponding to position 272 of the amino acid sequence" or "a position comparable to position 272 of the amino acid sequence" signifies the fact that through insertion or deletion of a codon coding for an amino acid in the N-terminal region (relative to position 272 of SEQ ID NO:6 or 8) of the encoded polypeptide the stated position and stated length is formally increased by one unit in the case of an insertion or is reduced by one unit in the case of a deletion. For example, deletion of the GAG codon coding for the amino acid L-glutamic acid at position 19 of SEQ ID NO:6 or 8 shifts the L-leucine from position 272 to position 271. The stated length would then be: 497 amino acids. In the same way, insertion or deletion of a codon coding for an amino acid in the C-terminal region (relative to position 272) of the encoded polypeptide formally increases the stated length by one unit in the case of an insertion or reduces it by one unit in the case of a deletion. Such comparable positions can easily be identified by comparing the amino acid sequences in the form of an alignment for example with the aid of the Clustal programme or of the MAFFT programme.

The enzymatic activity is substantially unaffected by such insertions and deletions. "Substantially unaffected" means that the enzymatic activity of said variants differs by not

more than 10%, not more than 7.5%, not more than 5%, not more than 2.5% or not more than 1% from the activity of the polypeptide having the amino acid sequence of SEQ ID NO:6 or 8.

5

One method for determining the enzymatic activity of 2-methylcitrate dehydratase is described in Horswill and Escalante-Semerena (Biochemistry 40(15), 4703-4713 (2001)).

10 The invention accordingly also relates to prpD1 alleles which code for polypeptide variants of SEQ ID NO:6 or 8 which comprise one or more insertion(s) or deletion(s). The polypeptide preferably comprises not more than 5, not more than 4, not more than 3 or not more than 2 insertions or
15 deletions of amino acids.

The sequence motifs Arg-Glu-Leu-Asp-Phe-His-Asp-Thr-Phe-Leu-Ala-Ala-Asp/Glu-Tyr-Ser-His-Pro, Gly-Ile-Cys-Leu-His-Glu-His-Lys-Ile-Asp-His-Val-Ala-His-Leu-Gly-Pro and Pro-Ala-Pro-Ile-
20 Trp-Glu-Gly-Glu-Asp-Gly-Val-Ile-Ala-Trp-Leu-Leu are preferably not disrupted by such insertions/deletions.

The mutants according to the invention can be produced by using conventional in-vivo mutagenesis methods with cell
25 populations of coryneform bacteria using mutagenic substances such as, for example, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), ethyl methanesulphonate (EMS), 5-bromouracil or ultraviolet light. Mutagenesis methods are described for example in the Manual of Methods for General Bacteriology
30 (Gerhard et al. (Eds.), American Society for Microbiology, Washington, DC, USA, 1981) or in Tosaka et al. (Agricultural and Biological Chemistry 42(4), 745-752 (1978)) or in Konicek et al. (Folia Microbiologica 33, 337-343 (1988)). Typical mutageneses using MNNG include concentrations of from 50 to
35 500 mg/l or even higher concentrations up to a maximum of 1 g/l, an incubation time of from 1 to 30 minutes at a pH of from 5.5 to 7.5. Under these conditions, the number of viable cells is reduced by a proportion of approximately 50% to 90%

or approximately 50% to 99% or approximately 50% to 99.9% or more.

Mutants or cells are removed from the mutagenized cell population and are propagated. In a further step, their ability to secrete amino acids, preferably L-lysine, L-tryptophan, L-valine, L-isoleucine or L-homoserine, in a batch culture using a suitable nutrient medium is preferably investigated. Suitable nutrient media and test conditions are described inter alia in US 6,221,636, in US 5,840,551, in US 5,770,409, in US 5,605,818, in US 5,275,940 and in US 4,224,409. In the case of uses of suitable robotic systems as described for example in Zimmermann et al. (VDI Berichte No. 1841, VDI-Verlag, Düsseldorf, Germany 2004, 439-443) or Zimmermann (Chemie Ingenieur Technik 77 (4), 426-428 (2005)), numerous mutants can be investigated in a short time. In general, a maximum of 3000, a maximum of 10 000, a maximum of 30 000 or also a maximum of 60 000 mutants, where appropriate also more, are investigated. Mutants which, compared with the parent strain or unmutagenized starting strain, secrete increased amino acids into the nutrient medium or into the interior of the cell are identified in this way. These include for example mutants whose amino acid secretion is increased by at least 0.5%.

DNA is then prepared or isolated from the mutants, and the corresponding polynucleotide is synthesized with the aid of the polymerase chain reaction using primer pairs which permit amplification of the prpD1 gene or of the prpD1 allele according to the invention or of the mutation according to the invention at position 272. The DNA is preferably isolated from mutants which secrete amino acids to an increased extent.

It is possible to select for this purpose any primer pairs from the nucleotide sequence located upstream and downstream of the mutation according to the invention, and the nucleotide sequence complementary thereto. One primer of a primer pair in this case preferably includes at least 15, at least 18, at

least 20, at least 21 or at least 24 consecutive nucleotides selected from the nucleotide sequence between position 1 and 1563 of SEQ ID NO:3 or SEQ ID NO:7. The second primer belonging to a primer pair includes at least 15, at least 18, at least 20, at least 21 or at least 24 consecutive nucleotides selected from the complementary nucleotide sequence between position 3000 and 1567 of SEQ ID NO:3 or SEQ ID NO:7. If amplification of the coding region is desired, the primer pair is preferably selected from the nucleotide sequence between position 1 and 750 of SEQ ID NO:3 or SEQ ID NO:7 and from the complementary nucleotide sequence between position 3000 and 2245 of SEQ ID NO:3 or SEQ ID NO:7. A suitable primer pair is for example the primer pair prpD1_XL_A1 and prpD1_XL_E1 represented by SEQ ID NO:9 and SEQ ID NO:10. A PCR product prepared using this primer pair is depicted in SEQ ID NO:13.

If amplification of part of the coding region, as depicted for example in SEQ ID NO:17 and 19, is desired, the primer pair is preferably selected from the nucleotide sequence between position 751 and 1563 of SEQ ID NO:3 or SEQ ID NO:7 and from the complementary nucleotide sequence between position 2244 and 1567 of SEQ ID NO:3 or SEQ ID NO:7.

The primer can moreover be equipped with recognition sites for restriction enzymes, with a biotin group or further accessories, as described in the prior art. The total length of the primer generally does not exceed 30, 40, 50 or 60 nucleotides.

Polynucleotides are prepared by amplification of selected sequences, such as the prpD1 allele according to the invention, from provided, for example chromosomal, DNA ("template DNA") by PCR amplification generally employing thermostable DNA polymerases. Examples of such DNA polymerases are the Taq polymerase from *Thermus aquaticus*, which is marketed inter alia by Qiagen (Hilden, Germany), the Vent polymerase from *Thermococcus litoralis*, which is marketed

inter alia by New England Biolabs (Frankfurt, Germany) or the Pfu polymerase from *Pyrococcus furiosus*, which is marketed inter alia by Stratagene (La Jolla, USA). Polymerases having proof-reading activity are preferred. Proof-reading activity means that these polymerases are able to recognize incorrectly incorporated nucleotides and to eliminate the error by renewed polymerization (Lottspeich and Zorbas, Bioanalytik, Spektrum Akademischer Verlag, Heidelberg, Germany (1998)). Examples of polymerases having proof-reading activity are the Vent polymerase and the Pfu polymerase.

The conditions in the reaction mixture are set according to the manufacturer's information. The polymerases are generally supplied by the manufacturer together with the usual buffer which normally has concentrations of 10-100 mM Tris/HCl and 6-55 mM KCl at pH 7.5-9.3. Magnesium chloride is added in a concentration of 0.5-10 mM unless it is present in the buffer supplied by the manufacturer. In addition, deoxynucleoside triphosphates are added in a concentration of 0.1-16.6 mM to the reaction mixture. The primers are introduced into the reaction mixture in a final concentration of 0.1-3 μ M, and the template DNA in the optimal case with 10^2 to 10^5 copies. It is also possible to employ 10^6 to 10^7 copies. The appropriate polymerase is added to the reaction mixture in an amount of 2-5 units. A typical reaction mixture has a volume of 20-100 μ l. Further additions which can be included in the reaction are bovine serum albumin, Tween 20, gelatin, glycerol, formamide or DMSO (Dieffenbach and Dveksler, PCR Primer - A Laboratory Manual, Cold Spring Harbor Laboratory Press, USA 1995).

A typical PCR run consists of three different, consecutively repeating temperature steps. At the outset, the reaction is started by raising the temperature to 92°C-98°C for 4 to 10 minutes in order to denature the introduced DNA. This is followed by repetitions firstly of a step for denaturation of the introduced DNA at approximately 92-98°C for 10-60 seconds, then a step to bind the primers to the introduced DNA at a particular temperature which depends on the primers

("annealing temperature") and which, from experience, is 50°C to 60°C and can be calculated individually for each primer pair, for 10-60 seconds. Detailed information concerning this is to be found by the skilled person in Rychlik et al.

5 (Nucleic Acids Research 18 (21): 6409-6412). This is subsequently followed by a synthesis step to extend the introduced primers ("extension") at the activity optimum indicated in each case for the polymerase, normally in the range from 73°C to 67°C, preferably 72°C to 68°C, depending on
10 the polymerase. The duration of this extension step depends on the efficiency of the polymerase and length of the PCR product to be amplified. In a typical PCR, this step takes 0.5-8 minutes, preferably 2-4 minutes. These three steps are repeated 30 to 35 times, where appropriate up to 50 times. A
15 final "extension" step of 4-10 minutes terminates the reaction. The polynucleotides prepared in this way are also referred to as amplicons; the term nucleic acid fragment is likewise in use.

20 A PCR product prepared using the primer pair prpD1_XL_A1 / prpD1_XL_E1 (see SEQ ID NO:9 and SEQ ID NO:10) is depicted in SEQ ID NO:13.

Further instructions and information on PCR are to be found by
25 the skilled person for example in the handbook "PCR-Strategies" (Innis, Felfand and Sninsky, Academic Press, Inc., 1995), in the handbook by Diefenbach and Dveksler "PCR Primer - a laboratory manual" (Cold Spring Harbor Laboratory Press, 1995), in the handbook by Gait "Oligonucleotide
30 synthesis: A Practical Approach" (IRL Press, Oxford, UK, 1984) and in Newton and Graham "PCR" (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

The nucleotide sequence is subsequently determined for example
35 by the chain termination method of Sanger et al. (Proceedings of the National Academies of Sciences, U.S.A., 74, 5463-5467 (1977)) with the modifications indicated by Zimmermann et al. (Nucleic Acids Research 18, 1067 (1990)), and the polypeptide

encoded by this nucleotide sequence is analysed in particular in relation to the amino acid sequence. For this purpose, the nucleotide sequence is entered into a program for translating DNA sequence into an amino acid sequence. Suitable programs are for example the "Patentin" program which is obtainable from patent offices, for example the US Patent Office (USPTO), or the "Translate Tool" which is available on the ExPASy Proteomics Server in the World Wide Web (Gasteiger et al., Nucleic Acids Research 31, 3784-3788 (2003)).

Mutants whose prpD1 alleles code for polypeptides having 2-methylcitrate dehydratase enzymic activity, which comprise at position 272 of the amino acid sequence, or the corresponding or comparable position, one of the proteinogenic amino acids except L-proline, are identified in this way. Exchange for L-leucine is preferred.

The complete chromosome of the mutant is determined where appropriate. It is possible in this connection to employ the method described by Margulies et al. (Nature, 437(7057): 376-2720 (2005)) and Velicer et al. (Proceedings of the National Academy of Sciences, U.S.A., 103(21), 8107-8112 (2006)) which is known to those skilled in the art by the phrase "pyrosequencing" and enables complete genomes to be sequenced rapidly.

The invention accordingly relates to a mutant of a coryneform bacterium, characterized in that it is obtainable by the following steps:

- a) treatment of a coryneform bacterium which has the ability to secrete amino acids with a mutagenic agent,
- b) isolation and propagation of the mutant generated in a),
- c) preferably determination of the ability of the mutant to secrete in a medium, or enrich in the interior of the cell, at least 0.5% more amino acid than the coryneform bacterium employed in a),
- d) preparation of nucleic acid from the mutant obtained in b),

- e) preparation of a nucleic acid molecule (or amplicon or nucleic acid fragment) using the polymerase chain reaction, of the nucleic acid from d), and of a primer pair consisting of a first primer including at least 15 consecutive nucleotides selected from the nucleotide sequence between position 1 and 1563, preferably 1 to 750 of SEQ ID NO:3 or SEQ ID NO:7 and a second primer including at least 15 consecutive nucleotides selected from the complementary nucleotide sequence between position 3000 and 1567, preferably 3000 and 2245 of SEQ ID NO:3 or 7,
- f) determination of the nucleotide sequence of the nucleic acid molecule obtained in e), and determination of the encoded amino acid sequence,
- g) where appropriate comparison of the amino acid sequence determined in f) with SEQ ID NO:6 or 8, and
- h) identification of a mutant which comprises a polynucleotide which codes for a polypeptide which comprises L-leucine at position 272 or at comparable position.

The mutants generated in this way typically comprise one (1) copy of the described prpD1 allele.

SEQ ID NO:5 represents by way of example the coding region of the prpD1 allele of a mutant according to the invention. The coding region of the wild-type gene is represented as SEQ ID NO:1. SEQ ID NO:1 comprises at position 814 the nucleobase Cytosinee, at position 815 the nucleobase Cytosinee and at position 816 the nucleobase Cytosinee. SEQ ID NO:1 thus comprises from position 814 to 816 the CCC codon coding for the amino acid L-proline. SEQ ID NO:5 comprises at position 815 the nucleobase thymine. This Cytosinee-thymine transition results at position 814 to 816 in the CTC codon coding for the amino acid L-leucine.

In addition, the nucleotide sequences depicted in SEQ ID NO: 5 and 7 may comprise further base exchanges which have resulted from the mutagenesis treatment but are not expressed by an altered amino acid sequence. Mutations of this type are also

referred to by those skilled in the art as silent or neutral mutations. These silent mutations may likewise already be present in the coryneform bacterium employed for the metagenesis treatment.

5

The coryneform bacteria used for the mutagenesis preferably already have the ability to secrete the desired amino acid into the nutrient medium or fermentation broth surrounding them, and/or enrich it in the interior of the cells.

10

L-Lysine-producing coryneform bacteria typically have a feedback-resistant or desensitized aspartate kinase. Feedback-resistant aspartate kinases mean aspartate kinases (LysC) which by comparison with the wild form exhibit less sensitivity to inhibition by mixtures of lysine and threonine or mixtures of AEC (aminoethylcysteine) and threonine or lysine alone or AEC alone. The genes or alleles coding for these desensitized aspartate kinases are also referred to as lysC^{FBR} alleles. Numerous lysC^{FBR} alleles are described in the state of the art and code for aspartate kinase variants which have amino acid exchanges by comparison with the wild-type protein. The coding region of the wild-type lysC gene of *Corynebacterium glutamicum* corresponding to the access number AX756575 of the NCBI database is depicted in SEQ ID NO:11, and the polypeptide encoded by this gene is depicted in SEQ ID NO:12.

The L-lysine-producing coryneform bacteria employed for the measures of the invention preferably have a lysC allele which codes for an aspartate kinase variant which has the amino acid sequence of SEQ ID NO:12, the latter including one or more of the amino acid exchanges selected from the group:

LysC A279T (L-alanine at position 279 of the encoded aspartate kinase protein according to SEQ ID NO: 12 exchanged for L-threonine; see US 5,688,671 and access numbers E06825, E06826, E08178 and I74588 to I74597),

LysC A279V (L-alanine at position 279 of the encoded aspartate kinase protein according to SEQ ID NO: 12 exchanged for L-

valine, see JP 6-261766 and access number E08179),

LysC L297Q (L-leucine at position 297 of the encoded aspartate kinase protein according to SEQ ID NO: 12 exchanged for L-glutamine; see DE 102006026328,

5 LysC S301F (L-serine at position 301 of the encoded aspartate kinase protein according to SEQ ID NO: 12 exchanged for L-phenylalanine; see US 6,844,176 and access number E08180),

LysC S301Y (L-serine at position 301 of the encoded aspartate kinase protein according to SEQ ID NO: 12 exchanged for L-tyrosine, see Kalinowski et al. (Molecular and General
10 Genetics 224, 317-324 (1990)) and access number X57226),

LysC T308I (L-threonine at position 308 of the encoded aspartate kinase protein according to SEQ ID NO: 12 exchanged for L-isoleucine; see JP 6-261766 and access number E08181)

15 LysC T311I (L-threonine at position 311 of the encoded aspartate kinase protein according to SEQ ID NO: 12 exchanged for L-isoleucine; see WO 00/632728 and US 6,893,848),

LysC S317A (L-serine at position 317 of the encoded aspartate kinase protein according to SEQ ID NO: 12 exchanged for L-alanine; see US 5,688,671 and access number I74589),
20

LysC R320G (L-arginine at position 320 of the encoded aspartate kinase protein according to SEQ ID NO: 12 exchanged for glycine; see Jetten et al. (Applied Microbiology and Biotechnology 43, 76-82 (1995)) and access number L27125),

25 LysC G345D (glycine at position 345 of the encoded aspartate kinase protein according to SEQ ID NO: 12 exchanged for L-aspartic acid; see Jetten et al. (Applied Microbiology and Biotechnology 43, 76-82 (1995)) and access number L16848),

LysC T380I (L-threonine at position 380 of the encoded aspartate kinase protein according to SEQ ID NO: 12 exchanged for L-isoleucine; see WO 01/49854 and access number AX192358),
30 and

LysC S381F (L-serine at position 381 of the encoded aspartate kinase protein according to SEQ ID NO: 12 exchanged for L-phenylalanine; see EP 0435132).
35

Particular preference is given to the lysC^{FBR} allele lysC T311I (threonine at position 311 of the encoded aspartate kinase

protein according to SEQ ID NO: 12 exchanged for isoleucine) and a lysC^{FBR} allele comprising at least one exchange selected from the group of a A279T (alanine at position 279 of the encoded aspartate kinase protein according to SEQ ID NO: 12
5 exchanged for threonine), S381F (serine at position 381 of the encoded aspartate kinase protein according to SEQ ID NO: 12 exchanged for phenylalanine) and S317A (serine at position 317 of the encoded aspartate kinase protein according to SEQ ID NO: 12 exchanged for alanine).

10 The lysC^{FBR} allele lysC T311I (threonine at position 311 of the encoded aspartate kinase protein according to SEQ ID NO: 12 exchanged for isoleucine) is very particularly preferred.

The strain DSM 16833 (WO 06/063660) has, just like the strain
15 ATCC 21543 (US 3,708,395), a lysC^{FBR} allele which codes for an aspartate kinase protein which comprises the amino acid exchange T311I.

The strain NRRL B-11474 (US 4,275,157) has a lysC^{FBR} allele
20 which codes for an aspartate kinase protein which comprises the amino acid exchanges A279T and S381F.

In the manner described above, starting from the strain ATCC 21543, a mutant designated DM1916 which comprises a prpD1
25 allele which codes for a polypeptide in which L-leucine is present at position 272 of the amino acid sequence was isolated. The nucleotide sequence of the coding region of the prpD1 allele of the mutant DM1916 is depicted as SEQ ID NO:5 and the amino acid sequence of the encoded polypeptide is
30 depicted as SEQ ID NO:6 or 8.

It is additionally possible to use L-lysine-secreting coryneform bacteria which have properties known in the state of the art.

35 L-Tryptophan-producing coryneform bacteria typically have a feedback-resistant or desensitized anthranilate synthase. Feedback-resistant anthranilate synthase (TrpE) means

anthranilate synthases which, by comparison with the wild form, have a sensitivity to inhibition by tryptophan or 5-fluorotryptophan (Matsui et al., Journal of Bacteriology 169 (11): 5330 - 5332(1987)) or similar analogues which is less by at least 5 to 10%, or at least 10% to 15% or at least 10% to 20%. The genes or alleles coding for these desensitized anthranilate synthases are also referred to as trpE^{FBR} alleles. Examples of such mutants and alleles are for example described in US 6180373 and EP0338474.

L-Valine-producing coryneform bacteria typically have a feedback-resistant or desensitized acetolactate synthase (acetohydroxyacid synthase) [EC No. 2.2.1.6].

Feedback-resistant acetolactate synthase means an acetolactate synthase which, by comparison with the wild form, shows a lower sensitivity to inhibition by one or more of the amino acids selected from the group of L-valine, L-isoleucine and L-leucine, preferably L-valine.

The acetolactate synthase (IlvB, IlvN) of Corynebacterium consists of a so-called large subunit encoded by the ilvB gene and of a so-called small subunit encoded by the ilvN gene (Keilhauer et al., Journal of Bacteriology 175(17), 5595-5603 (1993)). WO 05/003357 and Elisakova et al. (Applied and Environmental Microbiology 71(1):207-13 (2005)) report on variants of the IlvN subunit which confer resistance to L-valine, L-isoleucine and L-leucine on the acetolactate synthase. One variant comprises at position 21 of the amino acid sequence L-aspartic acid instead of L-isoleucine (IlvN I21D) and at position 22 L-phenylalanine instead of L-isoleucine (IlvN I22F). The second variant comprises at position 20 of the amino acid sequence L-aspartic acid instead of glycine (IlvN G20D), at position 21 of the amino acid sequence L-aspartic acid instead of L-isoleucine (IlvN I21D) and at position 22 L-phenylalanine instead of L-isoleucine (IlvN I22F).

L-Isoleucine-producing coryneform bacteria typically have a feedback-resistant or desensitized threonine dehydratase (= threonine deaminase).

5 Feedback-resistant threonine dehydratase means a threonine dehydratase (EC No. 4.3.1.19) which, by comparison with the wild form, shows a lower sensitivity to inhibition by L-isoleucine. The genes or alleles coding for this desensitized threonine dehydratase are also referred to as
10 *ilvA*^{FBR} alleles.

The coding region of the wild-type *ilvA* gene of *Corynebacterium glutamicum* corresponding to access number L01508 of the NCBI database is depicted in SEQ ID NO:17 and
15 the polypeptide encoded by this gene is depicted in SEQ ID NO:18.

The threonine dehydratase variants described in US 6,107,063 and in Morbach et al. (Applied and Environmental Microbiology
20 61 (12), 4315-4320 (1995)) comprise one or more of the amino acid exchanges selected from the group:

IlvA M199V (L-methionine at position 199 of the encoded threonine dehydratase protein according to SEQ ID NO: 18 exchanged for L-valine; see US 6,107,063),

25 *IlvA* A257G (L-alanine at position 257 of the encoded threonine dehydratase protein according to SEQ ID NO: 18 exchanged for L-arginine; see US 6,107,063),

IlvA H278R (L-histidine at position 278 of the encoded threonine dehydratase protein according to SEQ ID NO: 18
30 exchanged for L-arginine; see US 6,107,063),

IlvA V323A (L-valine at position 323 of the encoded threonine dehydratase protein according to SEQ ID NO: 18 exchanged for L-alanine; see Morbach et al.),

IlvA L351S (L-leucine at position 351 of the encoded threonine
35 dehydratase protein according to SEQ ID NO: 18 exchanged for L-serine; see US 6,107,063)

IlvA D378G (L-aspartic acid at position 378 of the encoded threonine dehydratase protein according to SEQ ID NO: 18

exchanged for glycine; see Morbach et al.)

The resulting mutants show, by comparison with the starting strain or parent strain employed, increased excretion or
5 production of the desired amino acid in a fermentation process.

The invention likewise relates to an isolated polynucleotide which codes for a polypeptide having 2-methylcitrate
10 dehydratase enzymic activity, which comprises L-leucine at the position corresponding to position 272 of the amino acid sequence of SEQ ID NO:2

or which includes the amino acid sequence of SEQ ID NO:2, with L-leucine being present at position 272.

15

The polynucleotide according to the invention can be isolated from a mutant according to the invention.

It is additionally possible to employ in vitro methods for the
20 mutagenesis of the prpD1 gene. On use of in vitro methods, isolated polynucleotides which comprise a prpD1 gene of a coryneform bacterium, preferably the wild-type gene of Corynebacterium glutamicum described in the prior art, are subjected to a mutagenic treatment.

25

The isolated polynucleotides may be for example isolated total DNA or chromosomal DNA or else amplicons of the prpD1 gene which have been prepared with the aid of the polymerase chain reaction (PCR). Amplicons of this type are also referred to as
30 PCR products. Instructions for amplifying DNA sequences with the aid of the polymerase chain reaction are to be found by the skilled person inter alia in the handbook by Gait: Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum
35 Akademischer Verlag, Heidelberg, Germany, 1994). It is likewise possible for the prpD1 gene which is to be mutagenized firstly to be incorporated into a vector, for example into a bacteriophage or into a plasmid.

Suitable methods for in vitro mutagenesis are inter alia treatment with hydroxylamine according to Miller (Miller, J. H.: A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1992), the use of mutagenic oligonucleotides (T. A. Brown: Gentechnologie für Einsteiger, Spektrum Akademischer Verlag, Heidelberg, 1993 and R. M. Horton: PCR-Mediated Recombination and Mutagenesis, Molecular Biotechnology 3, 93-99 (1995)) and the use of a polymerase chain reaction utilizing a DNA polymerase which shows a high error rate. A DNA polymerase of this type is for example the Mutazyme DNA polymerase (GeneMorph PCR Mutagenesis Kit, No.600550) from Stratagene (LaJolla, CA, USA).

Further instructions and reviews on the generation of mutations in vivo or in vitro can be found in the prior art and known textbooks of genetics and molecular biology such as, for example, the textbook of Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that of Winnacker ("Gene and Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that of Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

The invention further relates to an isolated polynucleotide which codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which includes the amino acid sequence of SEQ ID NO:2, with L-leucine being present at position 272 of the amino acid sequence.

The invention further relates to an isolated polynucleotide that codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which includes an amino acid sequence with a length of 498 amino acids, and where the polypeptide comprises L-leucine at the position corresponding to position 272 of the amino acid sequence of SEQ ID NO:2 or where the polypeptide includes the amino acid sequence of SEQ ID NO:2 in which L-leucine is present at position 272.

The invention further relates to an isolated polynucleotide which codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which comprises at position 252 to 291 of the amino acid sequence the amino acid sequence corresponding to position 252 to 291 of SEQ ID NO:6 or 8. The amino acid sequence of the encoded polypeptide preferably comprises an amino acid sequence corresponding to position 202 to 341 of SEQ ID NO:6 or 8 or position 152 to 391 of SEQ ID NO:6 or 8 or position 102 to 441 of SEQ ID NO:6 or 8 or position 52 to 491 of SEQ ID NO:6 or 8 or position 2 to 495 of SEQ ID NO:6 or 8 or position 2 to 496 of SEQ ID NO:6 or 8 or position 2 to 497 of SEQ ID NO:6 or 8. The length of the encoded polypeptide very particularly preferably includes 498 amino acids.

The invention further relates to an isolated polynucleotide which codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which comprises L-leucine at the position corresponding to position 272 of the amino acid sequence of SEQ ID NO:2 or which includes the amino acid sequence of SEQ ID NO:2, with L-leucine being present at position 272, and which includes a nucleotide sequence which is identical to the nucleotide sequence of a polynucleotide which is obtainable by polymerase chain reaction (PCR) using the primer pair whose nucleotide sequences in each case include at least 15 consecutive nucleotides which are selected from the nucleotide sequence between position 1 and 750 of SEQ ID NO:3 or SEQ ID NO:7 and from the complementary nucleotide sequence between position 3000 and 2245 of SEQ ID NO:3 or SEQ ID NO:7. One example of such a primer pair is depicted in SEQ ID NO:9 and SEQ ID NO:10. Preferred starting material ("template" DNA) is chromosomal DNA of coryneform bacteria which have been treated in particular with a mutagen. The chromosomal DNA is particularly preferably of the genus *Corynebacterium* and very particularly preferably that of the species *Corynebacterium glutamicum*.

The invention further relates to an isolated polynucleotide which hybridizes with the nucleotide sequence complementary to SEQ ID NO:5 under stringent conditions and codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which comprises L-leucine at the position corresponding to position 272 of the amino acid sequence of SEQ ID NO:2

or which includes the amino acid sequence of SEQ ID NO:2, with L-leucine being present at position 272.

Instructions for hybridizing nucleic acids or polynucleotides are to be found by the skilled person inter alia in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology 41: 255-260 (1991)). The hybridization takes place under stringent conditions, meaning that the only hybrids formed are those in which the probe, i.e. a polynucleotide including the nucleotide sequence complementary to SEQ ID NO:5, and the target sequence, i.e. the polynucleotides treated or identified with the probe, are at least 90% identical. It is known that the stringency of hybridization including the washing steps is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is generally carried out at relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

It is possible to employ for the hybridization reaction for example a buffer corresponding to 5x SSC buffer at a temperature of approx. 50°C-68°C. In this case, probes may also hybridize with polynucleotides which show less than 90% identity to the nucleotide sequence of the probe employed. Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved for example by reducing the salt concentration to 2x SSC and, where

appropriate, subsequently 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995), setting a temperature of approx. 50°C-68°C, approx. 52°C-68°C, approx. 54°C-68°C, approx. 56°C-68°C, approx. 58°C-68°C, approx. 60°C-68°C, approx. 62°C-68°C, approx. 64°C-68°C, approx. 66°C-68°C. Temperature ranges of approx. 64°C-68°C or approx. 66°C-68°C are preferred. It is possible where appropriate to reduce the salt concentration to a concentration corresponding to 0.2x SSC or 0.1x SSC. The SSC buffer comprises where appropriate sodium dodecyl sulphate (SDS) in a concentration of 0.1%. It is possible by increasing the hybridization temperature stepwise in steps of approx. 1-2°C from 50°C to 68°C to isolate polynucleotide fragments which have at least 90% or at least 91%, preferably at least 92% or at least 93% or at least 94% or at least 95% or at least 96% and very particularly preferably at least 97% or at least 98% or at least 99% identity to the sequence or complementary sequence of the probe employed, and code for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which comprises the amino acid exchange according to the invention. The nucleotide sequence of the polynucleotide obtained in this way is determined by known methods. Further instructions for hybridization are obtainable in the form of so-called kits on the market (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, catalogue No. 1603558). The nucleotide sequences obtained in this way code for polypeptides having 2-methylcitrate dehydratase enzymic activity, which are at least 90%, preferably at least 92% or at least 94% or at least 96%, and very particularly preferably at least 97% or at least 98% or at least 99% identical to the amino acid sequence of SEQ ID NO:6 or SEQ ID NO:8 and comprise the amino acid exchange according to the invention.

The invention further relates to an isolated polynucleotide which codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which comprises L-leucine at the position corresponding to position 272 of the amino acid sequence of SEQ ID NO:2

or which includes the amino acid sequence of SEQ ID NO:2, with L-leucine being present at position 272, and which includes an amino acid sequence which is additionally at least 90%, preferably at least 92% or at least 94% or at least 96%, and very particularly preferably at least 97% or at least 98% or at least 99% identical to the amino acid sequence of SEQ ID NO:6 or SEQ ID NO:8.

The invention further relates to an isolated polynucleotide which codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which comprises L-leucine at the position corresponding to position 272 of the amino acid sequence of SEQ ID NO:2

or which includes the amino acid sequence of SEQ ID NO:2, with L-leucine being present at position 272 and which includes a nucleotide sequence which is additionally at least 90%, preferably at least 92% or at least 94% or at least 96%, and very particularly preferably at least 97% or at least 98% or at least 99% identical to the nucleotide sequence of SEQ ID NO:5.

In addition, preferred isolated polynucleotides code for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which comprises L-leucine at the position corresponding to position 272 of the amino acid sequence of SEQ ID NO:2

or which includes the amino acid sequence of SEQ ID NO:2, with L-leucine being present at position 272, and which comprise at least one sequence motif or an amino acid sequence selected from the group of Arg-Glu-Leu-Asp-Phe-His-Asp-Thr-Phe-Leu-Ala-Ala-Asp/Glu-Tyr-Ser-His-Pro, Gly-Ile-Cys-Leu-His-Glu-His-Lys-Ile-Asp-His-Val-Ala-His-Leu-Gly-Pro and Pro-Ala-Pro-Ile-Trp-Glu-Gly-Glu-Asp-Gly-Val-Ile-Ala-Trp-Leu-Leu.

The designations "Asp/Glu" mean that "Asp or Glu" may be present at the corresponding position.

The invention further relates to an isolated polynucleotide

which codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which includes the amino acid sequence of SEQ ID NO:6 or 8. The encoded polypeptide comprises where appropriate one (1) or more conservative amino acid exchange(s). The polypeptide preferably comprises not more than two (2), not more than three (3), not more than four (4) or not more than five (5) conservative amino acid exchanges.

The invention further relates to an isolated polynucleotide which codes for a polypeptide having 2-methylcitrate dehydratase enzymic activity, which includes the amino acid sequence of SEQ ID NO:6 or 8 including an extension at the N or C terminus by at least one (1) amino acid. This extension comprises not more than 50, 40, 30, 20, 10, 5, 3 or 2 amino acids or amino acid residues.

The invention finally relates also to prpD1 alleles which code for polypeptide variants of SEQ ID NO:6 or 8, which comprise one or more insertions or deletions. These preferably comprise a maximum of 5, a maximum of 4, a maximum of 3 or a maximum of 2 insertions or deletions of amino acids. The sequence motif Arg-Glu-Leu-Asp-Phe-His-Asp-Thr-Phe-Leu-Ala-Ala-Asp/Glu-Tyr-Ser-His-Pro and/or Gly-Ile-Cys-Leu-His-Glu-His-Lys-Ile-Asp-His-Val-Ala-His-Leu-Gly-Pro and/or Pro-Ala-Pro-Ile-Trp-Glu-Gly-Glu-Asp-Gly-Val-Ile-Ala-Trp-Leu-Leu is/are preferably not disrupted by such insertions/deletions.

The invention further relates to an isolated polynucleotide which includes the nucleotide sequence shown in SEQ ID NO:5 or 7.

The invention finally relates to an isolated polynucleotide comprising the prpD1 allele of the mutant DM1916.

The invention additionally relates to an isolated polynucleotide which includes part of the coding region of a prpD1 allele according to the invention, where the isolated

polynucleotide includes in every case the part of the coding region which comprises the amino acid exchange at position 272 of the amino acid sequence of the encoded polypeptide.

- 5 Included in particular is a nucleic acid molecule or DNA fragment which codes for at least one amino acid sequence corresponding to position 252 to 291 of SEQ ID NO:2, or which codes for at least one amino acid sequence corresponding to position 202 to 341 of SEQ ID NO:2, or which codes for at least one amino acid sequence corresponding to position 152 to 391 of SEQ ID NO:2, or which codes for at least one amino acid sequence corresponding to position 102 to 441 of SEQ ID NO:2, or which codes for at least one amino acid sequence corresponding to position 52 to 491 of SEQ ID NO:2, or which codes for at least one amino acid sequence corresponding to position 2 to 495 of SEQ ID NO:2, or which codes for at least one amino acid sequence corresponding to position 2 to 496 of SEQ ID NO:2, or which codes for at least one amino acid sequence corresponding to position 2 to 497 of SEQ ID NO:2, where L-leucine is present at the position corresponding to 20 272 of SEQ ID NO:2.

One example of a reading frame including a polynucleotide which codes for at least the amino acid sequence from position 25 252 to 291 corresponding to SEQ ID NO:2, where one of the proteinogenic amino acids (Xaa) except L-proline is present at the position corresponding to 272 of the amino acid sequence, is detailed below:

```
gcg tgg ctg tta tgg ggc aaa gat cat gtt tat cat gtg cca
Ala Trp Leu Leu Ser Gly Lys Asp His Val Tyr His Val Pro
    255                260                265

ttg cgg gaa cac ggc gag nnn aag ctg ggg att cta gag
Leu Pro Glu His Gly Glu Xaa Lys Leu Gly Ile Leu Glu
    270                275

act tac aca aag gaa cat tca gcg gaa tat caa tgg cag
Thr Tyr Thr Lys Glu His Ser Ala Glu Tyr Gln Ser Gln
    280                285                290
```

30

It is likewise depicted as SEQ ID NO:17. The amino acid sequence encoded by this reading frame is depicted as SEQ ID NO:18. Position 21 in SEQ ID NO:18 corresponds to position 272 of SEQ ID NO:2, 4, 6 or 8.

Preferred nucleic acid molecules code for at least one amino acid sequence corresponding to position 252 to 291 of SEQ ID NO:6 or 8, or at least corresponding to position 202 to 341 of SEQ ID NO: 6 or 8, or at least corresponding to position 152 to 391 of SEQ ID NO: 6 or 8, or at least corresponding to position 102 to 441 of SEQ ID NO: 6 or 8, or at least corresponding to position 52 to 491 of SEQ ID NO: 6 or 8, or at least corresponding to position 2 to 495 of SEQ ID NO: 6 or 8, or at least corresponding to position 2 to 496 of SEQ ID NO: 6 or 8, or at least corresponding to position 2 to 497 of SEQ ID NO: 6 or 8.

One example of a reading frame according to the invention including a polynucleotide which codes for at least the amino acid sequence corresponding to position 252 to 291 of SEQ ID NO:6 or 8, is detailed below:

```
gcg tgg ctg tta tcg ggc aaa gat cat gtt tat cat gtg cca
Ala Trp Leu Leu Ser Gly Lys Asp His Val Tyr His Val Pro
      255                      260                      265
```

```
ttg ccg gaa cac ggc gag ctc aag ctg ggg att cta gag
Leu Pro Glu His Gly Glu Leu Lys Leu Gly Ile Leu Glu
      270                      275
```

```
act tac aca aag gaa cat tca gcg gaa tat caa tcg cag
Thr Tyr Thr Lys Glu His Ser Ala Glu Tyr Gln Ser Gln
      280                      285                      290
```

The reading frame is likewise depicted as SEQ ID NO:19. SEQ ID NO:20 shows the amino acid sequence encoded by this reading frame. Position 21 in SEQ ID NO:20 corresponds to position 272 of SEQ ID NO:2, 4, 6 or 8.

Very particularly preferred nucleic acid molecules include at least a nucleotide sequence corresponding to position 1504 to 1623 of SEQ ID NO:7, or at least a nucleotide sequence corresponding to position 1354 to 1773 of SEQ ID NO: 7, or at least a nucleotide sequence corresponding to position 1204 to 1923 of SEQ ID NO:7, or at least a nucleotide sequence corresponding to position 1054 to 2073 of SEQ ID NO:7, or at least a nucleotide sequence corresponding to position 904 to 2223 of SEQ ID NO:7, or at least a nucleotide sequence corresponding to position 754 to 2235 of SEQ ID NO:7, or at least a nucleotide sequence corresponding to position 754 to

2238 of SEQ ID NO:7, or at least a nucleotide sequence corresponding to position 754 to 2241 of SEQ ID NO:7.

5 The reading frames according to the invention, as shown by way of example in SEQ ID NO:17 and 19 as nucleotide sequence and in SEQ ID NO:18 and SEQ ID NO:20 in the form of the encoded amino acid sequence, may additionally comprise one or more mutations which lead to one or more conservative amino acid exchanges. The mutations preferably lead to a maximum of 4%,
10 to a maximum of 2% or to a maximum of 1% conservative amino acid exchanges. The reading frames according to the invention may further comprise one or more silent mutations. The reading frames according to the invention preferably comprise not more than 4% and particularly preferably not more than 2% to not
15 more than 1% silent mutations.

The isolated polynucleotides according to the invention can be used to produce recombinant strains of microorganisms which, in an improved manner compared with the initial or parent
20 strain, release amino acids into the medium surrounding them or accumulate them in the interior of cells.

A widespread method for incorporating mutations into genes of coryneform bacteria is that of allele exchange, which is also
25 known under the name "gene replacement". In this method, a DNA fragment which comprises the mutation of interest is transferred into the desired strain of a coryneform bacterium, and the mutation is incorporated by at least two recombination events or crossover events into the chromosome of the desired
30 strain, or the sequence of a gene present in the relevant strain is exchanged for the mutated sequence.

Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991) used this method in order to incorporate a lysA allele which harboured a
35 deletion, and in order to incorporate a lysA allele which harboured an insertion, into the chromosome of *C. glutamicum* instead of the wild-type gene. Schäfer et al. (Gene 145, 69-73 (1994)) employed this method to incorporate a deletion into

the hom-thrB operon of *C. glutamicum*. Nakagawa et al. (EP 1108790) and Ohnishi et al. (Applied Microbiology and Biotechnology 58(2), 217-223 (2002)) employed this method to incorporate various mutations starting from the isolated
5 alleles into the chromosome of *C. glutamicum*. Nakagawa et al. were able in this way to incorporate a mutation referred to as Val59Ala into the homoserine dehydrogenase gene (hom), a mutation referred to as Thr311Ile into the aspartate kinase gene (lysC or ask), a mutation referred to as Pro458Ser into
10 the pyruvate carboxylase gene (pyc) and a mutation referred to as Ala213Thr into the glucose-6-phosphate dehydrogenase gene (zwf) of *C. glutamicum* strains.

A polynucleotide according to the invention which can be used
15 for a method according to the invention includes the complete region as shown for example in SEQ ID NO:5, or includes part of the coding region such as, for example, the nucleotide sequence which codes for at least the amino acid sequence corresponding to position 252 to 291 of SEQ ID NO:6 or 8 and
20 which is depicted as SEQ ID NO:17 and 19. The part of the coding region corresponding to SEQ ID NO:17 and 19 includes a length of ≥ 120 nucleobases. The parts of SEQ ID NO:7 which are preferred are those including at least the sequence between position 1354 and 1773, or at least between position
25 1204 and 1923, or at least between position 1054 and 2073, and correspondingly have a length of ≥ 420 , ≥ 720 or ≥ 1020 nucleobases.

The DNA fragment comprising the mutation of interest is in
30 this method typically present in a vector, in particular a plasmid, which preferably undergoes only limited or no replication by the strain to be provided with the mutation. In general, a bacterium of the genus *Escherichia*, preferably of the species *Escherichia coli*, is used as auxiliary or
35 intermediate host in which the vector can be replicated.

Examples of such plasmid vectors are the pK*mob and pK*mobsacB vectors described by Schäfer et al. (Gene 145, 69-73 (1994)),

such as, for example, pK18mobsacB, and the vectors described in WO 02/070685 and WO 03/014362. These are replicative in *Escherichia coli*, but not in coryneform bacteria. Particularly suitable vectors are those comprising a gene with a conditionally negatively dominant effect such as, for example, the sacB gene (laevan sucrose gene) of, for example, *Bacillus* or the galK gene (galactose kinase gene) of, for example, *Escherichia coli*. (A gene with a conditionally negatively dominant effect means a gene which under certain conditions is disadvantageous, for example toxic, for the host but, under other conditions, has no negative effects on the host harbouring the gene.) These make it possible to select for recombination events in which the vector is eliminated from the chromosome. In addition, Nakamura et al. (US-A-6,303,2723) described a temperature-sensitive plasmid for coryneform bacteria which is able to replicate only at temperatures below 31°C.

The vector is then transferred into the coryneform bacterium by conjugation, for example by the method of Schäfer (Journal of Bacteriology 172, 1663-1666 (1990)) or transformation for example by the method of Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) or the method of Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)). The transfer of the DNA can where appropriate also be achieved by particle bombardment.

Homologous recombination by means of a first crossover event which brings about integration, and of a suitable second crossover event which brings about an excision in the target gene or in the target sequence achieves incorporation of the mutation and results in a recombinant bacterium.

Methods which can be employed for identifying and characterizing the resulting strains are inter alia those of Southern blotting hybridization, of the polymerase chain reaction, of sequence determination, the method of fluorescence resonance energy transfer (FRET) (Lay et al.

Clinical Chemistry 43, 2262-2267 (1997)) or methods of enzymology.

The description accordingly discloses a method for producing a coryneform bacterium, in which

a) a polynucleotide according to the invention is transferred into a coryneform bacterium,

b) the 2-methylcitrate dehydratase gene which is present in the chromosome of the coryneform bacterium and which codes for an amino acid sequence with L-proline at position 272 or at a comparable position of the amino acid sequence is exchanged

for the polynucleotide from a) which codes for an amino acid sequence which has at position 272 or at a comparable position of the amino acid sequence another proteinogenic L-amino acid, preferably L-leucine, and

c) the coryneform bacterium obtained as in step a) and b) is propagated.

A recombinant coryneform bacterium which comprises instead of the wild-type prpD1 gene one (1) prpD1 allele according to the invention is obtained in this way.

A method for producing a microorganism consists of

a) transferring a polynucleotide according to the invention which codes a polypeptide having 2-methylcitrate dehydratase enzymic activity into a microorganism,

b) replicating the polynucleotide in the microorganism, and

c) propagating the microorganism obtained as in step a) and b).

A recombinant microorganism which comprises at least one (1) copy or a plurality of copies of a polynucleotide according to the invention is obtained in this way.

The invention further accordingly relates to hosts or host cells, preferably microorganisms, particularly preferably coryneform bacteria and bacteria of the genus Escherichia, which comprise the polynucleotides according to the invention.

The invention likewise relates to microorganisms which have been produced using the isolated polynucleotides. Microorganisms or bacteria of this type are also referred to as recombinant microorganisms or recombinant bacteria. The invention likewise relates to vectors which comprise the polynucleotides according to the invention. Finally, the invention likewise relates to hosts which comprise these vectors.

It may additionally be advantageous for improved production of L-amino acids, in particular L-lysine, L-tryptophan, L-valine, L-isoleucine and L-homoserine, to overexpress various genes in the mutants or recombinant strains according to the invention. The use of endogenous genes is generally preferred.

"Endogenous genes" or "endogenous nucleotide sequences" mean the genes or nucleotide sequences, or alleles, present in the population of a species.

Thus, to produce L-lysine, it is possible to overexpress one or more of the genes selected from the group of

- a dapA gene coding for a dihydrodipicolinate synthase (DapA, EC No. 4.2.1.52), such as, for example, the dapA gene described in EP 0 197 335 of the wild type of Corynebacterium glutamicum,
- a lysA gene coding for a diaminopimelate decarboxylase (LysA, EC No. 4.1.1.20), such as, for example, the lysA gene described in US 6,090,597 of Corynebacterium glutamicum ATCC13869,
- a zwf gene coding for a glucose-6-phosphate dehydrogenase (Zwf, EC No. 1.1.1.49), such as, for example, the zwf gene described in JP-A-09224661 and EP-A-1108790 of the wild type of Corynebacterium glutamicum,
- the zwf alleles of Corynebacterium glutamicum which are described in US-2003-0175911-A1 and which code for a protein having glucose-6-phosphate dehydrogenase activity, in which for example the L-alanine at position 243 of the amino acid sequence is replaced by L-threonine, or in which the L-

aspartic acid at position 245 is replaced by L-serine,

- a *pyc* gene coding for a pyruvate carboxylase (Pyc, EC No. 6.4.1.1), such as, for example, the *pyc* gene described in DE-A-198 31 609 and EP 1108790 of the wild type of

5 *Corynebacterium glutamicum*,

- the *pyc* allele of *Corynebacterium glutamicum* which is described in EP 1 108 790 and which codes for a protein having pyruvate carboxylase activity in which L-proline at position 458 of the amino acid sequence is replaced by L-serine,

- 10 • the *pyc* alleles of *Corynebacterium glutamicum* which are described in WO 02/31158 and in particular EP1325135B1 and which code for proteins having pyruvate carboxylase activity which harbour one or more of the amino acid exchanges selected from the group of L-valine at position 1 replaced by L-methionine, L-glutamic acid at position 153 replaced by L-asparatic acid, L-alanine at position 182 replaced by L-serine, L-alanine at position 206 replaced by L-serine, L-histidine at position 227 replaced by L-arginine, L-alanine at position 455 replaced by glycine and L-aspartic acid at

15 position 1120 replaced by L-glutamic acid,

- 20 • a *lysC* gene coding for an aspartate kinase (LysC, EC No. 2.7.2.4), such as, for example, the *lysC* gene described as SEQ ID NO:281 in EP-A-1108790 (see also access number AX120085 and 120365) and the *lysC* gene described as SEQ ID NO:25 in WO

25 01/00843 (see access number AX063743) of the wild type of *Corynebacterium glutamicum*,

- a *lysC*^{FBR} allele coding for a feedback-resistant aspartate kinase variant, in particular corresponding to Table 1,

- 30 • a *lysE* gene coding for a lysine export protein (LysE), such as, for example, the *lysE* gene described in DE-A-195 48 222 of the wild-type *Corynebacterium glutamicum*,

- the *aat* gene coding for an aspartate aminotransferase (Aat, EC No. 2.6.1.1) (the *aat* gene of *Corynebacterium glutamicum* ATCC13032 is described for example in Kalinowski et al. (Journal of Biotechnology 104 (1-3), 5-25 (2003); see also

35 access number NC_006958). It is referred to therein as *aspB* gene. In US 6,004,773, a gene coding for an aspartate aminotransferase is referred to as *aspC*. Marienhagen et al.

(Journal of Bacteriology 187 (22), 7693-7646 (2005)) referred to the *aat* gene as *aspT* gene, and

- the *zwf* gene coding for the *Zwf* protein of the wild type of *Corynebacterium glutamicum* (US 6,632,644).

5

Overexpression means in general an increase in the intracellular concentration or activity of a ribonucleic acid, of a protein or of an enzyme. In the case of the present invention, *prpD1* alleles or polynucleotides which code for 2-methylcitrate dehydratases which comprise at position 272 of the amino acid sequence of the encoded polypeptide a proteinogenic amino acid except L-proline, with preference for exchange for L-leucine, are overexpressed. It is known that N-terminal amino acids, especially the N-terminal methionine, can be eliminated from the polypeptide formed, by the host's own enzymes - called aminopeptidases. The said increase in the concentration or activity of a gene product can be achieved for example by increasing the copy number of the appropriate polynucleotides by at least one copy.

20

A widely used method for increasing the copy number consists of incorporating the appropriate gene or allele into a vector, preferably a plasmid, which is replicated by a coryneform bacterium. Suitable plasmid vectors are for example *pZ1* (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554) or the *pSELF* vectors described by Tauch et al. (Journal of Biotechnology 99, 79-91 (2002)). A review article on the topic of plasmids in *Corynebacterium glutamicum* is to be found in Tauch et al. (Journal of Biotechnology 104, 27-40 (2003)).

30

Another widely used method for achieving overexpression is the method of chromosomal gene amplification. In this method, at least one additional copy of the gene or allele of interest is introduced into the chromosome of a coryneform bacterium.

35

In one embodiment, as described for example in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132

(1994)) for the hom-thrB operon, a plasmid which is non-replicative in *C. glutamicum* and which comprises the gene of interest is transferred into a coryneform bacterium. The strain resulting after homologous recombination by means of a crossover event comprises at least two copies of the relevant gene or allele.

In another embodiment, which is described in WO 03/040373 and US-2003-0219881-A1, one or more copy(ies) of the gene of interest is introduced by means of at least two recombination events into a desired site in the chromosome of *C. glutamicum*. In this way, for example, a copy of a lysC allele which codes for an L-lysine-insensitive aspartate kinase was incorporated into the gluB gene of *C. glutamicum*.

In a further embodiment, which is described in WO 03/014330 and US-2004-0043458-A1, at least one further copy of the gene of interest is incorporated, preferably in tandem arrangement to the previously present gene or allele, at the natural site by means of at least two recombination events. In this way, for example, a tandem duplication of a lysC^{FBR} allele was achieved at the natural lysC gene site.

Finally, it is possible to increase the copy number with the aid of transposons and IS elements (see: US 5,804,414, US 5,591,577).

A further method for achieving an overexpression consists of linking the appropriate gene or allele in a functional manner (operably linked) to a promoter or to an expression cassette. Suitable promoters for *Corynebacterium glutamicum* are described for example in the review article by Patek et al. (Journal of Biotechnology 104(1-3), 311-323 (2003)). It is possible in the same way to use the variants of the dapA promoter described by Vasicova et al. (Journal of Bacteriology 181, 6188-6191 (1999)), for example the promoter A25. A further possibility is to use the gap promoter of

Corynebacterium glutamicum (EP 06007373). Finally, the well-known promoters T3, T7, SP6, M13, lac, tac and trc described by Amann et al. (Gene 69(2), 301-315 (1988)) and Amann and Brosius (Gene 40(2-3), 183-190 (1985)) can be used.

5

A further possibility is to mutate the promoter region and regulatory region or the ribosome binding site which is located upstream of the structural gene. Expression is likewise improved by measures to prolong the lifespan of the mRNA. The enzymic activity is likewise enhanced in addition by preventing breakdown of the enzyme protein. A further possible alternative is overexpression of the relevant gene or allele through alteration of the composition of the media and management of the culture.

15

The activity or concentration of the appropriate protein is generally increased by the overexpression measures by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, maximally up to 1000% or 2000%, relative to the activity or concentration of the protein in the starting microorganism or parent strain. A starting microorganism or parent strain means a microorganism on which the measures of the invention are carried out.

25 The concentration of the protein can be determined by 1- and 2-dimensional protein gel fractionation and subsequent optical identification of the protein concentration using appropriate evaluation software in the gel. A useful method for preparing the protein gel in the case of coryneform bacteria and for identifying the proteins is the procedure described by Hermann et al. (Electrophoresis, 22:1712-23 (2001)). The protein concentration can likewise be determined by Western blot hybridization with an antibody specific for the protein to be detected (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) and subsequent optical evaluation with appropriate software to determine the concentration (Lohaus and Meyer (1998) Biospektrum 5:32-39; Lottspeich,

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Angewandte Chemie 272: 2630-2647 (1999)).

It may further be advantageous for the production of L-lysine, besides the use of the alleles according to the invention of the prpD1 gene, where appropriate with simultaneous overexpression of at least one of the genes selected from the aforementioned group of genes, simultaneously to attenuate or switch off one or more of the endogenous genes selected from the group of

- a pgi gene coding for glucose-6-phosphate isomerase (Pgi, EC No. 5.3.1.9), such as, for example, the pgi gene described in US 6,586,214 and US 6,465,238 of Corynebacterium glutamicum,
- a hom gene coding for homoserine dehydrogenase (Hom, EC No. 1.1.1.3), such as, for example, the hom gene described in EP-A-0131171 of Corynebacterium glutamicum,
- a thrB gene coding for homoserine kinase (ThrB, EC No. 2.7.1.39), such as, for example, the thrB gene described by Peoples et al. (Molecular Microbiology 2 (1988): 63-72) of Corynebacterium glutamicum and
- a pfkB gene coding for phosphofructokinase (PfkB, EC No. 2.7.1.56), such as, for example, the pfkB gene described in WO 01/00844 (sequence No. 57) of Corynebacterium glutamicum,
- an mdh gene coding for malate dehydrogenase (Mdh, EC No. 1.1.1.37) as described for example in WO 02/02778,
- an mqo gene coding for malate-quinone oxidoreductase (Mqo, EC No. 1.1.99.16), as described for example in US 7,094,106 and PCT/EP2005/057216.

The term "attenuation" describes in this connection the reduction or switching off of the intracellular activity of one or more enzymes (proteins) in a microorganism which are encoded by the appropriate DNA, by for example using a weak promoter or using a gene or allele which codes for a corresponding enzyme with a low activity, or inactivates the corresponding gene or enzyme (protein) and, where appropriate, combining these measures.

The attenuation measures generally reduce the activity or

concentration of the corresponding protein to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein, or of the activity or concentration of the protein in the starting microorganism.

5 Mutations suitable for generating an attenuation are transitions, transversions, insertions and deletions of at least one (1) base pair or nucleotide. Depending on the effect of the amino acid exchange caused by the mutation on the enzymic activity, reference is made to missense mutations or
10 nonsense mutations. The missense mutation leads to exchange of a given amino acid in a protein for another one, the amino acid exchange being in particular a non-conservative one. This impairs the ability to function or activity of the protein and reduces it to a value of from 0 to 75%, 0 to 50%, 0 to 25%, 0
15 to 10% or 0 to 5%. The nonsense mutation leads to a stop codon in the coding region of the gene and thus to premature termination of translation. Insertions or deletions of at least one base pair in a gene lead to frame shift mutations which lead to incorrect amino acids being incorporated or the
20 translation being terminated prematurely. If the mutation results in a stop codon in the coding region, this likewise leads to premature termination of translation. The said measures are preferably carried out in the 5'-terminal part of the coding region which codes for the N terminus of the
25 polypeptide. If the total length of a polypeptide (measured as number of chemically linked L-amino acids) is referred to as 100%, the part of the amino acid sequence belonging to the N terminus of the polypeptide - in the context of the present invention - comprises 80% of the L-amino acids following the
30 start amino acid L-formylmethionine.

Further instructions for generating such mutations belong to the state of the art and can be found in known textbooks of genetics and molecular biology such as, for example, the
35 textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that of Winnacker ("Gene and Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that of Hagemann ("Allgemeine Genetik", Gustav

Fischer Verlag, Stuttgart, 1986). Further measures are described in the prior art.

One method for targeted reduction of gene expression consists of placing the gene to be attenuated under the control of a promoter which can be induced by addition of metered amounts of IPTG (isopropyl β -D-thiogalactopyranoside), such as, for example, the trc promoter or the tac promoter. Suitable for this purpose are vectors such as, for example, the Escherichia coli expressions vector pXK99E (W00226787; deposited in accordance with the Budapest Treaty on 31 July 2001 in DH5alpha/pXK99E as DSM14440 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Brunswick, Germany)) or pVWEx2 (Wendisch, Ph. D. thesis, Berichte des Forschungszentrums Jülich, Jül-3397, ISSN 0994-2952, Jülich, Germany (1997)), which make IPTG-dependent expression of the cloned gene possible in Corynebacterium glutamicum.

This method has been employed for example in the Patent W00226787 for the regulated expression of the dead gene by integration of the vector pXK99Edead into the genome of Corynebacterium glutamicum and by Simic et al. (Applied and Environmental Microbiology 68: 3321-3327 (2002)) for the regulated expression of the glyA gene by integration of the vector pK18mobglyA' into Corynebacterium glutamicum.

A further method for specifically reducing gene expression is the antisense technique, where short oligodeoxynucleotides or vectors are brought into the target cells to synthesize longer antisense RNA. The antisense RNA is able to bind there to complementary segments of specific mRNAs and reduce their stability, or block translatability. One example thereof is to be found by the skilled person in Srivastava et al. (Applied Environmental Microbiology 2000 Oct; 66 (10): 4366 - 4371).

The isolated coryneform bacteria obtained by the measures of the invention show a secretion or production of the desired amino acid in a fermentation process which is increased by

comparison with the starting strain or parent strain employed.

Isolated bacteria mean the isolated and generated mutants and recombinant bacteria, especially coryneform bacteria, according to the invention which comprise a prpD1 allele which codes for a 2-methylcitrate dehydratase which comprises the described amino acid exchange at position 272 of the amino acid sequence.

The output of the isolated bacteria or of the fermentation process using the same in relation to one or more of the parameters selected from the group of the product concentration (product per volume), the product yield (product formed per carbon source consumed) and the product formation (product formed per volume and time) or else other process parameters and combinations thereof is improved by at least 0.5%, at least 1%, at least 1.5% or at least 2% relative to the starting strain or parent strain or to the fermentation process using the same.

The isolated coryneform bacteria according to the invention can be cultured continuously - as described for example in PCT/EP2004/008882 - or discontinuously in a batch process (batch cultivation) or in fed or repeated fed batch processes for the purpose of producing L-amino acids. A summary of a general nature about known cultivation methods is available in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Brunswick/Wiesbaden, 1994)).

The culture medium or fermentation medium to be used must satisfy in a suitable manner the demands of the respective strains. Descriptions of culture media of various microorganisms are present in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981). The terms culture medium, fermentation medium and nutrient medium or medium are

mutually exchangeable.

Carbon sources which can be used are sugars and carbohydrates such as, for example, glucose, sucrose, lactose, fructose, maltose, molasses, sucrose-containing solutions from sugarbeet or sugarcane production, starch, starch hydrolysate and cellulose, oils and fats such as, for example, soya oil, sunflower oil, peanut oil and coconut fat, fatty acids such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols such as, for example, glycerol, methanol and ethanol and organic acids such as, for example, acetic acid. These substances can be used singly or as mixture.

Nitrogen sources which can be used are organic nitrogen-containing compounds such as peptone, yeast extract, meat extract, malt extract, corn steep liquor, soybean flour and urea or inorganic compounds such as ammonium sulphate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources can be used singly or as mixture.

Phosphorus sources which can be used are phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts.

The culture medium must additionally comprise salts for example in the form of chlorides or sulphates of metals such as, for example, sodium, potassium, magnesium, calcium and iron, such as, for example, magnesium sulphate or iron sulphate, which are necessary for growth. Finally, essential growth factors such as amino acids, for example homoserine and vitamins, for example thiamine, biotin or pantothenic acid, can be employed in addition to the abovementioned substances. It is moreover possible to add to the culture medium suitable precursors of the respective amino acid.

The said starting materials can be added to the culture in the form of a single batch or be fed during the culturing in a

suitable manner.

To control the pH of the culture, basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia or acidic compounds such as phosphoric acid or sulphuric acid are employed in a suitable manner. The pH is generally adjusted to a value of from 6.0 to 9.0, preferably 6.5 to 8. To control foaming, it is possible to employ antifoams such as, for example, fatty acid polyglycol esters.

To maintain the stability of plasmids, it is possible to add to the medium suitable selectively acting substances such as, for example, antibiotics. In order to maintain aerobic conditions, oxygen or oxygen-containing gas mixtures such as, for example, air are introduced into the culture. It is likewise possible to use liquids enriched with hydrogen peroxide. The fermentation is carried out where appropriate with excess pressure, for example with a gauge pressure of from 0.03 to 0.2 MPa. The temperature of the culture is normally 20°C to 45°C and preferably 25°C to 40°C. In batch processes, the culturing is continued until a maximum of the desired amino acid has formed. This aim is normally reached within 10 hours to 160 hours. In continuous processes, longer culturing times are possible.

Suitable fermentation media are described inter alia in US 6,221,636, in US 5,840,551, in US 5,770,409, in US 5,605,818, in US 5,275,940, in US 4,275,157 and in US 4,224,409.

Methods for determining L-amino acids are known in the state of the art. The analysis can take place for example as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by anion exchange chromatography with subsequent ninhydrin derivatization, or it can take place by reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The invention accordingly relates to a process for producing

L-lysine acid in which

a) an isolated coryneform bacterium is fermented in a suitable medium, where the bacterium comprises a gene coding for a polypeptide having 2-methylcitrate dehydratase enzymic activity, where the polypeptide comprises L-leucine at the position corresponding to position 272 of the amino acid sequence of SEQ ID NO:2

or where the polypeptide includes the amino acid sequence of SEQ ID NO:2 in which L-leucine is present at position 272, and

b) the L-amino acid is accumulated in the fermentation broth or in the cells of the isolated coryneform bacterium.

This is generally followed by collecting the L-amino acid which has accumulated in the nutrient medium or in the fermentation broth and/or in the cells of the bacteria in order to obtain a solid or liquid product.

A fermentation broth means a fermentation medium in which a microorganism has been cultured for a certain time and at a certain temperature. The fermentation medium, or the media employed during the fermentation, comprise(s) all the substances and components ensuring growth of the microorganism and formation of the desired amino acid.

When the fermentation is complete, the resulting fermentation broth accordingly comprises a) the biomass of the microorganism which has been produced as a result of the growth of the cells of the microorganism, b) the desired amino acid formed during the fermentation, c) the organic by-products formed during the fermentation, and d) the constituents of the fermentation medium/media employed or of the starting materials such as, for example, vitamins such as biotin, amino acids such as homoserine or salts such as magnesium sulphate, which have not been consumed by the fermentation.

The organic by-products include substances which are produced by the microorganisms employed in the fermentation where

appropriate in addition to the respective desired L-amino acid and are secreted where appropriate. These include L-amino acids which account for less than 30%, 20% or 10% compared with the desired amino acid. These further include organic acids which have one to three carboxyl groups, such as, for example, acetic acid, lactic acid, citric acid, malic acid or fumaric acid. Also included finally are sugars such as, for example, trehalose.

Typical fermentation broths suitable for industrial purposes typically have an amino acid content of 30 g/kg to 200 g/kg or 40 g/kg to 180 g/kg or 50 g/kg to 150 g/kg. The biomass content (as dry biomass) is generally 20 to 50 g/kg.

In the case of the amino acid L-lysine, substantially four different product forms are known in the state of the art.

One group of L-lysine-containing products includes concentrated aqueous alkaline solutions of purified L-lysine (EP-B-0534865). A further group as described for example in US 6,340,486 and US 6,465,025 includes aqueous acidic biomass-containing concentrates of L-lysine-containing fermentation broths. The best-known group of solid products includes powder or crystalline forms of purified or pure L-lysine which is typically in the form of a salt such as, for example, L-lysine monohydrochloride. A further group of solid product forms is described for example in EP-B-0533039. Besides L-lysine, the product form described therein comprises most of the starting materials which were used during the fermentative production and were not consumed and, where appropriate, the biomass of the microorganism employed with a content of > 0%-100%.

In the case of the amino acids L-valine, L-isoleucine, L-proline, L-tryptophan and L-homoserine, the product forms known in the prior art are substantially those containing the relevant amino acids in purified or pure form ($\geq 95\%$ by weight or $\geq 98\%$ by weight).

Corresponding to the different product forms, a wide variety of processes are known with which the L-amino acid is collected, isolated or purified from the fermentation broth in order to produce the L-amino acid-containing product or the purified L-amino acid.

Solid pure L-amino acids are produced substantially by using methods of ion exchange chromatography, where appropriate with use of activated carbon, and methods of crystallization. In the case of lysine, the corresponding base or a corresponding salt such as, for example, the monohydrochloride (Lys-HCl) or lysine sulphate (Lys₂-H₂SO₄) is obtained in this way.

In the case of lysine, EP-B-0534865 describes a process for producing aqueous, basic L-lysine-containing solutions from fermentation broths. In the process described therein, the biomass is removed from the fermentation broth and discarded. A base such as, for example, sodium, potassium or ammonium hydroxide is used to adjust a pH of between 9 to 11. The mineral constituents (inorganic salts) are removed from the broth after concentration and cooling by crystallization and either used as fertilizers or discarded.

In processes for producing lysine using the bacteria according to the invention, processes resulting in products which comprise components of the fermentation broth are also employed. These are used in particular as animal feed additives.

Depending on requirements, the biomass can be removed wholly or partly from the fermentation broth by separation methods such as, for example, centrifugation, filtration, decantation or a combination thereof, or be left completely therein. Where appropriate, the biomass or the biomass-containing fermentation broth is inactivated during a suitable process step, for example by thermal treatment (heating) or by addition of acid.

The chemical constituents of the biomass are inter alia the cell envelope, for example the peptidoglycan and the arabinogalactan, the protein or polypeptide, for example the 2-methylcitrate dehydratase polypeptide, lipids and phospholipids and nucleic acids (DNA and RNA), for example polynucleotides comprising the mutation according to the invention. As a result of the measures of inactivation and/or of the further process steps (for example acidification, spray drying, granulation etc.), nucleic acids are typically present as fragments with a length of inter alia $\geq 40 - 60$ bp, $> 60 - 80$ bp, $> 80 - 100$ bp, $> 100 - 200$ bp, $> 200 - 300$ bp, $> 300 - 400$ bp, $> 400 - 500$ bp, $> 500 - 750$ bp, $> 750 - 1000$ bp, $> 1000 - 1250$ bp, $> 1250 - 1500$ bp, $> 1500 - 1750$ bp, $> 1750 - 2000$ bp, $> 2000 - 2500$ bp, $> 2500 - 3000$ bp, $> 3000 - 4000$ bp, $> 4000 - 5000$ bp.

In one procedure, the biomass is removed completely or almost completely so that no (0%) or not more than 30%, not more than 20%, not more than 10%, not more than 5%, not more than 1% or not more than 0.1% biomass remains in the product produced. In a further procedure, the biomass is not removed, or is removed only in small proportions, so that all (100%) or more than 70%, 80%, 90%, 95%, 99% or 99.9% biomass remains in the product produced. In one process according to the invention, accordingly, the biomass is removed in proportions $\geq$ to 0% to $\leq 100\%$.

Finally, the fermentation broth obtained after the fermentation can be adjusted, before or after the complete or partial removal of the biomass, to an acidic pH with an inorganic acid such as, for example, hydrochloric acid, sulphuric acid or phosphoric acid or organic acid such as, for example, propionic acid (GB 1,439,728 or EP 1 331 220). It is likewise possible to acidify the fermentation broth with the complete content of biomass (US 6,340,486 or US 6,465,025). Finally, the broth can also be stabilized by adding sodium bisulphite (NaHSO_3 , GB 1,439,728) or another salt, for example ammonium, alkali metal or alkaline earth metal salt of

sulphurous acid.

During the removal of the biomass, organic or inorganic solids present where appropriate in the fermentation broth are partially or completely removed. The organic by-products dissolved in the fermentation broth and the dissolved unconsumed components of the fermentation medium (starting materials) remain at least partly ($> 0\%$), preferably to the extent of at least 25%, particularly preferably to the extent of at least 50% and very particularly preferably to the extent of at least 75% in the product. Where appropriate, they also remain completely (100%) or almost completely, meaning $> 95\%$ or $> 98\%$, in the product. In this sense, the term "based on fermentation broth" means that the product comprises at least part of the components of the fermentation broth.

Subsequently, water is removed or thickened or concentrated from the broth by known methods such as, for example, with the aid of a rotary evaporator, thin-film evaporator, falling film evaporator, by reverse osmosis or by nanofiltration. This concentrated fermentation broth can then be worked up to free-flowing products, in particular to a fine-particle powder or preferably coarse granules, by methods of freeze drying, of spray drying, of spray granulation or by other processes as described for example in the circulating fluidized bed according to PCT/EP2004/006655. A desired product is isolated where appropriate from the resulting granules by screening or dust removal.

It is likewise possible to dry the fermentation broth directly, i.e. without previous concentration by spray drying or spray granulation.

"Free-flowing" means powders which flow unimpeded out of a series of glass orifice vessels with orifices of different sizes at least out of the vessel with a 5 mm (millimetres) orifice (Klein: Seifen, Öle, Fette, Wachse 94, 12 (1968)).

"Fine-particle" means a powder predominantly (> 50%) of a particle size of diameter from 20 to 200 µm.

5 "Coarse" means a product predominantly (> 50%) of a particle size of diameter from 200 to 2000 µm.

The particle size determination can be carried out by methods of laser diffraction spectrometry. Corresponding methods are described in the textbook on "Teilchengrößenmessung in der
10 Laborpraxis" by R. H. Müller and R. Schuhmann, Wissenschaftliche Verlagsgesellschaft Stuttgart (1996) or in the textbook "Introduction to Particle Technology" by M. Rhodes, published by Wiley & Sons (1998).

15 The free-flowing, fine-particle powder can in turn be converted by suitable compaction or granulation processes into a coarse, very free-flowing, storable and substantially dust-free product.

20 The term "dust-free" means that the product comprises only small proportions (<5%) of particle sizes below 100 µm in diameter.

"Storable" in the sense of this invention means a product
25 which can be stored for at least one (1) year or longer, preferably at least 1.5 years or longer, particularly preferably two (2) years or longer, in a dry and cool environment without any substantial loss (<5%) of the respective amino acid occurring.

30

The invention accordingly further relates to a process for producing an L-lysine containing product, preferably animal feed additive, from fermentation broths, characterized by the steps

35 a) culturing and fermentation of an L-amino acid-secreting coryneform bacterium which comprises at least one prpD1 allele which codes for a polypeptide having 2-methylcitrate dehydratase activity, where the polypeptide comprises L-

leucine at the position corresponding to position 272 of the amino acid sequence of SEQ ID NO:2

or where the polypeptide includes the amino acid sequence of SEQ ID NO:2 in which L-leucine is present at position 272, in a fermentation medium,

b) removal of the biomass formed during the fermentation in an amount of from 0 to 100% by weight, and

c) drying of the fermentation broth obtained as in a) and/or

b) in order to obtain the product in the desired powder or granular form,

where an acid selected from the group of sulphuric acid, phosphoric acid or hydrochloric acid is added where appropriate before step b) or c). Step a) or b) is preferably followed by removal of water from the L-amino acid-containing fermentation broth (concentration).

The description also discloses a process for producing a lysine sulphate-containing product which is described in principle in DE 102006016158, and in which the fermentation broth obtained using the microorganisms according to the invention, from which the biomass has been removed completely or partly where appropriate, is further processed by carrying out a process which includes at least the following steps:

a) the pH is reduced by adding sulphuric acid to 4.0 to 5.2, in particular 4.9 to 5.1, and a molar sulphate/L-lysine ratio of from 0.85 to 1.2, preferably 0.9 to 1.0, particularly preferably > 0.9 to < 0.95 , is adjusted in the broth, where appropriate by adding a further or a plurality of sulphate-containing compound(s) and

b) the mixture obtained in this way is concentrated by removal of water, and granulated where appropriate, where one or both of the following measures is/are carried out where appropriate before step a):

c) measurement of the molar sulphate/L-lysine ratio to ascertain the required amount of sulphate-containing compound(s)

d) addition of a sulphate-containing compound selected from the group of ammonium sulphate, ammonium bisulphate and

sulphuric acid in appropriate ratios.

Where appropriate, also before step b), a salt of sulphurous acid, preferably alkali metal bisulphite, particularly preferably sodium bisulphite, is added in a concentration of 0.01 to 0.5 by weight, preferably 0.1 to 0.3% by weight, particularly preferably 0.1 to 0.2% by weight, based on the fermentation broth.

Preferred sulphate-containing compounds which should be mentioned in the context of the abovementioned process steps are in particular ammonium sulphate and/or ammonium bisulphate or corresponding mixtures of ammonia and sulphuric acid and sulphuric acid itself.

The molar sulphate/L-lysine ratio V is calculated by the formula: $V = 2 \times [\text{SO}_4^{2-}] / [\text{L-lysine}]$. This formula takes account of the fact that the SO_4^{2-} anion has two charges. A ratio of $V = 1$ means that the stoichiometric composition $\text{Lys}_2(\text{SO}_4)$ is present, whereas the finding with a ratio of $V = 0.9$ is a 10% sulphate deficit and with a ratio of $V = 1.1$ is a 10% sulphate excess.

It is advantageous to employ during the granulation or compaction the usual organic or inorganic auxiliaries or carriers such as starch, gelatin, cellulose derivatives or similar substances, as normally used in the processing of food products or feeds as binders, gelling agents or thickeners, or further substances such as, for example, silicas, silicates (EP0743016A) stearates.

It is further advantageous to provide the surface of the resulting granules with oils as described in WO 04/054381. Oils which can be used are mineral oils, vegetable oils or mixtures of vegetable oils. Examples of such oils are soya oil, olive oil, soya oil/lecithin mixtures. In the same way, silicone oils, polyethylene glycols or hydroxyethylcellulose are also suitable. Treatment of the surfaces with the said

oils achieves an increased abrasion resistance of the product and a reduction in the dust content. The oil content in the product is 0.02 to 2.0% by weight, preferably 0.02 to 1.0% by weight, and very particularly preferably 0.2 to 1.0% by weight based on the total amount of the feed additive.

Preferred products have a proportion of $\geq 97\%$ by weight of a particle size of from 100 to 1800 μm or a proportion of $\geq 95\%$ by weight of a particle size of from 300 to 1800 μm diameter. The proportion of dust, i.e. particles with a particle size $< 100 \mu\text{m}$, is preferably > 0 to 1% by weight, particularly preferably not exceeding 0.5% by weight.

However, alternatively, the product may also be absorbed on an organic or inorganic carrier known and customary in the processing of feeds, such as, for example, silicas, silicates, meals, brans, flours, starches, sugars or others, and/or be mixed and stabilized with customary thickeners and binders. Examples of use and processes therefor are described in the literature (Die Mühle + Mischfüttertechnik 132 (1995) 49, page 817).

Finally, the product can also be brought by coating processes with film-formers such as, for example, metal carbonates, silicas, silicates, alginates, stearates, starches, gums and cellulose ethers as described in DE-C-4100920 to a state in which is stable to digestion by animal stomachs, especially the stomach of ruminants.

To adjust a desired amino acid concentration in the product it is possible, depending on requirements, to add the appropriate amino acid during the process in the form of a concentrate or, if appropriate, of a substantially pure substance or its salt in liquid or solid form. These can be added singly or as mixtures to the resulting or concentrated fermentation broth, or else during the drying or granulation process.

The invention further relates to a process for producing a

solid lysine-containing product as described in principle in US 20050220933, and which includes the working up of the fermentation broth obtained using the microorganisms according to the invention, in the following steps:

- 5 a) filtration of the fermentation broth, preferably with a membrane filter, to result in a biomass-containing sludge and a filtrate,
 - b) concentration of the filtrate, preferably so as to result in a solids content of from 48 to 52% by weight,
 - 10 c) granulation of the concentrate obtained in step b), preferably at a temperature of from 50°C to 62°C, and
 - d) coating of the granules obtained in c) with one or more of the coating agent(s)
- 15 The coating agents used for the coating in step d) are preferably selected from the group consisting of
- d1) the biomass obtained in step a),
 - d2) an L-lysine-containing compound, preferably selected from the group of L-lysine hydrochloride or L-lysine sulphate,
 - 20 d3) a substantially L-lysine-free substance with an L-lysine content of <1% by weight, preferably <0.5% by weight, preferably selected from the group of starch, carageenan, agar, silicas, silicates, meals, brans and flours, and
 - d4) a water-repellant substance, preferably selected from the
 - 25 group of oils, polyethylene glycols and liquid paraffins.

In the case of lysine, the ratio of the ions during the production of lysine-containing products is preferably adjusted so that the equivalent ion ratio corresponding to the

30 following formula

$$2x[\text{SO}_4^{2-}] + [\text{Cl}^-] - [\text{NH}_4^+] - [\text{Na}^+] - [\text{K}^+] - 2x[\text{Mg}^{2+}] - 2x[\text{Ca}^{2+}] / [\text{L-Lys}]$$

results in 0.68 to 0.95, preferably 0.68 to 0.90, as described by Kushiki et al. in US 20030152633 (the molar concentrations are to be given in the "[]").

35

In the case of lysine, the solid product produced in this way has, based on the fermentation broth, a lysine content (as lysine base) of 10% by weight to 70% by weight or 20% by

weight to 70% by weight, preferably 30% by weight to 70% by weight and very particularly preferably of 40% by weight to 70% by weight, based on the dry matter of the product. Maximum contents of lysine base of 71% by weight, 72% by weight, 73%
5 by weight are likewise possible.

In the case of an electrically neutral amino acid such as L-tryptophan, the solid product produced in this way has, based on the fermentation broth, an amino acid content of at least
10 5% by weight, 10% by weight, 20% by weight, 30% by weight and at most 50% by weight, 60% by weight, 70% by weight, 80% by weight, 90% by weight or up to 95% by weight.

The water content of the solid product is up to 5% by weight,
15 preferably up to 4% by weight, and particularly preferably less than 3% by weight.

The description also discloses an L-lysine-containing feed additive based on fermentation broth, which exhibits the
20 following features

- a) a lysine content (as base) of at least 10% by weight up to a maximum of 73% by weight,
- b) a water content not exceeding 5% by weight, and
- c) a biomass content corresponding to at least 0.1% of the
25 biomass present in the fermentation broth, where the biomass, inactivated where appropriate, is formed by coryneform bacteria according to the invention.

The description also discloses an L-tryptophan-containing feed
30 additive based on fermentation broth, which exhibits the following features

- a) a tryptophan content of at least 5% by weight up to a maximum of 95% by weight,
- b) a water content not exceeding 5% by weight, and
- 35 c) a biomass content corresponding to at least 0.1% of the biomass present in the fermentation broth, where the biomass, inactivated where appropriate, is formed by coryneform bacteria according to the invention.

The strain MH20-22B was deposited on 28 October 2004 at the Deutsche Sammlung für Mikroorganismen and Zellkulturen (DSMZ, Brunswick, Germany) as DSM 16835.

5

The *Corynebacterium glutamicum* mutant DM1916 according to the invention which comprises L-leucine at position 272 of the amino acid sequence of the PrpD1 polypeptide was deposited on 15 May 2006 at the Deutsche Sammlung für Mikroorganismen and Zellkulturen (DSMZ, Brunswick, Germany) as DSM 18258.

10

The present invention is explained in more detail below by means of exemplary embodiments.

15 Example 1

Mutagenesis of the L-lysine-producing strain DM416

The *Corynebacterium glutamicum* strain DM416 was employed as starting strain for the mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). The strain DM416 is a homoserine- and leucine-requiring mutant of *Corynebacterium glutamicum* and is deposited under the designation ATCC21543 at the American Type Culture Collection (Manassas, VA, USA).

20

The strain DM416 was cultured in 10 ml of LB broth (Merck, Darmstadt, Germany) which were present in a 100 ml Erlenmeyer flask at 33°C and 200 rpm on an orbital shaker of the Certomat BS-1 type (B. Braun Biotech International, Melsungen, Germany) for 24 hours. The culture was then centrifuged, the sediment was resuspended in 10 ml of 0.9% NaCl solution, the resulting suspension was again centrifuged, and the resulting sediment was taken up in 10 ml of 0.9% NaCl solution. 5 ml of this cell suspension were treated with 400 µg/ml MNNG at 30°C and 200 rpm on a shaker (see above) for 15 minutes. The mutagenesis mixture was then centrifuged and the sediment was taken up in 10 ml of 2% Na thiosulphate in 0.9% NaCl buffer (pH = 6.0). The cell suspension was then diluted in the ratio 1:1000,

25

30

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1:10 000 and 1:100 000 with 0.9% NaCl solution, and aliquots were plated on brain-heart agar (Merck, Darmstadt, Germany). Approximately 4000 mutants were isolated in this way.

5 Example 2

Output test on the mutants of the strain DM416

The mutants obtained in Example 1 were cultured in a nutrient medium suitable for producing lysine and the lysine content in
10 the culture supernatant was determined.

For this purpose, the clones were initially grown on brain-heart agar plates (Merck, Darmstadt, Germany) at 33°C for 24 hours. A preculture was inoculated (10 ml of medium in 100 ml
15 Erlenmeyer flask) starting from each of these agar plate cultures. The medium used for the preculture was the MM medium. The preculture was incubated at 33°C and 240 rpm on a shaker for 24 hours. A main culture was inoculated from this preculture in such a way that the initial OD (660 nm) of the
20 main culture was 0.1 OD. The MM medium was likewise used for the main culture.

MM medium

CSL	5 g/l
MOPS	20 g/l
Glucose (autoclaved separately)	50 g/l
Salts:	
(NH ₄) ₂ SO ₄	25 g/l
KH ₂ PO ₄	0.1 g/l
MgSO ₄ * 7 H ₂ O	1.0 g/l
CaCl ₂ * 2 H ₂ O	10 mg/l
FeSO ₄ * 7 H ₂ O	10 mg/l
MnSO ₄ * H ₂ O	5.0 mg/l
Biotin (sterilized by filtration)	0.3 mg/l
Thiamine * HCl (sterilized by filtration)	0.2 mg/l
L-Leucine (sterilized by filtration)	0.2 g/l

L-Homoserine (sterilized by filtration)	0.2 g/l
CaCO ₃	25 g/l

CSL (corn steep liquor), MOPS (morpholinopropanesulphonic acid) and the salt solution were adjusted to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions, and the dry autoclaved CaCO₃ were then added.

The culturing took place in volumes of 10 ml which were present in 100 ml Erlenmeyer flasks with baffles. The temperature was 33°C, the number of revolutions was 250 rpm and the humidity was 80%.

After 24 hours, the optical density (OD) at a measurement wavelength of 660 nm was determined using a Biomek 1000 (Beckmann Instruments GmbH, Munich, Germany). The amount of lysine formed was determined using an amino acid analyser from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection. A mutant distinguished by increased formation of lysine was called DM1916.

Table 1

Strain	OD(660)	Lysine HCl (g/l)
DM416	6.3	1.43
DM1916	6.3	1.61

Example 3

Sequencing of the prpD1 allele of the mutant DM1916

Chromosomal DNA was isolated from the clone DM1916 by the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)). The polymerase chain reaction was used to amplify a DNA segment which harbours the prpD1 gene or allele. On the basis of the known sequence of the prpD1 gene for *C. glutamicum* (sequence No. 770 from EP1108790), the following

primer oligonucleotides were selected for the PCR:

prpD1_XL_A1 (SEQ ID NO: 9):

5' gcgaattcta aactgcgtga ggttgtgg 3'

prpD1_XL_A2 (SEQ ID NO: 10):

5' gcgaattctc cccacatcaa caccattc 3'

The depicted primers were synthesized by MWG Biotech (Ebersberg, Germany) and the PCR reaction was carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers make it possible to amplify a DNA segment which is about 1.63 kb in length and which harbours the prpD1 gene or allele. In addition, the primers contain the sequence for a cleavage site of the restriction endonuclease EcoRI, which is marked by underlining in the nucleotide sequence depicted above.

The amplified DNA fragment which has a length of about 1.63 kb and which harbours the prpD1 allele of the strain DM1916 was identified by electrophoresis in a 0.8% agarose gel, isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

The nucleotide sequence of the amplified DNA fragment or PCR product was ascertained by sequencing by Agowa (Berlin, Germany). The sequence of the PCR product is depicted in SEQ ID NO: 13. The sequence of the coding region is additionally depicted in SEQ ID NO: 5. The amino acid sequence of the relevant methylcitrate dehydratase protein which was obtained with the aid of the Patentin program is depicted in SEQ ID NO: 6.

The base thymine is located at position 815 of the nucleotide sequence of the coding region of the prpD1 allele of the strain DM1916 (SEQ ID NO: 5). The base Cytosine is located at the corresponding position of the wild-type gene (SEQ ID NO: 1).

The amino acid leucine is located at position 272 of the amino acid sequence of the methylcitrate dehydratase protein of strain DM1916 (SEQ ID NO: 6). The amino acid proline is located at the corresponding position of the wild-type protein (SEQ ID NO: 2).

The prpD1 allele which comprises the base thymine at position 815 of the coding region, and accordingly codes for a methylcitrate dehydratase protein which comprises the amino acid leucine at position 272 of the amino acid sequence, is referred to hereinafter as prpD1_P272L allele. In the designation "prpD1_P272L" P stands for L-proline, L for L-leucine and 272 indicates the position of the amino acid exchange (see SEQ ID NO: 2 and 6).

The *Corynebacterium glutamicum* mutant DM1916 which comprises L-leucine at position 272 of the amino acid sequence of the PrpD1 polypeptide was deposited on 15 May 2006 at the Deutsche Sammlung für Mikroorganismen and Zellkulturen (DSMZ, Brunswick, Germany) as DSM 18258.

Example 4

Exchange of the prpD1 wild-type gene of strain DM416 for the prpD1_P272L allele

4.1 Construction of the exchange vector pK18mobsacB_prpD1_P272L

The DNA fragment which has a length of about 1.63 kb, which harbours the prpD1_P272L allele and which was prepared by PCR and described in Example 3 was incorporated by exchange mutagenesis with the aid of the sacB system described in Schäfer et al. (Gene, 14, 69-73 (1994)) into the chromosome of the *C. glutamicum* strain DM416 described in Example 1. This system makes it possible to produce and select allele exchanges accomplished by homologous recombination.

For this purpose the prpD1_P272L fragment about 1.63 kb in size was cleaved with the restriction endonuclease EcoRI, identified by electrophoresis in a 0.8% agarose gel and then
5 isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

The mobilizable cloning vector pK18mobsacB was digested with the restriction enzyme EcoRI, and the ends were
10 dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim, Germany). The vector prepared in this way was mixed with the approx. 1.63 kb prpD1_P272L fragment, and the mixture was treated with T4-DNA ligase (Amersham-Pharmacia, Freiburg, Germany).

15 The E. coli strain S17-1 (Simon et al., Bio/Technology 1: 784-791, 1993) was then transformed with the ligation mixture (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, IRL-Press, Cold Spring Harbor, New York, 1989). Selection of the
20 plasmid-harboring cells took place by plating out the transformation mixture on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989) which was supplemented by 25 mg/l kanamycin.

Plasmid DNA was isolated from a transformant with the aid of
25 the QIAprep Spin Miniprep Kit from Qiagen and examined by restriction cleavage with the enzyme PstI and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacB_prpD1_P272L and is depicted in Figure 1.

30 4.2 Allele exchange

The vector pK18mobsacB_prpD1_P272L mentioned in Example 4.1 was transferred by a protocol of Schäfer et al. (Journal of Microbiology 172: 1663-1666 (1990)) into the C. glutamicum
35 strain DM416 by conjugation. The vector is not capable of independent replication in DM416 and remains in the cell only if it is integrated into the chromosome as the result of a recombination event. Selection of transconjugants, i.e. of

clones with integrated pK18mobsacB_prpD1_P272L took place by plating out the conjugation mixture on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor, New York, 1989) which was supplemented with 15
5 mg/l kanamycin and 50 mg/l nalidixic acid. Kanamycin-resistant transconjugants were streaked onto LB agar plates with 25 mg/l kanamycin and incubated at 33°C for 24 hours. Mutants in which the plasmid had been excised as the result of a second recombination event were selected by culturing the clones
10 nonselectively in LB liquid medium for 30 hours, followed by streaking on LB agar with 10% sucrose and incubating for 16 hours.

The plasmid pK18mobsacB_prpD1_P272L comprises, just like the
15 initial plasmid pK18mobsacB, besides the kanamycin-resistance gene a copy of the sacB gene which codes for the laeA sucrase from *Bacillus subtilis*. The sucrose-inducible expression leads to the formation of laeA sucrase which catalyses the synthesis of the product laeA which is toxic
20 for *C. glutamicum*. Thus, the only clones to grow on LB agar with sucrose are those in which the integrated pK18mobsacB_prpD1_P272L has excised as a result of a second recombination event. Depending on the position of the second recombination event in relation to the site of mutation, the
25 allele exchange or incorporation of the mutation takes place on excision, or the original copy remains in the host's chromosome.

Approximately 40 to 50 colonies were tested for the phenotype
30 "growth in the presence of sucrose" and "no growth in the presence of kanamycin". For 4 colonies which exhibited the phenotype "growth in the presence of sucrose" and "no growth in the presence of kanamycin", a region of the prpD1 gene covering the P272L mutation and starting from the sequencing
35 primer pr1-2 (corresponds to nucleotide sequence position 424-443 of the coding region of the prpD1 gene from SEQ ID NO: 1) was sequenced by Agowa (Berlin, Germany) in order to demonstrate that the mutation of the prpD1_P272L allele is

present in the chromosome. The primer pr1-2 used was synthesized by Agowa for this purpose:

pr1-2:

5` ggt atc gcc act gcc tat ga 3`

A clone which contains the base thymine at position 815 of the coding region of the prpD1 gene, and thus has the prpD1_P272L allele, was identified in this way. This clone is called the DM416prpD1_P272L strain.

Example 6

Comparison of the output of the DM416prpD1_P272L strain with that of the starting strain DM416

The output test on the C. glutamicum strain DM416prpD1_P272L obtained in Example 5 was carried out as described in Example 2. The result of the test is depicted in Table 2.

Table 2

Strain	OD (660 nm)	Lysine HCl g/l
DM416	6.2	1.40
DM416prpD1_P272L	6.2	1.64

Brief description of the figure:

Figure 1: Map of the plasmid pK18mobsacB_prpD1_P272L.

The abbreviations and designations used have the following meaning. The stated base pair numbers are approximations obtained within the scope of the reproducibility of measurements.

Kan:	kanamycin-resistance gene
EcoRI:	cleavage site of the restriction enzyme EcoRI

PstI:	cleavage site of the restriction enzyme PstI
prpD1:	prpD1 P272L allele
sacB:	sacB gene
RP4-mob:	mob region with the origin of replication for transfer (oriT)
oriV:	origin of replication V

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 Val Ala Arg Gln Gln Ala Gln Ser His Pro Arg Glu Lys Gly Gly Lys
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 Val Phe Gly Ile Ser Gly Ser Tyr Ser Pro Glu Trp Ala Ala Phe Ala
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 Asn Gly Val Ala Val Arg Glu Leu Asp Phe His Asp Thr Phe Leu Ala
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 Ala Glu Tyr Ser His Pro Gly Asp Asn Ile Pro Pro Leu Leu Ala Val
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 Ala Gln Ala Gln Arg Ser Ser Gly Arg Asp Leu Ile Arg Gly Ile Ala
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 Thr Ala Tyr Glu Val Gln Val Glu Leu Val Arg Gly Ile Cys Leu His
 145 150 155 160

gag cac aaa att gat cac gta gcc cac ctt ggt ccc agc gct gca gcg Glu His Lys Ile Asp 165 His Val Ala His Leu 170 Gly Pro Ser Ala Ala 175	528
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atc ggc cag gca ttg cac acc acg acg gct acg agg cag tcg cga aaa Ile Gly Gln Ala Leu His Thr Thr 200 Thr Ala Thr Arg Gln Ser Arg Lys 205	624
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ggc aaa gat cat gtt tat cat gtg cca ttg ccg gaa cac ggc gag ccc Gly Lys Asp His Val Tyr His Val Pro Leu Pro Glu His Gly Glu Pro 270	816
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 Lys Met Asp Pro Gln Ala Ser Arg Glu Thr Leu Asp His Ser Ile Met
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 His Lys Ile Arg Thr Val Glu Asp Pro Glu Trp Thr Arg Arg Tyr His
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Glu His Lys Ile Asp His Val Ala His Leu Gly Pro Ser Ala Ala Ala
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Ile Gly Gln Ala Leu His Thr Thr Thr Ala Thr Arg Gln Ser Arg Lys
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<222> (1)..(1494)

<223> prpDl allele

<220>

<221> mutation

<222> (815)..(815)

<223> C -> T Transition

<400> 5

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1 5 10 15	
aaa gca gag cac ctt gcg tgg aaa ttc tcc gag ctt gcc acc gac ccc	96
Lys Ala Glu Val Leu Ala Trp Lys Phe Ser Glu Leu Ala Thr Asp Pro	
20 25 30	
gtg gag gtg aca ccg gat gtt tcg gag atg atc atc aac cgg atc atc	144
Val Glu Val Thr Pro Asp Val Ser Glu Met Ile Ile Asn Arg Ile Ile	
35 40 45	
gac aac gcg gcg gtg tct gcc gcg tcg gtg ttg cgc cgg cct gtg act	192
Asp Asn Ala Ala Val Ser Ala Ala Ser Val Leu Arg Arg Pro Val Thr	
50 55 60	
gtg gcc agg caa caa gcg cag tcc cat ccg cgg gaa aag ggc gga aaa	240
Val Ala Arg Gln Gln Ala Gln Ser His Pro Arg Glu Lys Gly Gly Lys	
65 70 75 80	
gtt ttt gga att tca ggc agc tac tca cca gag tgg gct gcc ttt gct	288
Val Phe Gly Ile Ser Gly Ser Tyr Ser Pro Glu Trp Ala Ala Phe Ala	
85 90 95	
aat ggt gtg gcc gta cgt gaa ttg gac ttc cac gat aca ttt tta gca	336
Asn Gly Val Ala Val Arg Glu Leu Asp Phe His Asp Thr Phe Leu Ala	
100 105 110	
gct gaa tac tcc cat ccc ggc gac aat att cca cca ctt ctt gca gta	384
Ala Glu Tyr Ser His Pro Gly Asp Asn Ile Pro Pro Leu Leu Ala Val	
115 120 125	
gcg cag gct cag aga agc agc ggc agg gat ctc att cgg ggt atc gcc	432
Ala Gln Ala Gln Arg Ser Ser Gly Arg Asp Leu Ile Arg Gly Ile Ala	
130 135 140	
act gcc tat gag gtg cag gtg gaa ttg gtg agg ggg atc tgc ttg cat	480
Thr Ala Tyr Glu Val Gln Val Glu Leu Val Arg Gly Ile Cys Leu His	
145 150 155 160	

gag Glu	cac His	aaa Lys	att Ile	gat Asp 165	cac His	gta Val	gcc Ala	cac His	ctt Leu 170	ggt Gly	ccc Pro	agc Ser	gct Ala 175	gca Ala	gcg Ala	528
ggt Gly	ttg Leu	ggc Gly	acg Thr 180	ttg Leu	ttg Leu	cat His	gta Val	gat Asp 185	gag Glu	gaa Glu	acc Thr	atc Ile	tac Tyr 190	cag Gln	gcg Ala	576
atc Ile	ggc Gly	cag Gln 195	gca Ala	ttg Leu	cac His	acc Thr	acg Thr 200	acg Thr	gct Ala	acg Thr	agg Arg	cag Gln 205	tcg Ser	cga Arg	aaa Lys	624
ggt Gly	gag Glu 210	att Ile	tcc Ser	agc Ser	tgg Trp	aag Lys 215	gcg Ala	ttc Phe	gcg Ala	cca Pro	gcg Ala 220	ttt Phe	gcg Ala	gga Gly	aag Lys	672
atg Met 225	gcc Ala	att Ile	gag Glu	gcg Ala	atg Met 230	gat Asp	cgt Arg	gcg Ala	atg Met	cgt Arg 235	ggg Gly	gag Glu	ggt Gly	tcg Ser	ccc Pro 240	720
gca Ala	ccg Pro	att Ile	tgg Trp	gag Glu 245	ggc Gly	gaa Glu	gac Asp	ggg Gly	gtc Val 250	atc Ile	gcg Ala	tgg Trp	ctg Leu	tta Leu 255	tcg Ser	768
ggc Gly	aaa Lys	gat Asp	cat His 260	gtt Val	tat Tyr	cat His	gtg Val	cca Pro 265	ttg Leu	ccg Pro	gaa Glu	cac His	ggc Gly 270	gag Glu	ctc Leu	816
aag Lys	ctg Leu	ggg Gly 275	att Ile	cta Leu	gag Glu	act Thr	tac Tyr 280	aca Thr	aag Lys	gaa Glu	cat His	tca Ser 285	gcg Ala	gaa Glu	tat Tyr	864
caa Gln 290	tcg Ser	cag Gln	gca Ala	ccg Pro	att Ile	gat Asp 295	ctg Leu	gcg Ala	cgc Arg	agg Arg	atg Met 300	aag Lys	cca Pro	ctg Leu	gtt Val	912
gac Asp 305	gcg Ala	gct Ala	ggc Gly	gga Gly	acg Thr 310	gaa Glu	cac His	att Ile	gca Ala	gag Glu 315	att Ile	gtg Val	ctg Leu	cgc Arg	acc Thr 320	960
agt Ser	cac His	cac His	acg Thr	cat His 325	tat Tyr	gtg Val	att Ile	ggc Gly	act Thr 330	ggg Gly	gcg Ala	aac Asn	gat Asp	ccg Pro 335	cag Gln	1008
aag Lys	atg Met	gat Asp	ccg Pro 340	cag Gln	gcc Ala	tcg Ser	cgt Arg	gaa Glu 345	acc Thr	ctg Leu	gat Asp	cat His	tcc Ser 350	atc Ile	atg Met	1056
tac Tyr	att Ile	ttc Phe 355	gcc Ala	gtc Val	gcg Ala	ctt Leu	caa Gln 360	gat Asp	ggc Gly	gtg Val	tgg Trp	cac His 365	cac His	gag Glu	ttt Phe	1104
tcc Ser	tac Tyr 370	acc Thr	cgc Arg	aag Lys	cgt Arg	tcc Ser 375	acc Thr	cgc Arg	ccg Pro	gaa Glu	act Thr 380	gtg Val	gag Glu	ctg Leu	tgg Trp	1152
cac His 385	aag Lys	att Ile	cgc Arg	acc Thr	gtg Val 390	gag Glu	gat Asp	cct Pro	gaa Glu	tgg Trp 395	acg Thr	cgc Arg	cga Arg	tac Tyr	cat His 400	1200
tct Ser	gat Asp	gat Asp	cct Pro	gca Ala 405	aaa Lys	aag Lys	gcc Ala	ttt Phe	ggt Gly 410	gcg Ala	aaa Lys	gca Ala	gtg Val	atc Ile 415	aca Thr	1248
atg Met	gct Ala	gat Asp	ggc Gly 420	acc Thr	gtg Val	att Ile	gag Glu	gat Asp 425	gaa Glu	ttg Leu	gct Ala	gtc Val	gcg Ala 430	gat Asp	gcc Ala	1296

cac ccg ctg ggt gct cgg ccg ttt gcg cgg gag aat tac att gaa aaa 1344
His Pro Leu Gly Ala Arg Pro Phe Ala Arg Glu Asn Tyr Ile Glu Lys
435 440 445

ttc cgc aca ctc gcg cag ggg att gtc att gat tca gaa cag gaa cgc 1392
Phe Arg Thr Leu Ala Gln Gly Ile Val Ile Asp Ser Glu Gln Glu Arg
450 455 460

ttc ttg cat gcc gtg caa agc ctg cct gac ctg gat gat ctt gat cag 1440
Phe Leu His Ala Val Gln Ser Leu Pro Asp Leu Asp Asp Leu Asp Gln
465 470 475 480

ctc aac atc gaa gtc gac ata agc aac cag gcc gcg acg aaa gcg ggg 1488
Leu Asn Ile Glu Val Asp Ile Ser Asn Gln Ala Ala Thr Lys Ala Gly
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ctg tta tga 1497
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<210> 6

<211> 498

<212> PRT

<213> Corynebacterium glutamicum

<400> 6

Met Arg Ile His Asp Val Tyr Thr His Leu Ser Ala Asp Asn Phe Pro
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Lys Ala Glu His Leu Ala Trp Lys Phe Ser Glu Leu Ala Thr Asp Pro
20 25 30

Val Glu Val Thr Pro Asp Val Ser Glu Met Ile Ile Asn Arg Ile Ile
35 40 45

Asp Asn Ala Ala Val Ser Ala Ala Ser Val Leu Arg Arg Pro Val Thr
50 55 60

Val Ala Arg Gln Gln Ala Gln Ser His Pro Arg Glu Lys Gly Gly Lys
65 70 75 80

Val Phe Gly Ile Ser Gly Ser Tyr Ser Pro Glu Trp Ala Ala Phe Ala
85 90 95

Asn Gly Val Ala Val Arg Glu Leu Asp Phe His Asp Thr Phe Leu Ala
100 105 110

Ala Glu Tyr Ser His Pro Gly Asp Asn Ile Pro Pro Leu Leu Ala Val
115 120 125

Ala Gln Ala Gln Arg Ser Ser Gly Arg Asp Leu Ile Arg Gly Ile Ala
130 135 140

Thr Ala Tyr Glu Val Gln Val Glu Leu Val Arg Gly Ile Cys Leu His
145 150 155 160

Glu His Lys Ile Asp His Val Ala His Leu Gly Pro Ser Ala Ala Ala

165	170	175
Gly Leu Gly Thr 180	Leu Leu His Val Asp 185	Glu Glu Thr Ile Tyr Gln Ala 190
Ile Gly Gln 195	Ala Leu His Thr Thr Thr Ala Thr Arg 200	Gln Ser Arg Lys 205
Gly Glu Ile Ser Ser Trp 210	Lys Ala Phe Ala Pro Ala Phe Ala Gly Lys 215	
Met Ala Ile Glu Ala 225	Met Asp Arg Ala Met Arg 230	Gly Glu Gly Ser Pro 240
Ala Pro Ile Trp 245	Glu Gly Glu Asp Gly Val Ile Ala Trp Leu Leu Ser 250	
Gly Lys Asp His Val Tyr His Val 260	Pro Leu Pro Glu His Gly Glu Leu 265	
Lys Leu Gly Ile Leu Glu Thr 275	Tyr Thr Lys Glu His Ser Ala Glu Tyr 280	
Gln Ser Gln Ala Pro Ile Asp 290	Leu Ala Arg Arg Met Lys Pro Leu Val 295	
Asp Ala Ala Gly Gly Thr Glu His Ile Ala 305	Glu Ile Val Leu Arg Thr 310	
Ser His His Thr His Tyr Val Ile Gly Thr Gly Ala Asn Asp Pro Gln 325		335
Lys Met Asp Pro Gln Ala Ser Arg Glu Thr Leu Asp His Ser Ile Met 340		345
Tyr Ile Phe Ala Val Ala Leu Gln Asp Gly Val Trp His His Glu Phe 355		360
Ser Tyr Thr Arg Lys Arg Ser Thr Arg Pro Glu Thr Val Glu Leu Trp 370		375
His Lys Ile Arg Thr Val Glu Asp Pro Glu Trp Thr Arg Arg Tyr His 385		390
Ser Asp Asp Pro Ala Lys Lys Ala Phe Gly Ala Lys Ala Val Ile Thr 405		410
Met Ala Asp Gly Thr Val Ile Glu Asp Glu Leu Ala Val Ala Asp Ala 420		425
His Pro Leu Gly Ala Arg Pro Phe Ala Arg Glu Asn Tyr Ile Glu Lys 435		440
		445

Phe Arg Thr Leu Ala Gln Gly Ile Val Ile Asp Ser Glu Gln Glu Arg
 450 455 460

Phe Leu His Ala Val Gln Ser Leu Pro Asp Leu Asp Asp Leu Asp Gln
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Leu Asn Ile Glu Val Asp Ile Ser Asn Gln Ala Ala Thr Lys Ala Gly
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Leu Leu

<210> 7

<211> 3000

<212> DNA

<213> Corynebacterium glutamicum

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<221> misc feature

<222> (1)..(750)

<223> sequence located upstream of the CDS

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<221> primer_bind

<222> (661)..(680)

<223> Binding site primer prpD1_XL_A1

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<221> CDS

<222> (751)..(2244)

<223> prpD1 allele

<220>

<221> mutation

<222> (1565)..(1565)

<223> C -> T Transition

<220>

<221> misc_feature

<222> (2245)..(3000)

<223> sequence located upstream of the CDS

<220>

<221> primer_bind

<222> (2258)..(2277)

<223> Binding site primer prpD1_XL_E1

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accaaagtgt tgcactgctc ttactgccac ggctttgagg cccgcgattc tgaaatcgtc    180
gtggtgggta cctcgcccat ggctgcgcac caagcgttga tgttctcgca gttgtccaaa    240
actgtcagct tgggtgggcac gatcgacatt gatgaacaaa ccagcgagag cctagatagt    300
gctggagtaa aagtgttggg caccaatgct gtgcgcgtat ccgccgaagg tgatggcctg    360
tctgtggaac tgtccgaagg cgatcattta agctgcgaca acatcgtggt ggcattctcg    420
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			Met	Arg	Ile	His	Asp	Val	Tyr	Thr							
			1				5										
cac	ctt	tcg	gcc	gat	aac	ttt	ccc	aaa	gca	gag	cac	ctt	gcg	tgg	aaa		822
His	Leu	Ser	Ala	Asp	Asn	Phe	Pro	Lys	Ala	Glu	His	Leu	Ala	Trp	Lys		
	10					15						20					
ttc	tcc	gag	ctt	gcc	acc	gac	ccc	gtg	gag	gtg	aca	ccg	gat	gtt	tcg		870
Phe	Ser	Glu	Leu	Ala	Thr	Asp	Pro	Val	Glu	Val	Thr	Pro	Asp	Val	Ser		
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gag	atg	atc	atc	aac	cgg	atc	atc	gac	aac	gcg	gcg	gtg	tct	gcc	gcg		918
Glu	Met	Ile	Ile	Asn	Arg	Ile	Ile	Asp	Asn	Ala	Ala	Val	Ser	Ala	Ala		
				45					50					55			
tcg	gtg	ttg	cg	cgg	cct	gtg	act	gtg	gcc	agg	caa	caa	gcg	cag	tcc		966
Ser	Val	Leu	Arg	Arg	Pro	Val	Thr	Val	Ala	Arg	Gln	Gln	Ala	Gln	Ser		
			60					65					70				
cat	ccg	cgg	gaa	aag	ggc	gga	aaa	gtt	ttt	gga	att	tca	ggc	agc	tac		1014
His	Pro	Arg	Glu	Lys	Gly	Gly	Lys	Val	Phe	Gly	Ile	Ser	Gly	Ser	Tyr		
		75					80					85					
tca	cca	gag	tgg	gct	gcc	ttt	gct	aat	ggg	gtg	gcc	gta	cgt	gaa	ttg		1062
Ser	Pro	Glu	Trp	Ala	Ala	Phe	Ala	Asn	Gly	Val	Ala	Val	Arg	Glu	Leu		
	90					95					100						
gac	ttc	cac	gat	aca	ttt	tta	gca	gct	gaa	tac	tcc	cat	ccc	ggc	gac		1110
Asp	Phe	His	Asp	Thr	Phe	Leu	Ala	Ala	Glu	Tyr	Ser	His	Pro	Gly	Asp		
					110					115					120		
aat	att	cca	cca	ctt	ctt	gca	gta	gcg	cag	gct	cag	aga	agc	agc	ggc		1158
Asn	Ile	Pro	Pro	Leu	Leu	Ala	Val	Ala	Gln	Ala	Gln	Arg	Ser	Ser	Gly		
				125					130					135			
agg	gat	ctc	att	cgg	ggt	atc	gcc	act	gcc	tat	gag	gtg	cag	gtg	gaa		1206
Arg	Asp	Leu	Ile	Arg	Gly	Ile	Ala	Thr	Ala	Tyr	Glu	Val	Gln	Val	Glu		
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ttg	gtg	agg	ggg	atc	tgc	ttg	cat	gag	cac	aaa	att	gat	cac	gta	gcc		1254
Leu	Val	Arg	Gly	Ile	Cys	Leu	His	Glu	His	Lys	Ile	Asp	His	Val	Ala		
		155					160					165					
cac	ctt	ggt	ccc	agc	gct	gca	gcg	ggt	ttg	ggc	acg	ttg	ttg	cat	gta		1302
His	Leu	Gly	Pro	Ser	Ala	Ala	Ala	Gly	Leu	Gly	Thr						

gcg atg cgt ggg gag ggt tcg ccc gca ccg att tgg gag ggc gaa gac Ala Met Arg Gly Glu Gly Ser Pro Ala Pro Ile Trp Glu Gly Glu Asp	1494
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ggg gtc atc gcg tgg ctg tta tcg ggc aaa gat cat gtt tat cat gtg Gly Val Ile Ala Trp Leu Leu Ser Gly Lys Asp His Val Tyr His Val	1542
250 255 260	
cca ttg ccg gaa cac ggc gag ctc aag ctg ggg att cta gag act tac Pro Leu Pro Glu His Gly Glu Leu Lys Leu Gly Ile Leu Glu Thr Tyr	1590
265 270 275 280	
aca aag gaa cat tca gcg gaa tat caa tcg cag gca ccg att gat ctg Thr Lys Glu His Ser 285 Ala Glu Tyr Gln Ser 290 Gln Ala Pro Ile Asp 295 Leu	1638
gcg cgc agg atg aag cca ctg gtt gac gcg gct ggc gga acg gaa cac Ala Arg Arg Met 300 Lys Pro Leu Val Asp 305 Ala Ala Gly Gly Thr 310 Glu His	1686
att gca gag att gtg ctg cgc acc agt cac cac acg cat tat gtg att Ile Ala Glu Ile Val Leu Arg Thr 320 Ser His His Thr His Tyr Val Ile	1734
315 320 325	
ggc act ggg gcg aac gat ccg cag aag atg gat ccg cag gcc tcg cgt Gly Thr 330 Gly Ala Asn Asp 335 Pro Gln Lys Met Asp 340 Pro Gln Ala Ser Arg	1782
gaa acc ctg gat cat tcc atc atg tac att ttc gcc gtc gcg ctt caa Glu Thr 345 Leu Asp His 350 Ile Met Tyr Ile Phe 355 Ala Val Ala Leu 360 Gln	1830
gat ggc gtg tgg cac cag gag ttt tcc tac acc cgc aag cgt tcc acc Asp Gly Val Trp 365 His Glu Phe Ser 370 Tyr Thr Arg Lys Arg 375 Ser Thr	1878
cgc ccg gaa act gtg gag ctg tgg cac aag att cgc acc gtg gag gat Arg Pro Glu Thr Val Glu Leu Trp His 385 Lys Ile Arg Thr Val Glu Asp	1926
380 385 390	
cct gaa tgg acg gcg cga tac cat tct gat gat cct gca aaa aag gcc Pro Glu Trp Thr Arg Arg Tyr His 400 Ser Asp Asp Pro Ala Lys Lys Ala	1974
395 400 405	
ttt ggt gcg aaa gca gtg atc aca atg gct gat ggc acc gtg att gag Phe Gly 410 Ala Lys Ala Val Ile 415 Thr Met Ala Asp 420 Gly Thr Val Ile Glu	2022
gat gaa ttg gct gtc gcg gat gcc cac ccg ctg ggt gct cgg ccg ttt Asp Glu Leu Ala Val 430 Ala Asp Ala His Pro Leu Gly Ala Arg Pro Phe	2070
425 430 435 440	
gcg cgg gag aat tac att gaa aaa ttc cgc aca ctc gcg cag ggg att Ala Arg Glu Asn Tyr Ile Glu Lys Phe Arg Thr Leu Ala Gln Gly Ile	2118
445 450 455	
gtc att gat tca gaa cag gaa cgc ttc ttg cat gcc gtg caa agc ctg Val Ile Asp Ser Glu Gln Glu Arg Phe Leu His Ala Val Gln Ser Leu	2166
460 465 470	
cct gac ctg gat gat ctt gat cag ctc aac atc gaa gtc gac ata agc Pro Asp 475 Leu Asp Asp Leu Asp 480 Gln Leu Asn Ile Glu Val 485 Asp Ile Ser	2214
aac cag gcc gcg acg aaa gcg ggg ctg tta tgaatctctt ttcgaatggt Asn Gln Ala Ala Thr Lys Ala 495 Gly Leu Leu	2264
490	

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<210> 8

<211> 498

<212> PRT

<213> *Corynebacterium glutamicum*

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Met Arg Ile His Asp Val Tyr Thr His Leu Ser Ala Asp Asn Phe Pro
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Lys Ala Glu His Leu Ala Trp Lys Phe Ser Glu Leu Ala Thr Asp Pro
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Val Glu Val Thr Pro Asp Val Ser Glu Met Ile Ile Asn Arg Ile Ile
35 40 45

Asp Asn Ala Ala Val Ser Ala Ala Ser Val Leu Arg Arg Pro Val Thr
50 55 60

Val Ala Arg Gln Gln Ala Gln Ser His Pro Arg Glu Lys Gly Gly Lys
65 70 75 80

Val Phe Gly Ile Ser Gly Ser Tyr Ser Pro Glu Trp Ala Ala Phe Ala
85 90 95

Asn Gly Val Ala Val Arg Glu Leu Asp Phe His Asp Thr Phe Leu Ala
100 105 110

Ala Glu Tyr Ser His Pro Gly Asp Asn Ile Pro Pro Leu Leu Ala Val
115 120 125

Ala Gln Ala Gln Arg Ser Ser Gly Arg Asp Leu Ile Arg Gly Ile Ala
130 135 140

Thr Ala Tyr Glu Val Gln Val Glu Leu Val Arg Gly Ile Cys Leu His
145 150 155 160

Glu His Lys Ile Asp His Val Ala His Leu Gly Pro Ser Ala Ala Ala
165 170 175

Gly Leu Gly Thr Leu Leu His Val Asp Glu Glu Thr Ile Tyr Gln Ala
180 185 190

Ile Gly Gln Ala Leu His Thr Thr Thr Ala Thr Arg Gln Ser Arg Lys
195 200 205

Gly Glu Ile Ser Ser Trp Lys Ala Phe Ala Pro Ala Phe Ala Gly Lys
210 215 220

Met Ala Ile Glu Ala Met Asp Arg Ala Met Arg Gly Glu Gly Ser Pro
225 230 235 240

Ala Pro Ile Trp Glu Gly Glu Asp Gly Val Ile Ala Trp Leu Leu Ser
245 250 255

Gly Lys Asp His Val Tyr His Val Pro Leu Pro Glu His Gly Glu Leu
260 265 270

Lys Leu Gly Ile Leu Glu Thr Tyr Thr Lys Glu His Ser Ala Glu Tyr
275 280 285

Gln Ser Gln Ala Pro Ile Asp Leu Ala Arg Arg Met Lys Pro Leu Val
290 295 300

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

Ser His His Thr His Tyr Val Ile Gly Thr Gly Ala Asn Asp Pro Gln
325 330 335

Lys Met Asp Pro Gln Ala Ser Arg Glu Thr Leu Asp His Ser Ile Met
340 345 350

Tyr Ile Phe Ala Val Ala Leu Gln Asp Gly Val Trp His His Glu Phe
355 360 365

Ser Tyr Thr Arg Lys Arg Ser Thr Arg Pro Glu Thr Val Glu Leu Trp
370 375 380

His Lys Ile Arg Thr Val Glu Asp Pro Glu Trp Thr Arg Arg Tyr His
385 390 395 400

Ser Asp Asp Pro Ala Lys Lys Ala Phe Gly Ala Lys Ala Val Ile Thr
405 410 415

Met Ala Asp Gly Thr Val Ile Glu Asp Glu Leu Ala Val Ala Asp Ala
 420 425 430

His Pro Leu Gly Ala Arg Pro Phe Ala Arg Glu Asn Tyr Ile Glu Lys
 435 440 445

Phe Arg Thr Leu Ala Gln Gly Ile Val Ile Asp Ser Glu Gln Glu Arg
 450 455 460

Phe Leu His Ala Val Gln Ser Leu Pro Asp Leu Asp Asp Leu Asp Gln
 465 470 475 480

Leu Asn Ile Glu Val Asp Ile Ser Asn Gln Ala Ala Thr Lys Ala Gly
 485 490 495

Leu Leu

<210> 9

<211> 28

<212> DNA

<213> Artificial Sequence

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<223> Primer prpD1_xL_A1

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<221> misc_feature

<222> (1)..(2)

<220>

<221> misc_structure

<222> (3)..(8)

<223> EcoRI restriction cleavage site

<400> 9

gcgaattcta aactgcgtga ggttgtgg 28

<210> 10

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer prpD1_XL_E1

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<221> misc_feature

<222> (1)..(2)

<220>

<221> misc_structure

<222> (3)..(8)

<223> EcoRI restriction cleavage site

<400> 10

gcgaattctc. cccacatcaa caccattc 28

<210> 11

<211> 1263

<212> DNA

<213> Corynebacterium glutamicum

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<221> CDS

<222> (1)..(1263)

<223> lysC wild-type gene

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gga aat gat gtc gtg gtt gtc tgc tcc gca atg gga gac acc acg gat Gly Asn Asp Val Val Val Val Cys Ser Ala Met Gly Asp Thr Thr Asp 35 40 45	144
gaa ctt cta gaa ctt gca gcg gca gtg aat ccc gtt ccg cca gct cgt Glu Leu Leu Glu Leu Ala Ala Val Asn Pro Val Pro Pro Ala Arg 50 55 60	192
gaa atg gat atg ctc ctg act gct ggt gag cgt att tct aac gct ctc Glu Met Asp Met Leu Leu Thr Ala Gly Glu Arg Ile Ser Asn Ala Leu 65 70 80	240
gtc gcc atg gct att gag tcc ctt ggc gca gaa gcc caa tct ttc acg Val Ala Met Ala Ile Glu Ser Leu Gly Ala Glu Ala Gln Ser Phe Thr 85 90 95	288
ggc tct cag gct ggt gtg ctc acc acc gag cgc cac gga aac gca cgc Gly Ser Gln Ala Gly Val Leu Thr Thr Glu Arg His Gly Asn Ala Arg 100 105 110	336
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aag atc tgc att gtt gct ggt ttc cag ggt gtt aat aaa gaa acc cgc Lys Ile Cys Ile Val Ala Gly Phe Gln Gly Val Asn Lys Glu Thr Arg 130 135 140	432
gat gtc acc acg ttg ggt cgt ggt ggt tct gac acc act gca gtt gcg Asp Val Thr Thr Leu Gly Arg Gly Gly Ser Asp Thr Thr Ala Val Ala 145 150 155 160	480
ttg gca gct gct ttg aac gct gat gtg tgt gag att tac tcg gac gtt Leu Ala Ala Ala Leu Asn Ala Asp Val Cys Glu Ile Tyr Ser Asp Val 165 170 175	528
gac ggt gtg tat acc gct gac ccg cgc atc gtt cct aat gca cag aag Asp Gly Val Tyr Thr Ala Asp Pro Arg Ile Val Pro Asn Ala Gln Lys 180 185 190	576
ctg gaa aag ctc agc ttc gaa gaa atg ctg gaa ctt gct gct gtt ggc Leu Glu Lys Leu Ser Phe Glu Glu Met Leu Glu Leu Ala Ala Val Gly 195 200 205	624
tcc aag att ttg gtg ctg cgc agt gtt gaa tac gct cgt gca ttc aat Ser Lys Ile Leu Val Leu Arg Ser Val Glu Tyr Ala Arg Ala Phe Asn 210 215 220	672
gtg cca ctt cgc gta cgc tcg tct tat agt aat gat ccc ggc act ttg Val Pro Leu Arg Val Arg Ser Ser Tyr Ser Asn Asp Pro Gly Thr Leu 225 230 235 240	720

225	230	235	240	
att gcc ggc tct atg gag gat att cct gtg gaa gaa gca gtc ctt acc Ile Ala Gly Ser Met Glu Asp Ile Pro Val Glu Glu Ala Val Leu Thr	245	250	255	768
ggt gtc gca acc gac aag tcc gaa gcc aaa gta acc gtt ctg ggt att Gly Val Ala Thr Asp Lys Ser Glu Ala Lys Val Thr Val Leu Gly Ile	260	265	270	816
tcc gat aag cca ggc gag gct gcg aag gtt ttc cgt gcg ttg gct gat Ser Asp Lys Pro Gly Glu Ala Ala Lys Val Phe Arg Ala Leu Ala Asp	275	280	285	864
gca gaa atc aac att gac atg gtt ctg cag aac gtc tct tct gta gaa Ala Glu Ile Asn Ile Asp Met Val Leu Gln Asn Val Ser Ser Val Glu	290	295	300	912
gac ggc acc acc gac atc acc ttc acc tgc cct cgt tcc gac ggc cgc Asp Gly Thr Thr Asp Ile Thr Phe Thr Cys Pro Arg Ser Asp Gly Arg	305	310	315	960
cgc gcg atg gag atc ttg aag aag ctt cag gtt cag ggc aac tgg acc Arg Ala Met Glu Ile Leu Lys Lys Leu Gln Val Gln Gly Asn Trp Thr	325	330	335	1008
aat gtg ctt tac gac gac cag gtc ggc aaa gtc tcc ctc gtg ggt gct Asn Val Leu Tyr Asp Asp Gln Val Gly Lys Val Ser Leu Val Gly Ala	340	345	350	1056
ggc atg aag tct cac cca ggt gtt acc gca gag ttc atg gaa gct ctg Gly Met Lys Ser His Pro Gly Val Thr Ala Glu Phe Met Glu Ala Leu	355	360	365	1104
cgc gat gtc aac gtg aac atc gaa ttg att tcc acc tct gag att cgt Arg Asp Val Asn Val Asn Ile Glu Leu Ile Ser Thr Ser Glu Ile Arg	370	375	380	1152
att tcc gtg ctg atc cgt gaa gat gat ctg gat gct gct gca cgt gca Ile Ser Val Leu Ile Arg Glu Asp Asp Leu Asp Ala Ala Ala Arg Ala	385	390	395	1200
ttg cat gag cag ttc cag ctg ggc ggc gaa gac gaa gcc gtc gtt tat Leu His Glu Gln Phe Gln Leu Gly Gly Glu Asp Glu Ala Val Val Tyr	405	410	415	1248
gca ggc acc gga cgc Ala Gly Thr Gly Arg	420			1263
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Glu Arg Ile Arg Asn val Ala Glu Arg Ile val Ala Thr Lys Lys Ala	20	25	30	
Gly Asn Asp Val val val Val Cys Ser Ala Met Gly Asp Thr Thr Asp	35	40	45	

Glu Leu Leu Glu Leu Ala Ala Ala Val Asn Pro Val Pro Pro Ala Arg
 50 55 60
 Glu Met Asp Met Leu Leu Thr Ala Gly Glu Arg Ile Ser Asn Ala Leu
 65 70 75 80
 Val Ala Met Ala Ile Glu Ser Leu Gly Ala Glu Ala Gln Ser Phe Thr
 85 90 95
 Gly Ser Gln Ala Gly Val Leu Thr Thr Glu Arg His Gly Asn Ala Arg
 100 105 110
 Ile Val Asp Val Thr Pro Gly Arg Val Arg Glu Ala Leu Asp Glu Gly
 115 120 125
 Lys Ile Cys Ile Val Ala Gly Phe Gln Gly Val Asn Lys Glu Thr Arg
 130 135 140
 Asp Val Thr Thr Leu Gly Arg Gly Gly Ser Asp Thr Thr Ala Val Ala
 145 150 155 160
 Leu Ala Ala Ala Leu Asn Ala Asp Val Cys Glu Ile Tyr Ser Asp Val
 165 170 175
 Asp Gly Val Tyr Thr Ala Asp Pro Arg Ile Val Pro Asn Ala Gln Lys
 180 185 190
 Leu Glu Lys Leu Ser Phe Glu Glu Met Leu Glu Leu Ala Ala Val Gly
 195 200 205
 Ser Lys Ile Leu Val Leu Arg Ser Val Glu Tyr Ala Arg Ala Phe Asn
 210 215 220
 Val Pro Leu Arg Val Arg Ser Ser Tyr Ser Asn Asp Pro Gly Thr Leu
 225 230 235 240
 Ile Ala Gly Ser Met Glu Asp Ile Pro Val Glu Glu Ala Val Leu Thr
 245 250 255
 Gly Val Ala Thr Asp Lys Ser Glu Ala Lys Val Thr Val Leu Gly Ile
 260 265 270
 Ser Asp Lys Pro Gly Glu Ala Ala Lys Val Phe Arg Ala Leu Ala Asp
 275 280 285
 Ala Glu Ile Asn Ile Asp Met Val Leu Gln Asn Val Ser Ser Val Glu
 290 295 300
 Asp Gly Thr Thr Asp Ile Thr Phe Thr Cys Pro Arg Ser Asp Gly Arg
 305 310 315 320

Arg Ala Met Glu Ile Leu Lys Lys Leu Gln Val Gln Gly Asn Trp Thr
 325 330 335

Asn Val Leu Tyr Asp Asp Gln Val Gly Lys Val Ser Leu Val Gly Ala
 340 345 350

Gly Met Lys Ser His Pro Gly Val Thr Ala Glu Phe Met Glu Ala Leu
 355 360 365

Arg Asp Val Asn Val Asn Ile Glu Leu Ile Ser Thr Ser Glu Ile Arg
 370 375 380

Ile Ser Val Leu Ile Arg Glu Asp Asp Leu Asp Ala Ala Ala Arg Ala
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Leu His Glu Gln Phe Gln Leu Gly Gly Glu Asp Glu Ala Val Val Tyr
 405 410 415

Ala Gly Thr Gly Arg
 420

<210> 13

<211> 1633

<212> DNA

<213> Corynebacterium glutamicum

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<222> (1)..(1633)

<223> PCR-Product

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<221> misc_structure

<222> (3)..(8)

<223> EcoRI restriction cleavage site

<220>

<221> CDS

<222> (99)..(1592)

<220>

<221> mutation

<222> (913)..(913)

<223> C -> T Transition

<220>

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<222> (1626)..(1631)

<223> EcoRI restriction cleavage site

<220>

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<222> (1632)..(1633)

<400> 13

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gggtgggact

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				Met	Arg	Ile	His	Asp	Val	
				1				5		

tat	acc	cac	ctt	tcg	gcc	gat	aac	ttt	ccc	aaa	gca	gag	cac	ctt	gcg	164
Tyr	Thr	His	Leu	Ser	Ala	Asp	Asn	Phe	Pro	Lys	Ala	Glu	His	Leu	Ala	
			10					15					20			

tgg	aaa	ttc	tcc	gag	ctt	gcc	acc	gac	ccc	gtg	gag	gtg	aca	ccg	gat	212
Trp	Lys	Phe	Ser	Glu	Leu	Ala	Thr	Asp	Pro	Val	Glu	Val	Thr	Pro	Asp	
		25					30					35				

gtt	tcg	gag	atg	atc	atc	aac	cgg	atc	atc	gac	aac	gcg	gcg	gtg	tct	260
Val	Ser	Glu	Met	Ile	Ile	Asn	Arg	Ile	Ile	Asp	Asn	Ala	Ala	Val	Ser	
	40					45					50					

gcc	gcg	tcg	gtg	ttg	cgc	cgg	cct	gtg	act	gtg	gcc	agg	caa	caa	gcg	308
Ala	Ala	Ser	Val	Leu	Arg	Arg	Pro	Val	Thr	Val	Ala	Arg	Gln	Gln	Ala	
	55				60					65					70	

cag	tcc	cat	ccg	cgg	gaa	aag	ggc	gga	aaa	gtt	ttt	gga	att	tca	ggc	356
Gln	Ser	His	Pro	Arg	Glu	Lys	Gly	Gly	Lys	Val	Phe	Gly	Ile	Ser	Gly	
				75					80					85		

agc	tac	tca	cca	gag	tgg	gct	gcc	ttt	gct	aat	ggt	gtg	gcc	gta	cgt	404
Ser	Tyr	Ser	Pro	Glu	Trp	Ala	Ala	Phe	Ala	Asn	Gly	Val	Ala	Val	Arg	
			90					95					100			

gaa	ttg	gac	ttc	cac	gat	aca	ttt	tta	gca	gct	gaa	tac	tcc	cat	ccc	452
Glu	Leu	Asp	Phe	His	Asp	Thr	Phe	Leu	Ala	Ala	Glu	Tyr	Ser	His	Pro	
		105					110					115				

ggc	gac	aat	att	cca	cca	ctt	ctt	gca	gta	gcg	cag	gct	cag	aga	agc	500
Gly	Asp	Asn	Ile	Pro	Pro	Leu	Leu	Ala	Val	Ala	Gln	Ala	Gln	Arg	Ser	
	120					125					130					

agc	ggc	agg	gat	ctc	att	cgg	ggt	atc	gcc	act	gcc	tat	gag	gtg	cag	548
Ser	Gly	Arg	Asp	Leu	Ile	Arg	Gly	Ile	Ala	Thr	Ala	Tyr	Glu	Val	Gln	
	135				140					145					150	

gtg	gaa	ttg	gtg	agg	ggg	atc	tgc	ttg	cat	gag	cac	aaa	att	gat	cac	596
Val	Glu	Leu	Val	Arg	Gly	Ile	Cys	Leu	His	Glu	His	Lys	Ile	Asp	His	
				155				160						165		

gta	gcc	cac	ctt	ggt	ccc	agc	gct	gca	gcg	ggt	ttg	ggc	acg	ttg	ttg	644
Val	Ala	His	Leu	Gly	Pro	Ser	Ala	Ala	Ala	Gly	Leu	Gly	Thr	Leu	Leu	
			170					175					180			

cat	gta	gat	gag	gaa	acc	atc	tac	cag	gcg	atc	ggc	cag	gca	ttg	cac	692
His	Val	Asp	Glu	Glu	Thr	Ile	Tyr	Gln	Ala	Ile	Gly	Gln	Ala	Leu	His	
		185					190					195				

acc	acg	acg	gct	acg	agg	cag	tcg	cga	aaa	ggt	gag	att	tcc	agc	tgg	740
Thr	Thr	Thr	Ala	Thr	Arg	Gln	Ser	Arg	Lys	Gly	Glu	Ile	Ser	Ser	Trp	
						205					210					

aag	gcg	ttc	gcg	cca	gcg	ttt	gcg	gga	aag	atg	gcc	att	gag	gcg	atg	788
Lys	Ala	Phe	Ala	Pro	Ala	Phe	Ala	Gly	Lys	Met	Ala	Ile	Glu	Ala	Met	
	215				220					225					230	

gat	cgt	gcg	atg	cgt	ggg	gag	ggt	tcg	ccc	gca	ccg	att	tgg	gag	ggc	836
Asp	Arg	Ala	Met	Arg	Gly	Glu	Gly	Ser	Pro	Ala	Pro	Ile	Trp	Glu	Gly	
				235					240					245		

gaa	gac	ggg	gtc	atc	gcg	tgg	ctg	tta	tcg	ggc	aaa	gat	cat	gtt	tat	884
Glu	Asp	Gly	Val	Ile	Ala	Trp	Leu	Leu	Ser	Gly	Lys	Asp	His	Val	Tyr	
			250					255					260			

cat	gtg	cca	ttg	ccg	gaa	cac	ggc	gag	ctc	aag	ctg	ggg	att	cta	gag	932
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His Val	Pro	Leu	Pro	Glu	His	Gly	Glu	Leu	Lys	Leu	Gly	Ile	Leu	Glu		
	265					270					275					
act	tac	aca	aag	gaa	cat	tca	gcg	gaa	tat	caa	tcg	cag	gca	ccg	att	980
Thr	Tyr	Thr	Lys	Glu	His	Ser	Ala	Glu	Tyr	Gln	Ser	Gln	Ala	Pro	Ile	
	280					285				290						
gat	ctg	gcg	cgc	agg	atg	aag	cca	ctg	gtt	gac	gcg	gct	ggc	gga	acg	1028
Asp	Leu	Ala	Arg	Arg	Met	Lys	Pro	Leu	Val	Asp	Ala	Ala	Gly	Gly	Thr	
	295				300					305					310	
gaa	cac	att	gca	gag	att	gtg	ctg	cgc	acc	agt	cac	cac	acg	cat	tat	1076
Glu	His	Ile	Ala	Glu	Ile	Val	Leu	Arg	Thr	Ser	His	His	Thr	His	Tyr	
				315					320					325		
gtg	att	ggc	act	ggg	gcg	aac	gat	ccg	cag	aag	atg	gat	ccg	cag	gcc	1124
Val	Ile	Gly	Thr	Gly	Ala	Asn	Asp	Pro	Gln	Lys	Met	Asp	Pro	Gln	Ala	
			330					335					340			
tcg	cgt	gaa	acc	ctg	gat	cat	tcc	atc	atg	tac	att	ttc	gcc	gtc	gcg	1172
Ser	Arg	Glu	Thr	Leu	Asp	His	Ser	Ile	Met	Tyr	Ile	Phe	Ala	Val	Ala	
		345					350					355				
ctt	caa	gat	ggc	gtg	tgg	cac	cac	gag	ttt	tcc	tac	acc	cgc	aag	cgt	1220
Leu	Gln	Asp	Gly	Val	Trp	His	His	Glu	Phe	Ser	Tyr	Thr	Arg	Lys	Arg	
	360					365					370					
tcc	acc	cgc	ccg	gaa	act	gtg	gag	ctg	tgg	cac	aag	att	cgc	acc	gtg	1268
Ser	Thr	Arg	Pro	Glu	Thr	Val	Glu	Leu	Trp	His	Lys	Ile	Arg	Thr	Val	
	375				380					385					390	
gag	gat	cct	gaa	tgg	acg	cgc	cga	tac	cat	tct	gat	gat	cct	gca	aaa	1316
Glu	Asp	Pro	Glu	Trp	Thr	Arg	Arg	Tyr	His	Ser	Asp	Asp	Pro	Ala	Lys	
				395				400						405		
aag	gcc	ttt	ggt	gcg	aaa	gca	gtg	atc	aca	atg	gct	gat	ggc	acc	gtg	1364
Lys	Ala	Phe	Gly	Ala	Lys	Ala	Val	Ile	Thr	Met	Ala	Asp	Gly	Thr	Val	
			410					415					420			
att	gag	gat	gaa	ttg	gct	gtc	gcg	gat	gcc	cac	ccg	ctg	ggt	gct	cgg	1412
Ile	Glu	Asp	Glu	Leu	Ala	Val	Ala	Asp	Ala	His	Pro	Leu	Gly	Ala	Arg	
		425					430					435				
ccg	ttt	gcg	cgg	gag	aat	tac	att	gaa	aaa	ttc	cgc	aca	ctc	gcg	cag	1460
Pro	Phe	Ala	Arg	Glu	Asn	Tyr	Ile	Glu	Lys	Phe	Arg	Thr	Leu	Ala	Gln	
	440					445					450					
ggg	att	gtc	att	gat	tca	gaa	cag	gaa	cgc	ttc	ttg	cat	gcc	gtg	caa	1508
Gly	Ile	Val	Ile	Asp	Ser	Glu	Gln	Glu	Arg	Phe	Leu	His	Ala	Val	Gln	
	455				460					465					470	
agc	ctg	cct	gac	ctg	gat	gat	ctt	gat	cag	ctc	aac	atc	gaa	gtc	gac	1556
Ser	Leu	Pro	Asp	Leu	Asp	Asp	Leu	Asp	Gln	Leu	Asn	Ile	Glu	Val	Asp	
				475					480					485		
ata	agc	aac	cag	gcc	gcg	acg	aaa	gcg	ggg	ctg	tta	tgaatctctt				1602
Ile	Ser	Asn	Gln	Ala	Ala	Thr	Lys	Ala	Gly	Leu	Leu					
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<211> 498

<212> PRT

<213> Corynebacterium glutamicum

<400> 14

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 Lys Ala Glu His Leu Ala Trp Lys Phe Ser Glu Leu Ala Thr Asp Pro
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 Val Glu Val Thr Pro Asp Val Ser Glu Met Ile Ile Asn Arg Ile Ile
 35 40 45
 Asp Asn Ala Ala Val Ser Ala Ala Ser Val Leu Arg Arg Pro Val Thr
 50 55 60
 Val Ala Arg Gln Gln Ala Gln Ser His Pro Arg Glu Lys Gly Gly Lys
 65 70 75 80
 Val Phe Gly Ile Ser Gly Ser Tyr Ser Pro Glu Trp Ala Ala Phe Ala
 85 90 95
 Asn Gly Val Ala Val Arg Glu Leu Asp Phe His Asp Thr Phe Leu Ala
 100 105 110
 Ala Glu Tyr Ser His Pro Gly Asp Asn Ile Pro Pro Leu Leu Ala Val
 115 120 125
 Ala Gln Ala Gln Arg Ser Ser Gly Arg Asp Leu Ile Arg Gly Ile Ala
 130 135 140
 Thr Ala Tyr Glu Val Gln Val Glu Leu Val Arg Gly Ile Cys Leu His
 145 150 155 160
 Glu His Lys Ile Asp His Val Ala His Leu Gly Pro Ser Ala Ala Ala
 165 170 175
 Gly Leu Gly Thr Leu Leu His Val Asp Glu Glu Thr Ile Tyr Gln Ala
 180 185 190
 Ile Gly Gln Ala Leu His Thr Thr Thr Ala Thr Arg Gln Ser Arg Lys
 195 200 205
 Gly Glu Ile Ser Ser Trp Lys Ala Phe Ala Pro Ala Phe Ala Gly Lys
 210 215 220
 Met Ala Ile Glu Ala Met Asp Arg Ala Met Arg Gly Glu Gly Ser Pro
 225 230 235 240
 Ala Pro Ile Trp Glu Gly Glu Asp Gly Val Ile Ala Trp Leu Leu Ser
 245 250 255
 Gly Lys Asp His Val Tyr His Val Pro Leu Pro Glu His Gly Glu Leu
 260 265 270
 Lys Leu Gly Ile Leu Glu Thr Tyr Thr Lys Glu His Ser Ala Glu Tyr

275 280 285
 Gln Ser Gln Ala Pro Ile Asp Leu Ala Arg Arg Met Lys Pro Leu Val
 290 295 300
 Asp Ala Ala Gly Gly Thr Glu His Ile Ala Glu Ile Val Leu Arg Thr
 305 310 315 320
 Ser His His Thr His Tyr Val Ile Gly Thr Gly Ala Asn Asp Pro Gln
 325 330 335
 Lys Met Asp Pro Gln Ala Ser Arg Glu Thr Leu Asp His Ser Ile Met
 340 345 350
 Tyr Ile Phe Ala Val Ala Leu Gln Asp Gly Val Trp His His Glu Phe
 355 360 365
 Ser Tyr Thr Arg Lys Arg Ser Thr Arg Pro Glu Thr Val Glu Leu Trp
 370 375 380
 His Lys Ile Arg Thr Val Glu Asp Pro Glu Trp Thr Arg Arg Tyr His
 385 390 395 400
 Ser Asp Asp Pro Ala Lys Lys Ala Phe Gly Ala Lys Ala Val Ile Thr
 405 410 415
 Met Ala Asp Gly Thr Val Ile Glu Asp Glu Leu Ala Val Ala Asp Ala
 420 425 430
 His Pro Leu Gly Ala Arg Pro Phe Ala Arg Glu Asn Tyr Ile Glu Lys
 435 440 445
 Phe Arg Thr Leu Ala Gln Gly Ile Val Ile Asp Ser Glu Gln Glu Arg
 450 455 460
 Phe Leu His Ala Val Gln Ser Leu Pro Asp Leu Asp Asp Leu Asp Gln
 465 470 475 480
 Leu Asn Ile Glu Val Asp Ile Ser Asn Gln Ala Ala Thr Lys Ala Gly
 485 490 495

Leu Leu

<210> 15

<211> 1311

<212> DNA

<213> Corynebacterium glutamicum

<220>

<221> CDS

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gga Gly	gcg Ala	gag Glu	ctg Leu 20	att Ile	cgt Arg	gcc Ala	gcc Ala	gac Asp 25	att Ile	caa Gln	acg Thr	gcg Ala	cag Gln 30	gca Ala	cga Arg	96
att Ile	tcc Ser	tcc Ser 35	gtc Val	att Ile	gca Ala	cca Pro	act Thr 40	cca Pro	ttg Leu	cag Gln	tat Tyr	tgc Cys 45	cct Pro	cgt Arg	ctt Leu	144
tct Ser	gag Glu 50	gaa Glu	acc Thr	gga Gly	gcg Ala	gaa Glu 55	atc Ile	tac Tyr	ctt Leu	aag Lys	cgt Arg 60	gag Glu	gat Asp	ctg Leu	cag Gln	192
gat Asp 65	gtt Val	cgt Arg	tcc Ser	tac Tyr	aag Lys 70	atc Ile	cgc Arg	ggg Gly	gag Ala	ctg Leu 75	aac Asn	tct Ser	gga Gly	gcg Ala	cag Gln 80	240
ctc Leu	acc Thr	caa Gln	gag Glu	cag Gln 85	cgc Arg	gat Asp	gca Ala	ggg Gly	atc Ile 90	gtt Val	gcc Ala	gca Ala	tct Ser	gca Ala 95	ggg Gly	288
aac Asn	cat His	gcc Ala	cag Gln 100	ggc Gly	gtg Val	gcc Ala	tat Tyr 105	gtg Val 105	tgc Cys	aag Lys	tcc Ser	ttg Leu	ggc Gly 110	gtt Val	cag Gln	336
gga Gly	gag Arg	atc Ile 115	tat Tyr	gtt Val	cct Pro	gtg Val	cag Gln 120	act Thr	cca Pro	aag Lys	caa Gln	aag Lys 125	cgt Arg	gac Asp	cgc Arg	384
atc Ile 130	atg Met	gtt Val	cac His	ggc Gly	gga Gly	gag Glu 135	ttt Phe	gtc Val	tcc Ser	ttg Leu	gtg Val 140	gtc Val	act Thr	ggc Gly	aat Asn	432
aac Asn 145	ttc Phe	gac Asp	gaa Glu	gca Ala	tcg Ser 150	gct Ala	gca Ala	gag Ala	cat His 155	gaa Glu	gat Asp	gca Ala	gag Glu	cgc Arg	acc Thr 160	480
ggc Gly	gca Ala	acg Thr	ctg Leu	atc Ile 165	gag Glu	cct Pro	ttc Phe	gat Asp	gct Ala 170	cgc Arg	aac Asn	acc Thr	gtc Val	atc Ile 175	ggg Gly	528
cag Gln	ggc Gly	acc Thr	gtg Val 180	gct Ala	gct Ala	gag Glu	atc Ile	ttg Leu 185	tcg Ser	cag Gln	ctg Leu	act Thr	tcc Ser 190	atg Met	ggc Gly	576
aag Lys	agt Ser	gca Ala 195	gat Asp	cac His	gtg Val	atg Met	gtt Val 200	cca Pro	gtc Val	ggc Gly	ggg Gly	ggc Gly 205	gga Gly	ctt Leu	ctt Leu	624
gca Ala 210	ggg Gly	gtg Val	gtc Val	agc Ser	tac Tyr	atg Met 215	gct Ala	gat Asp	atg Met	gca Ala	cct Pro 220	cgc Arg	act Thr	gag Ala	atc Ile	672
gtt Val 225	ggg Gly	atc Ile	gaa Glu	cca Pro	gag Ala 230	gga Gly	gca Ala	gca Ala	tcc Ser 235	atg Met	cag Gln	gct Ala	gca Ala	ttg Leu 240	cac His	720
aat Asn	ggg Gly	gga Gly	cca Pro	atc Ile 245	act Thr	ttg Leu	gag Glu	act Thr	gtt Val 250	gat Asp	ccc Pro	ttt Phe	gtg Val	gac Asp 255	ggc Gly	768
gca Ala	gca Ala	gtc Val	aaa Lys 260	cgt Arg	gtc Val	gga Gly	gat Asp	ctc Leu 265	aac Asn	tac Tyr	acc Thr	atc Ile	gtg Val 270	gag Glu	aag Lys	816

aac cag ggt cgc gtg cac atg atg agc gcg acc gag ggc gct gtg tgt 864
 Asn Gln Gly Arg Val His Met Met Ser Ala Thr Glu Gly Ala Val Cys
 275 280 285
 act gag atg ctc gat ctt tac caa aac gaa ggc atc atc gcg gag cct 912
 Thr Glu Met Leu Asp Leu Tyr Gln Asn Glu Gly Ile Ile Ala Glu Pro
 290 295 300
 gct ggc gcg ctg tct atc gct ggg ttg aag gaa atg tcc ttt gca cct 960
 Ala Gly Ala Leu Ser Ile Ala Gly Leu Lys Glu Met Ser Phe Ala Pro
 305 310 315 320
 ggt tct gtc gtg gtg tgc atc atc tct ggt ggc aac aac gat gtg ctg 1008
 Gly Ser Val Val Val Cys Ile Ile Ser Gly Gly Asn Asn Asp Val Leu
 325 330 335
 cgt tat gcg gaa atc gct gag cgc tcc ttg gtg cac cgc ggt ttg aag 1056
 Arg Tyr Ala Glu Ile Ala Glu Arg Ser Leu Val His Arg Gly Leu Lys
 340 345 350
 cac tac ttc ttg gtg aac ttc ccg caa aag cct ggt cag ttg cgt cac 1104
 His Tyr Phe Leu Val Asn Phe Pro Gln Lys Pro Gly Gln Leu Arg His
 355 360 365
 ttc ctg gaa gat atc ctg gga ccg gat gat gac atc acg ctg ttt gag 1152
 Phe Leu Glu Asp Ile Leu Gly Pro Asp Asp Ile Thr Leu Phe Glu
 370 375 380
 tac ctc aag cgc aac aac cgt gag acc ggt act gcg ttg gtg ggt att 1200
 Tyr Leu Lys Arg Asn Asn Arg Glu Thr Gly Thr Ala Leu Val Gly Ile
 385 390 395 400
 cac ttg agt gaa gca tca gga ttg gat tct ttg ctg gaa cgt atg gag 1248
 His Leu Ser Glu Ala Ser Gly Leu Asp Ser Leu Leu Glu Arg Met Glu
 405 410 415
 gaa tcg gca att gat tcc cgt cgc ctc gag ccg ggc acc cct gag tac 1296
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 Glu Tyr Leu Thr 435

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<211> 436

<212> PRT

<213> Corynebacterium glutamicum

<400> 16

Met Ser Glu Thr Tyr Val Ser Glu Lys Ser Pro Gly Val Met Ala Ser
 1 5 10 15

Gly Ala Glu Leu Ile Arg Ala Ala Asp Ile Gln Thr Ala Gln Ala Arg
 20 25 30

Ile Ser Ser Val Ile Ala Pro Thr Pro Leu Gln Tyr Cys Pro Arg Leu
 35 40 45

Ser Glu Glu Thr Gly Ala Glu Ile Tyr Leu Lys Arg Glu Asp Leu Gln
 50 55 60

Asp Val Arg Ser Tyr Lys Ile Arg Gly Ala Leu Asn Ser Gly Ala Gln
 65 70 75 80
 Leu Thr Gln Glu Gln Arg Asp Ala Gly Ile Val Ala Ala Ser Ala Gly
 85 90 95
 Asn His Ala Gln Gly Val Ala Tyr Val Cys Lys Ser Leu Gly Val Gln
 100 105 110
 Gly Arg Ile Tyr Val Pro Val Gln Thr Pro Lys Gln Lys Arg Asp Arg
 115 120 125
 Ile Met Val His Gly Gly Glu Phe Val Ser Leu Val Val Thr Gly Asn
 130 135 140
 Asn Phe Asp Glu Ala Ser Ala Ala Ala His Glu Asp Ala Glu Arg Thr
 145 150 155 160
 Gly Ala Thr Leu Ile Glu Pro Phe Asp Ala Arg Asn Thr Val Ile Gly
 165 170 175
 Gln Gly Thr Val Ala Ala Glu Ile Leu Ser Gln Leu Thr Ser Met Gly
 180 185 190
 Lys Ser Ala Asp His Val Met Val Pro Val Gly Gly Gly Gly Leu Leu
 195 200 205
 Ala Gly Val Val Ser Tyr Met Ala Asp Met Ala Pro Arg Thr Ala Ile
 210 215 220
 Val Gly Ile Glu Pro Ala Gly Ala Ala Ser Met Gln Ala Ala Leu His
 225 230 235 240
 Asn Gly Gly Pro Ile Thr Leu Glu Thr Val Asp Pro Phe Val Asp Gly
 245 250 255
 Ala Ala Val Lys Arg Val Gly Asp Leu Asn Tyr Thr Ile Val Glu Lys
 260 265 270
 Asn Gln Gly Arg Val His Met Met Ser Ala Thr Glu Gly Ala Val Cys
 275 280 285
 Thr Glu Met Leu Asp Leu Tyr Gln Asn Glu Gly Ile Ile Ala Glu Pro
 290 295 300
 Ala Gly Ala Leu Ser Ile Ala Gly Leu Lys Glu Met Ser Phe Ala Pro
 305 310 315 320
 Gly Ser Val Val Val Cys Ile Ile Ser Gly Gly Asn Asn Asp Val Leu
 325 330 335
 Arg Tyr Ala Glu Ile Ala Glu Arg Ser Leu Val His Arg Gly Leu Lys

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      340              345              350
His Tyr Phe Leu Val Asn Phe Pro Gln Lys Pro Gly Gln Leu Arg His
      355              360              365

Phe Leu Glu Asp Ile Leu Gly Pro Asp Asp Asp Ile Thr Leu Phe Glu
      370              375              380

Tyr Leu Lys Arg Asn Asn Arg Glu Thr Gly Thr Ala Leu Val Gly Ile
      385              390              395              400

His Leu Ser Glu Ala Ser Gly Leu Asp Ser Leu Leu Glu Arg Met Glu
      405              410              415

Glu Ser Ala Ile Asp Ser Arg Arg Leu Glu Pro Gly Thr Pro Glu Tyr
      420              425              430

Glu Tyr Leu Thr
      435

<210> 17
<211> 120
<212> DNA
<213> Corynebacterium glutamicum
<220>
<221> CDS
<222> (1)..(120)
<223> part of the coding region of the prpD1 allele
<220>
<221> misc_feature
<222> (61)..(63)
<223> n is a, c, g, or t
<400> 17
gcg tgg ctg tta tcg ggc aaa gat cat gtt tat cat gtg cca ttg ccg      48
Ala Trp Leu Leu Ser Gly Lys Asp His Val Tyr His Val Pro Leu Pro
1          5          10          15

gaa cac ggc gag nnn aag ctg ggg att cta gag act tac aca aag gaa      96
Glu His Gly Glu xaa Lys Leu Gly Ile Leu Glu Thr Tyr Thr Lys Glu
      20          25          30

cat tca gcg gaa tat caa tcg cag      120
His Ser Ala Glu Tyr Gln Ser Gln
      35          40

<210> 18
<211> 40
<212> PRT
<213> Corynebacterium glutamicum
<220>
<221> misc_feature
<222> (21)..(21)
<223> The 'Xaa' at location 21 stands for Lys, Asn, Arg, Ser,

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Thr, Ile, Met, Glu, Asp, Gly, Ala, Val, Gln, His, Leu, Tyr,
Trp, Cys, or Phe.

<400> 18

Ala Trp Leu Leu Ser Gly Lys Asp His Val Tyr His Val Pro Leu Pro
1 5 10 15

Glu His Gly Glu Xaa Lys Leu Gly Ile Leu Glu Thr Tyr Thr Lys Glu
20 25 30

His Ser Ala Glu Tyr Gln Ser Gln
35 40

<210> 19

<211> 120

<212> DNA

<213> Corynebacterium glutamicum

<220>

<221> CDS

<222> (1)..(120)

<223> part of the coding region of the prpD1 allele

<400> 19

gcg tgg ctg tta tcg ggc aaa gat cat gtt tat cat gtg cca ttg ccg 48
Ala Trp Leu Leu Ser Gly Lys Asp His Val Tyr His Val Pro Leu Pro
1 5 10 15

gaa cac ggc gag ctc aag ctg ggg att cta gag act tac aca aag gaa 96
Glu His Gly Glu Leu Lys Leu Gly Ile Leu Glu Thr Tyr Thr Lys Glu
20 25 30

cat tca gcg gaa tat caa tcg cag 120
His Ser Ala Glu Tyr Gln Ser Gln
35 40

<210> 20

<211> 40

<212> PRT

<213> Corynebacterium glutamicum

<400> 20

Ala Trp Leu Leu Ser Gly Lys Asp His Val Tyr His Val Pro Leu Pro
1 5 10 15

Glu His Gly Glu Leu Lys Leu Gly Ile Leu Glu Thr Tyr Thr Lys Glu
20 25 30

His Ser Ala Glu Tyr Gln Ser Gln
35 40

Patentkrav

1. Isolerede mutanter af coryneforme bakterier, som indeholder et gen, der koder for et polypeptid med 2-methylcitrat-dehydratase-aktivitet, kendetegnet ved, at polypeptidet på position 272 i den position, der svarer til aminosyresekvensen i SEQ ID NO:2, indeholder L-leucin, eller at polypeptidet omfatter aminosyresekvensen i SEQ ID NO: 2, idet der på position 272 er indeholdt L-leucin.

2. Mutanter af coryneforme bakterier ifølge krav 1, kendetegnet ved, at det i forbindelse med de coryneforme bakterier drejer sig om en bakterie udvalgt af gruppen *Corynebacterium efficiens*, *Corynebacterium glutamicum*, *Corynebacterium thermoaminogenes* og *Corynebacterium aminogenes*, fortrinsvis om *Corynebacterium glutamicum*.

3. Mutanter af coryneforme bakterier ifølge krav 1 eller 2, kendetegnet ved, at det drejer sig om bakterier, der udskiller L-aminosyre, fortrinsvis om bakterier, der udskiller L-lysin, L-valin, L-isoleucin, L-tryptophan eller L-homoserin.

4. Mutanter af coryneforme bakterier ifølge krav 1 til 3, kendetegnet ved, at genet omfatter en nukleotidsekvens, som er identisk med nukleotidsekvensen i et polynukleotid, som er opnåeligt ved hjælp af en polymerasekædereaktion (PCR) under anvendelse af DNA udvundet af en coryneform bakterie og under anvendelse af et primerpar bestående af en første primer omfattende i det mindste 15 på hinanden følgende nukleotider udvalgt af nukleotidsekvensen mellem position 1 og 750 i SEQ ID NO:3 eller SEQ ID NO:7 og en anden primer omfattende i det mindste 15 på hinanden følgende nukleotider udvalgt af den komplementære nukleotidsekvens mellem position 3000 og 2245 i SEQ ID NO:3 eller SEQ ID NO: 7, eller at det kodede polypeptid omfatter en aminosyresekvens med en længde svarende til 498 L-aminosyrer, eller at det kodede polypeptid fra position 252 til 291 i aminosyresekvensen indeholder aminosyresekvensen svarende til position 252 til 291 i SEQ ID NO:6, eller at det

kodede polypeptid omfatter en aminosyresekvens, som er i det mindste 90 % identisk med aminosyresekvensen i SEQ ID NO:6 og på position 272 indeholder L-leucin,

5 eller at genet omfatter en nukleotidsekvens, som er i det mindste 90 % identisk med nukleotidsekvensen i SEQ ID NO:5.

5. Mutanter af coryneforme bakterier ifølge krav 1, kendetegnet ved, at det kodede protein omfatter en aminosyresekvens udvalgt af gruppen

- 10 a) aminosyresekvens ifølge SEQ ID NO:6,
b) aminosyresekvens ifølge SEQ ID NO:6 inklusiv maksimalt 5 konservative aminosyreudskiftninger og
c) aminosyresekvens ifølge SEQ ID NO:6 inklusiv maksimalt 5 insertioner eller deletioner af aminosyrer.

15

6. Mutanter af coryneforme bakterier ifølge krav 4, kendetegnet ved, at genet omfatter nukleotidsekvensen i SEQ ID NO:5 eller SEQ ID NO:7.

- 20 7. Et isoleret polynukleotid, som koder for et polypeptid med 2-methylcitrat-dehydratase-enzymaktivitet, som på den position, der svarer til position 272 i aminosyresekvensen i SEQ ID NO:2, indeholder L-leucin, eller som omfatter aminosyresekvensen i SEQ ID NO: 2, idet der på position 272 er
25 indeholdt L-leucin.

8. Isoleret polynukleotid ifølge krav 7, kendetegnet ved, at det kodede polypeptid omfatter en aminosyresekvens med en længde på 498 aminosyrer, eller at det kodede polypeptid fra
30 position 252 til 291 i aminosyresekvensen indeholder aminosyresekvensen svarende til position 252 til 291 i SEQ ID NO:6, eller at det isolerede polynukleotid er identisk med nukleotidsekvensen i et polynukleotid, som opnås ved hjælp af en polymerasekædereaktion (PCR) under anvendelse af DNA
35 udvundet af en coryneform bakterie og under anvendelse af et primerpar bestående af en første primer omfattende i det mindste 15 på hinanden følgende nukleotider udvalgt af nukleotidsekvensen mellem position 1 og 750 i SEQ ID NO:3

- eller SEQ ID NO:7 og en anden primer omfattende i det mindste 15 på hinanden følgende nukleotider udvalgt af den komplementære nukleotidsekvens mellem position 3000 og 2245 i SEQ ID NO:3 eller SEQ ID NO: 7, eller at det isolerede
- 5 polynukleotid hybridiserer med den til SEQ ID NO:5 komplementære nukleotidsekvens under stringente betingelser, eller at det kodede polypeptid omfatter en aminosyresekvens, som er i det mindste 90 % identisk med aminosyresekvensen i SEQ ID NO:6, eller
- 10 at polynukleotidet omfatter en nukleotidsekvens, som er i det mindste 90 % identisk med nukleotidsekvensen i SEQ ID NO:5, eller at det kodede protein omfatter en aminosyresekvens udvalgt af gruppen
- a) aminosyresekvens ifølge SEQ ID NO:6,
- 15 b) aminosyresekvens ifølge SEQ ID NO:6 inklusiv maksimalt 5 konservative aminosyreudskiftninger og
- c) aminosyresekvens ifølge SEQ ID NO:6 inklusiv maksimalt 5 insertioner eller deletioner af aminosyrer.
- 20 9. Isoleret polynukleotid ifølge krav 7 eller 8 kendetegnet ved, at det kodede polypeptid omfatter aminosyresekvensen i SEQ ID NO:6.
10. Isoleret polynukleotid ifølge krav 9, kendetegnet ved, at
- 25 det omfatter nukleotidsekvensen SEQ ID NO:5.
11. Et isoleret polynukleotid, som omfatter et nukleinsyremolekyle, som i det mindste koder for en læseramme med en aminosyresekvens svarende til position 252 til 291 i
- 30 SEQ ID NO:2, idet der på den position, der svarer til position 272 i SEQ ID NO:2, er indeholdt L-leucin.
12. Isoleret polynukleotid ifølge krav 11, kendetegnet ved, at det i det mindste koder for en læseramme med en
- 35 aminosyresekvens, der svarer til position 252 til 291 i SEQ ID NO:6, eller at aminosyresekvensen indeholder en eller flere konservative aminosyreudskiftninger, idet de konservative aminosyreudskiftninger ikke vedrører position 272, eller at

det isolerede polynukleotid indeholder en eller flere stille mutationer, eller at det isolerede polynukleotid omfatter i det mindste en nukleotidsekvens svarende til position 1504 til 1623 i SEQ ID NO:7.

5

13. En rekombinant mikroorganisme, som indeholder det isolerede polynukleotid ifølge krav 7 til 12.

14. Rekombinant mikroorganisme ifølge krav 13, kendetegnet ved, at det drejer sig om en coryneform bakterie eller om en bakterie af slægten *Escherichia*, fortrinsvis om slægten *Corynebacterium* og særligt foretrukket om arten *Corynebacterium glutamicum*.

15. En vektor, som indeholder det isolerede polynukleotid ifølge krav 7 til 12.

16. En rekombinant mikroorganisme, som indeholder vektoren ifølge krav 15.

20

17. Rekombinant mikroorganisme ifølge krav 16, kendetegnet ved, at det drejer sig om en coryneform bakterie eller om en bakterie af slægten *Escherichia*, fortrinsvis om slægten *Corynebacterium* og særligt foretrukket om arten *Corynebacterium glutamicum*.

25

18. Fremgangsmåde til fremstilling af L-lysin kendetegnet ved, at man

a) fermenterer en isoleret coryneform bakterie i et egnet medie, idet bakterien indeholder i det mindste en kopi af et gen, som koder for et polypeptid med 2-methylcitrat-dehydratase-enzymaktivitet, idet polypeptidet på den position, der svarer til position 272 i aminosyresekvensen i SEQ ID NO:2, indeholder L-leucin,

35 eller idet polypeptidet omfatter den aminosyresekvens i SEQ ID NO: 2, hvor der på position 272 er indeholdt L-leucin,

b) og beriger L-aminosyren i fermentationsmassen eller i bakteriernes celler.

19. Fremgangsmåde ifølge krav 18, kendetegnet ved, det i forbindelse med den isolerede coryneforme bakterie drejer sig om en mutant ifølge krav 1 til 6 eller om en rekombinant coryneform bakterie, som indeholder et isoleret polynukleotid ifølge krav 7 til 12, eller som fremstilledes under anvendelsen heraf, eller at man isolerer eller samler, fortrinsvis oprenser L-aminosyren.

20. Fremgangsmåde ifølge krav 18, kendetegnet ved, at man isolerer eller samler L-lysin sammen med bestanddele fra fermentationsmassen og/eller biomassen (> 0 til 100 %), eller at man

a) fra den i trin b) i krav 18 opnåede fermentationsmasse fjerner den dannede biomasse i en mængde på 0 til 100 % og
b) fra den i trin a) opnåede masse fremstiller et i det væsentlige tørt og formet produkt ved hjælp af en fremgangsmåde udvalgt af gruppen granulering, kompaktering, spraytørring og ekstrudering.

21. Fremgangsmåde ifølge krav 20, kendetegnet ved, at man til fermentationsmassen før eller efter trin a) tilsætter en syre udvalgt af gruppen svovlsyre, saltsyre og phosphorsyre, eller at man fra den opnåede masse før eller i trin a) fjerner vand, eller at man påsprøjter det i eller under trin b) opnåede formede produkt med en olie.

22. Fremgangsmåde ifølge krav 18, kendetegnet ved, at man gennemfører følgende trin

a) filtrering af fermentationsmassen, fortrinsvis med et membranfilter, således at der opnås et biomasseholdigt slam og et filtrat,

b) opkoncentration af filtratet, fortrinsvis således at der opnås et faststofindhold på 48 til 52 vægt-%,

c) granulering af det i trin b) opnåede koncentrat, fortrinsvis ved en temperatur på 50 °C til 62 °C og

d) belægning af det i c) opnåede granulat med et eller flere belægningsmidler (coating agent(s)).

Figure 1: Plasmid pK18mobsacB_prpD1_P272L

