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(54) Title: PROCESS FOR THE PRODUCTION OF L-AMINO ACIDS USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY WHICH CONTAIN AN ENHANCE YFIDD ORF AND/OR PFLD GENE

(57) Abstract: The invention relates to a process for the production of L-amino acids by fermentation of recombinant microorganisms of the Enterobacteriaceae family, wherein a) the microorganisms producing the desired L-amino acid, in which the yfiD ORF and/or the pflB gene or nucleotide sequences coding for the gene products are overexpressed, are cultured in a medium under conditions in which the desired L-amino acid in the medium or in the cells is enriched, and b) the desired L-amino acid is isolated, whereby optionally constituents of the fermentation broth and/or the biomass in its entirety or in portions (> 0 to 100 %) remain in the isolated product or are completely removed.

PROCESS FOR THE PRODUCTION OF L-AMINOACIDS USING STRAINS OF THE ENTEROBACTERIACEA E FAMILY WHICH CONTAIN AN ENHANCE YFIDD ORF AND/OR PFLD GENE

Field of the Invention

This invention relates to a process for fermentative production of L-amino acids, in particular L-threonine, using strains of the Enterobacteriaceae family in which the open reading frame (ORF) having the designation yfiD and/or the pflB gene is/are enhanced.

Background of the Invention

L-amino acids, in particular L-threonine, find application in human medicine and in the pharmaceutical industry, in the food industry and, quite especially, in animal nutrition.

It is known that L-amino acids are produced by fermentation of strains of Enterobacteriaceae, in particular Escherichia coli (E. coli) and Serratia marcescens. On account of its great importance, work on improving the production process is constantly in progress. Process improvements may concern measures pertaining to fermentation technology, such as, for example, stirring and provision with oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during fermentation, or the processing into product-form, by ion-exchange chromatography for example, or the intrinsic output properties of the microorganism itself.

With a view to improving the output properties of these microorganisms, methods of mutagenesis, selection and mutant selection are adopted. In this way, strains are obtained that are resistant to antimetabolites such as, for example, the threonine analogue α-amino-β-hydroxyvaleric acid (AHV) or auxotrophic with respect to metabolites of regulatory significance and that produce L-amino acids such as L-threonine, for example.

Methods of recombinant DNA technology have also been employed for a number of years for strain improvement of L-amino-acid-producing strains of the Enterobacteriaceae family, by individual amino-acid-biosynthesis genes being amplified and by the effect on production being investigated. Summary information on the cellular and molecular biology of Escherichia coli and Salmonella can be found in Neidhardt (ed.): Escherichia coli and Salmonella, Cellular and Molecular Biology, 2nd edition, ASM Press, Washington, D.C., USA, (1996).

Object of the Invention

The inventors have set themselves the task of making available novel measures for improved fermentative production of L-amino acids, in particular L-threonine.

Summary of the Invention

The invention provides a process for production of L-amino acids, in particular L-threonine, using microorganisms of the Enterobacteriaceae family which, in particular, already produce L-amino acids and in which at least the open reading frame yfiD and/or the pflB gene, or nucleotide sequence(s) or alleles coding for the gene product thereof, is/are enhanced, in particular overexpressed.

Detailed Description of the Invention

When L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, selected from the group comprising L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-threonine is particularly preferred.

The term 'enhancement' in this context describes the increase in the intracellular activity or concentration of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, by, for example, the number of copies of the gene or genes, of the ORF or ORFs, being increased by at least one (1) copy, by use being made of a strong promoter or a gene or allele that codes for a corresponding enzyme or protein with a high activity, and by optionally combining these measures.

The expression 'open reading frame' (ORF) designates a segment of a nucleotide sequence that codes for or can code for a protein or, to be more exact, a polypeptide or ribonucleic acid, to which, according to the state of the art, no function can be assigned. After assignment of a function to the segment of the nucleotide sequence in question, one generally speaks of a gene. The term 'alleles' is generally understood to mean alternative forms of a given gene. The forms are distinguished by differences in the nucleotide sequence.

The expression 'gene product' designates, in general, the protein coded by a nucleotide sequence, i.e. an ORF, a gene or an allele, or the coded ribonucleic acid.

By the measures of enhancement, in particular overexpression, the activity or concentration of the corresponding protein is generally increased by at least 10 %, 25 %, 50 %, 75 %, 100 %, 150 %, 200 %, 300 %, 400 % or 500 %, up to a maximum of 1000 % or 2000 %, relative to that of the wild-type protein or, to be more exact, the activity or concentration of the protein in the initial microorganism. The expression 'initial microorganism' or 'parent strain' is understood to mean the microorganism in respect of which the measures according to the invention are carried out.

The invention provides a process for the production of L-amino acids by fermentation of recombinant microorganisms of the Enterobacteriaceae family, characterized in that

a) the microorganisms producing the desired L-amino-acid, in which the yfiD ORF and/or the pflB gene or nucleotide sequences or alleles coding for the gene products are enhanced, in particular overexpressed, are cultured in a medium under conditions in which the desired L-amino acid in the medium or in the cells is enriched, and

b) the desired L-amino acid is isolated, whereby optionally constituents of the fermentation broth and/or the biomass in its entirety or in portions (> 0 to 100 %) remain in the isolated product or are completely removed.

The microorganisms, in particular recombinant microorganisms, which are also provided by the present invention, are able to produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, in appropriate circumstances starch, in appropriate circumstances cellulose, or from glycerin and ethanol. Such microorganisms are representatives of the Enterobacteriaceae family, selected from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. In the case of the genus Escherichia, in particular the species Escherichia coli, and in the case of the genus Serratia, in particular the species Serratia marcescens, should be mentioned.

In general, recombinant microorganisms are generated by transformation, transduction or conjugation with a vector carrying the desired gene.

Suitable strains of the genus Escherichia, in particular of the species Escherichia coli, which in particular produce L-threonine, are, for example:

- Escherichia coli H4581 (EP 0 301 572)
- Escherichia coli KY10935 (Bioscience Biotechnology and Biochemistry 61(11):1877-1882 (1997)
- Escherichia coli VNIIgenetika MG442 (US-A-4278,765)
- Escherichia coli VNIIgenetika M1 (US-A-4,321,325)
- Escherichia coli VNIIgenetika 472T23 (US-A-5,631,157)
- Escherichia coli BKIIM B-3996 (US-A-5,175,107)
- Escherichia coli kat 13 (WO 98/04715)
- Escherichia coli KCCM-10132 (WO 00/09660)

Suitable L-threonine-producing strains of the genus Serratia, in particular of the species Serratia marcescens, are, for example:

- Serratia marcescens HNr21 (Applied and Environmental Microbiology 38(6): 1045-1051 (1979))
- Serratia marcescens TLr156 (Gene 57(2-3): 151-158 (1987))
- Serratia marcescens T-2000 (Applied Biochemistry and Biotechnology 37(3): 255-265 (1992))

L-threonine-producing strains from the Enterobacteriaceae family preferably possess, inter alia, one or more of the genetic or phenotypic features selected from the group comprising: resistance to α-amino-β-hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to α-methylserine, resistance to diaminosuccinic acid, resistance to α-aminobutyric acid, resistance to borrelidin, resistance to cyclopentanecarboxylic acid, resistance to rifampicin, resistance to valine analogues such as, for example, valine hydroxamate, resistance to purine analogues such as, for example, 6-

dimethylaminopurine, need for L-methionine, in appropriate circumstances partial and compensable need for L-isoleucine, need for meso-diaminopimelic acid, auxotrophy with respect to threonine-containing dipeptides, resistance to L-threonine, resistance to threonine raffinate, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid, resistance to L-aspartate, resistance to L-leucine, resistance to L-phenylalanine, resistance to L-serine, resistance to L-cysteine, resistance to L-valine, sensitivity to fluoropyruvate, defective threonine dehydrogenase, in appropriate circumstances ability to utilize sucrose, enhancement of the threonine operon, enhancement of homoserine dehydrogenase I-aspartate kinase I, preferably of the feedback-resistant form, enhancement of homoserine kinase, enhancement of threonine synthase, enhancement of aspartate kinase, optionally of the feedback-resistant form, enhancement of aspartate semialdehyde dehydrogenase, enhancement of phosphoenolpyruvate carboxylase, optionally of the feedback-resistant form, enhancement of phosphoenolpyruvate synthase, enhancement of transhydrogenase, enhancement of the RhtB gene product, enhancement of the RhtC gene product, enhancement of the YfiK gene product, enhancement of a pyruvate carboxylase, and attenuation of the formation of acetic acid.

It has been found that, after enhancement, in particular overexpression, of the open reading frame yfiD and/or of the pflB gene or nucleotide sequence(s) or alleles coding for the corresponding gene products, microorganisms of the Enterobacteriaceae family produce L-amino acids, in particular L-threonine, in improved manner.

The nucleotide sequences of the genes or open reading frames (ORFs) of Escherichia coli pertain to the state of the art and can be gathered from the genome sequence of

Escherichia coli published by Blattner et al. (Science 277: 1453-1462 (1997)).

The open reading frame yfiD and the protein coded by this ORF are described, inter alia, by the following data:

Designation: open reading frame
Function: putative formate acetyl transferase
Description: the open reading frame yfiD codes for a 14.3 kDa protein, the isoelectric point is situated at 5.1; localized chromosomally, it is situated, for example in the case of Escherichia coli K12 MG1655, in the intergenic region of the open reading frame yfiK, coding for a putative L-aspartate oxidase, and the ung gene, coding for uracil DNA glycosylase
Reference: Blankenhorn et al.; Journal of Bacteriology 181(7): 2209-2216 (1999)
Fountoulakis et al.; Electrophoresis 20(11): 2181-2195 (1999)
Kirkpatrick et al.; Journal of Bacteriology 183(21): 6466-6477 (2001)
Wyborn et al.; Microbiology 148: 1015-1026 (2002)
Accession No.: AE000344

The pflB gene and the protein coded by this gene are described, inter alia, by the following data:

Designation: formate acetyl transferase I, pyruvate formate lyase I
EC No.: 2.3.1.54
Reference: Rodel et al.; European Journal of Biochemistry 177(1): 153-158 (1988)
Wagner et al.; Proceedings of the National Academy of Sciences of the United States of America 89(3): 996-1000 (1992)

Accession No.: AE000192
Alternative gene name: pfl

The pyruvate formate lyase from Salmonella typhimurium is described, inter alia, in the following reference: Wong et al.; Journal of Bacteriology 171(9): 4900-4905 (1989).

The nucleic-acid sequences can be gathered from the databases of the National Center for Biotechnology Information (NCBI) of the National Library of Medicine (Bethesda, MD, USA), from the Nucleotide Sequence Database of the European Molecular Biology Laboratory (EMBL, Heidelberg, Germany, and Cambridge, UK) or from the DNA Data Bank of Japan (DDBJ, Mishima, Japan).

For the sake of better clarity, the known sequence relating to the yfiD ORF is represented under SEQ ID NO. 3. The protein coded by this reading frame is represented as SEQ ID No. 4.

The open reading frames described in the specified passages can be used in accordance with the invention. Moreover, use may be made of alleles of the genes or open reading frames that result from the degeneracy of the genetic code or by virtue of functionally neutral sense mutations. The use of endogenous genes or of endogenous open reading frames is preferred.

The expression 'endogenous genes' or 'endogenous nucleotide sequences' is understood to mean the genes or open reading frames or alleles or, to be more exact, nucleotide sequences that are present in the population of a species.

The alleles that contain functionally neutral sense mutations include, inter alia, those which result in at least one (1) conservative amino-acid exchange in the protein coded by them.

In the case of the aromatic amino acids, one speaks of conservative exchanges if phenylalanine, tryptophan and tyrosine are exchanged for one another. In the case of the hydrophobic amino acids, one speaks of conservative exchanges if leucine, isoleucine and valine are exchanged for one another. In the case of the polar amino acids, one speaks of conservative exchanges if glutamine and aparagine are exchanged for one another. In the case of the basic amino acids, one speaks of conservative exchanges if arginine, lysine and histidine are exchanged for one another. In the case of the acidic amino acids, one speaks of conservative exchanges if aspartic acid and glutamic acid are exchanged for one another. In the case of the amino acids containing hydroxyl groups, one speaks of conservative exchanges if serine and threonine are exchanged for one another.

In like manner, those nucleotide sequences can also be used which code for variants of the stated proteins which additionally contain at the N-terminus or C-terminus a lengthening or shortening by at least one (1) amino acid. This lengthening or shortening amounts to not more than 50, 40, 30, 20, 10, 5, 3 or 2 amino acids or amino-acid residues.

The suitable alleles also include those which code for proteins in which at least one (1) amino acid is inserted or deleted. The maximum number of such changes, which are designated as indels, may concern 2, 3, 5, 10, 20 but in no case more than 30 amino acids.

The suitable alleles include, furthermore, those which can be obtained by hybridization, in particular under stringent conditions using SEQ ID No. 3 or SEQ ID No. 7 or parts thereof, in particular the coding regions or the sequences complementary thereto.

Instructions on the identification of DNA sequences by means of hybridization can be found by a person skilled in the art, inter alia, in the manual entitled "The DIG System Users Guide for Filter Hybridization" produced by 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 - that is to say, only hybrids are formed in which the probe and the target sequence, i.e. the polynucleotides treated with the probe, are at least 70 % identical. It is known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the composition of the buffer, the temperature, and the salt concentration. The hybridization reaction is generally carried out with relatively low stringency in comparison with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

For the Hybridisation reaction, a buffer corresponding to 5x SSC buffer at a temperature of about 50 °C - 68 °C can be employed, for example. In this connection, probes can also hybridize with polynucleotides that exhibit less than 70 % identity to the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be attained, for example, by lowering the salt concentration to 2x SSC and optionally subsequently to 0.5x SSC (The DIG System User´s Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995), with a temperature of about 50 °C - 68 °C, about 52 °C - 68 °C, about 54 °C - 68 °C, about 56 °C - 68 °C, about 58 °C - 68 °C, about 60 °C - 68 °C, about 62 °C - 68 °C, about 64 °C - 68 °C or about 66 °C - 68 °C being adjusted. It is optionally possible to lower the salt concentration to a concentration corresponding to 0.2x SSC or 0.1x SSC. By stepwise increase of the Hybridisation temperature in steps of about 1 - 2 °C from 50 °C to 68 °C,

polynucleotide fragments can be isolated which, for example, possess at least 70 % or at least 80 % or at least 90 % to 95 % or at least 96 % to 99 % identity to the sequence of the probe employed. Further instructions on Hybridisation are commercially obtainable in the form of so-called kits (e.g. DIG Easy Hyb produced by Roche Diagnostics GmbH, Mannheim, Germany, Catalogue No. 1603558).

With a view to achieving an enhancement, the expression of the genes or open reading frames or alleles can be increased, or the catalytic or regulatory properties (activity) of the proteins can be enhanced, for example. Both measures may optionally be combined.

With a view to achieving an overexpression, the number of copies of the corresponding genes or open reading frames can be increased, for example, or the promoter and regulation region or the ribosome binding site, which is located upstream of the structural gene, can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in like manner. By means of inducible promoters it is additionally possible to heighten the expression in the course of fermentative production of L-threonine. By virtue of measures for prolonging the lifespan of mRNA, the expression is likewise improved. Moreover, by preventing the degradation of the enzyme protein the enzyme activity is likewise enhanced. The genes or gene constructs may either be present in extrachromosomally replicating plasmids with a different number of copies or may be integrated within the chromosome and amplified. Alternatively an overexpression of the genes in question can furthermore be obtained by changing the composition of the media and by culture management.

Instructions on this can be found by a person skilled in the art, inter alia, in Chang and Cohen (Journal of Bacteriology 134: 1141-1156 (1978)), in Hartley and Gregori

(Gene 13: 347-353 (1981)), in Amann and Brosius (Gene 40: 183-190 (1985)), in de Broer et al. (Proceedings of the National Academy of Sciences of the United States of America 80: 21-25 (1983)), in LaVallie et al. (BIO/TECHNOLOGY 11: 187-193 (1993)), in WO98/04715, in Llosa et al. (Plasmid 26: 222-224 (1991)), in Quandt and Klipp (Gene 80: 161-169 (1989)), in Hamilton et al. (Journal of Bacteriology 171: 4617-4622 (1989)), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)) and in known textbooks on genetics and molecular biology.

Use may be made of plasmid vectors capable of replicating in Enterobacteriaceae, such as, for example, cloning vectors derived from pACYC184 (Bartolomé et al.; Gene 102: 75-78 (1991)), pTrc99A (Amann et al.; Gene 69: 301-315 (1988)) or pSC101 derivatives (Vocke and Bastia, Proceedings of the National Academy of Sciences USA 80(21): 6557-6561 (1983)). A strain transformed with a plasmid vector can be employed in a process according to the invention, in which case the plasmid vector carries at least the yfiD ORF and/or the pflB gene or nucleotide sequences or alleles coding for the gene products thereof.

By the term 'transformation' one understands the uptake of an isolated nucleic acid by a host (microorganism).

It is likewise possible to transfer mutations that relate to the expression of the respective genes or open reading frames into various strains by sequence exchange (Hamilton et al.; Journal of Bacteriology 171: 4617-4622 (1989)), conjugation or transduction.

Detailed explanations relating to the terms of genetics and molecular biology can be found in known textbooks on genetics and molecular biology, such as, for example, the textbook by Birge (Bacterial and Bacteriophage Genetics, 4th ed., Springer Verlag, New York (USA), 2000) or the

textbook by Berg, Tymoczko and Stryer (Biochemistry, 5th ed., Freeman and Company, New York (USA), 2002) or the textbook by Sambrook et al. (Molecular Cloning, A Laboratory Manual, (3-volume set), Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001).

Moreover, for the production of L-amino acids, in particular L-threonine, with strains of the Enterobacteriaceae family it may be advantageous, in addition to the enhancement of the yfiD ORF and/or of the pflB gene, to enhance one or more enzymes of the known threonine-biosynthesis pathway or enzymes of anaplerotic metabolism or enzymes for the production of reduced nicotinamide adenine dinucleotide phosphate or enzymes of glycolysis or PTS enzymes or enzymes of sulfur metabolism. The use of endogenous genes is generally preferred.

Thus, for example, simultaneously one or more of the genes selected from the group comprising

- the thrABC operon coding for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),
- the pyc gene of Corynebacterium glutamicum coding for pyruvate carboxylase (WO 99/18228),
- the pps gene coding for phosphoenolpyruvate synthase (Molecular and General Genetics 231(2): 332-336 (1992)),
- the ppc gene coding for phosphoenolpyruvate carboxylase (Gene 31: 279-283 (1984)),
- the genes pntA and pntB coding for transhydrogenase (European Journal of Biochemistry 158: 647-653 (1986)),
- the gene rhtB imparting homoserine resistance (EP-A-0 994 190),

- the mqo gene coding for malate:quinine oxidoreductase (WO 02/06459),
- the rhtC gene imparting threonine resistance (EP-A-1 013 765),
- thrE gene of Corynebacterium glutamicum coding for the threonine-export protein (WO 01/92545),
- the gdhA gene coding for glutamate dehydrogenase (Nucleic Acids Research 11: 5257-5266 (1983); Gene 23: 199-209 (1983)),
- the hns gene coding for the DNA binding protein HLP-II (WO 03/004671),
- the pgm gene coding for phosphoglucomutase (WO 03/004598),
- the fba gene coding for fructose biphosphate aldolase (WO 03/004664),
- the ptsH gene of the ptsHIcrr operon coding for the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system PTS (WO 03/004674),
- the ptsI gene of the ptsHIcrr operon coding for enzyme I of the phosphotransferase system PTS (WO 03/004674),
- the crr gene of the ptsHIcrr operon coding for the glucose-specific IIA component of the phosphotransferase system PTS (WO 03/004674),
- the ptsG gene coding for the glucose-specific IIBC component (WO 03/004670),
- the lrp gene coding for the regulator of the leucine regulon (WO 03/004665),
- the csrA gene coding for the global regulator Csr (Journal of Bacteriology 175: 4744-4755 (1993)),

- the fadR gene coding for the regulator of the fad regulon (Nucleic Acids Research 16: 7995-8009 (1988)),
- the iclR gene coding for the regulator of central intermediary metabolism (Journal of Bacteriology 172: 2642-2649 (1990)),
- the mopB gene coding for the 10 kDa chaperon (WO 03/004669), which is also known under the designation 'groES',
- the ahpC gene of the ahpCF operon coding for the small subunit of alkyl hydroperoxide reductase (WO 03/004663),
- the ahpF gene of the ahpCF operon coding for the large subunit of alkyl hydroperoxide reductase (WO 03/004663),
- the cysK gene coding for cysteine synthase A (WO 03/006666),
- the cysB gene coding for the regulator of the cys regulon (WO 03/006666),
- the cysJ gene of the cysJIH operon coding for the flavoprotein of NADPH sulfite reductase (WO 03/006666),
- the cysI gene of the cysJIH operon coding for the haemoprotein of NADPH sulfite reductase (WO 03/006666),
- the cysH gene of the cysJIH operon coding for adenylyl sulfate reductase (WO 03/006666),
- the phoB gene of the phoBR operon coding for the positive regulator PhoB of the pho regulon (WO 03/008606),
- the phoR gene of the phoBR operon coding for the sensor protein of the pho regulon (WO 03/008606),
- the phoE gene coding for protein E of the outer cell membrane (WO 03/008608),

- the pykF gene coding for pyruvate kinase I which is stimulated by fructose (WO 03/008609),
- the pfkB gene coding for 6-phosphofructokinase II (WO 03/008610),
- the malE gene coding for the periplasmic binding protein of maltose transport (WO 03/008605),
- the sodA gene coding for superoxide dismutase (WO 03/008613),
- the rseA gene of the rseABC operon coding for a membrane protein with anti-sigmaE activity (WO 03/008612),
- the rseC gene of the rseABC operon coding for a global regulator of the sigmaE factors (WO 03/008612),
- the sucA gene of the sucABCD operon coding for the decarboxylase subunit of 2-ketoglutarate dehydrogenase (WO 03/008614),
- the sucB gene of the sucABCD operon coding for the dihydrolipoyl transsuccinase E2 subunit of 2-ketoglutarate dehydrogenase (WO 03/008614),
- the sucC gene of the suc ABCD operon coding for the β-subunit of succinyl-CoA synthetase (WO 03/008615),
- the sucD gene of the sucABCD operon coding for the α-subunit of succinyl-CoA synthetase (WO 03/008615),
- the adk gene coding for adenylate kinase (Nucleic Acids Research 13(19): 7139-7151 (1985)),
- the hdeA gene coding for a periplasmic protein with chaperonin-type function (Journal of Bacteriology 175(23): 7747-7748 (1993)),

- the hdeB gene coding for a periplasmic protein with chaperonin-type function (Journal of Bacteriology 175(23): 7747-7748 (1993)),
- the icd gene coding for isocitrate dehydrogenase (Journal of Biological Chemistry 262(22): 10422-10425 (1987)),
- the mglB gene coding for the periplasmic, galactose-binding transport protein (Molecular and General Genetics 229(3): 453-459 (1991)),
- the lpd gene coding for dihydrolipoamide dehydrogenase (European Journal of Biochemistry 135(3): 519-527 (1983)),
- the aceE gene coding for the E1 component of the pyruvate-dehydrogenase complex (European Journal of Biochemistry 133(1): 155-162 (1983)),
- the aceF gene coding for the E2 component of the pyruvate-dehydrogenase complex (European Journal of Biochemistry 133(3): 481-489 (1983)),
- the pepB gene coding for aminopeptidase B (Journal of Fermentation and Bioengineering 82: 392-397 (1996)),
- the aldH gene coding for aldehyde dehydrogenase (E.C. 1.2.1.3) (Gene 99(1): 15-23 (1991)),
- the bfr gene coding for the iron-storage homoprotein (bacterioferritin) (Journal of Bacteriology 171(7): 3940-3947 (1989)),
- the udp gene coding for uridine phosphorylase (Nucleic Acids Research 17(16): 6741 (1989)) and
- the rseB gene coding for the regulator of sigmaE-factor activity (Molecular Microbiology 24(2): 355-371 (1997))

are enhanced, in particular overexpressed.

Moreover, for the production of L-amino acids, in particular L-threonine, it can be advantageous, in addition to the enhancement of the yfiD ORF and/or of the pflB gene, to attenuate, in particular to eliminate or to diminish the expression of, one or more of the genes selected from the group comprising

- the tdh gene coding for threonine dehydrogenase (Journal of Bacteriology 169: 4716-4721 (1987)),
- the mdh gene coding for malate dehydrogenase (E.C. 1.1.1.37) (Archives in Microbiology 149: 36-42 (1987)),
- the gene product of the open reading frame (ORF) yjfA (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) WO 02/29080),
- the gene product of the open reading frame (ORF) ytfP (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)), WO 02/29080),
- the pckA gene coding for the enzyme phosphoenolpyruvate carboxykinase (WO 02/29080),
- the poxB gene coding for pyruvate oxidase (WO 02/36797),
- the aceA gene coding for the enzyme isocitrate lyase (WO 02/081722),
- the dgsA gene coding for the DgsA regulator of the phosphotransferase system (WO 02/081721), which is also known under the designation 'mlc gene',
- the fruR gene coding for the fructose repressor (WO 02/081698), which is also known under the designation 'cra gene',

- the rpoS gene coding for the sigma^{38} factor (WO 01/05939), which is also known under the designation 'katF gene',

- the aspA gene coding for aspartate ammonium lyase (WO 03/008603) and

- the aceB gene coding for malate synthase A (WO 03/008604).

The term 'attenuation' in this context describes the diminution or elimination of the intracellular activity or concentration of one or more enzymes or proteins in a microorganism that is/are coded by the corresponding DNA, by, for example, use being made of a weak promoter or a gene or allele that codes for a corresponding enzyme or protein with a low activity or that inactivates the corresponding enzyme or protein or the open reading frame or the gene, and by optionally combining these measures.

By the measures of attenuation, the activity or concentration of the corresponding protein is generally lowered 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, to be more exact, of the activity or concentration of the protein in the initial microorganism.

Moreover, for the production of L-amino acids, in particular L-threonine, it can be advantageous, in addition to the enhancement of the yfiD ORF and/or of the pflB gene, to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced in accordance with the invention can be cultured in the batch process, in the fed-batch process or in the repeated fed-batch process. A summary of known cultivation methods is described in the

textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren and periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium that is to be used has to satisfy the demands of the respective strains in suitable manner. Descriptions of culture media of various microorganisms are contained in the manual entitled "Manual of Methods for General Bacteriology" produced by The American Society for Bacteriology (Washington D.C., USA, 1981).

By way of carbon source, use may be made of sugar and carbohydrates such as, for example, glucose, sucrose, lactose, fructose, maltose, molasses, starch and, in appropriate circumstances, cellulose, oils and fats such as, for example, soybean oil, sunflower oil, peanut oil and copra oil, fatty acids such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols such as, for example, glycerin and ethanol, and organic acids such as, for example, acetic acid. These substances may be used individually or in the form of a mixture.

By way of nitrogen source, use may be made of organic nitrogenous compounds such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soybean flour and urea or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources may be used individually or in the form of a mixture.

By way of phosphorus source, use may be made of phosphoric acid, potassium dihydrogenphosphate or dipotassium hydrogenphosphate or the corresponding sodium-containing salts. The culture medium must furthermore contain salts of metals, such as, for example, magnesium sulfate or iron sulfate, that are necessary for growth. Finally, essential

growth-regulating substances such as amino acids and vitamins may be employed in addition to the aforementioned substances. Suitable precursors may, moreover, be added to the culture medium. The stated feed materials may be added to the culture in the form of a single batch or may be fed in during the cultivation in suitable manner.

The fermentation is generally carried out at a pH value from 5.5 to 9.0, in particular 6.0 to 8.0. For the purpose of controlling the pH of the culture, basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or ammoniacal liquor or acidic compounds such as phosphoric acid or sulfuric acid are employed in suitable manner. For the purpose of controlling the evolution of foam, anti-foaming agents such as, for example, fatty-acid polyglycol esters may be employed. For the purpose of maintaining the stability of plasmids, suitable substances acting selectively, for example antibiotics, may be added to the medium. In order to maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as air for example, are introduced into the culture. The temperature of the culture is normally around 25 °C to 45 °C and preferably around 30 °C to 40 °C. The culture is carried on until such time as a maximum of L-amino acids or, to be more exact, of L-threonine has formed. This objective is normally attained within 10 hours to 160 hours.

The analysis of L-amino acids can be undertaken by anion-exchange chromatography with subsequent ninhydrin derivation, as described in Spackman et al. (Analytical Chemistry 30: 1190-1206 (1958)), or it can be undertaken by reversed phase HPLC, as described in Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

The process according to the invention serves for fermentative production of L-amino acids, such as, for example, L-threonine, L-isoleucine, L-valine, L-methionine, L-homoserine and L-lysine, in particular L-threonine.

The present invention will be elucidated in more detail in the following on the basis of exemplary embodiments.

Minimal media (M9) and complete media (LB) that are used for Escherichia coli are described by J.H. Miller (A short course in bacterial genetics (1992), Cold Spring Harbor Laboratory Press). The isolation of plasmid DNA from Escherichia coli and also all techniques relating to restriction, ligation, Klenow treatment and alkaline phosphatase treatment are carried out in accordance with Sambrook et al. (Molecular Cloning - A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press). The transformation of Escherichia coli is carried out, unless described otherwise, in accordance with Chung et al. (Proceedings of the National Academy of Sciences of the United States of America 86: 2172-2175 (1989)).

The incubation temperature in the course of the production of strains and transformants is 37 °C.

Example 1

1.1 Construction of the expression plasmid pTrc99AyfiD

The open reading frame yfiD from E. coli K12 is amplified by using the polymerase chain reaction (PCR) and also synthetic oligonucleotides. Starting from the nucleotide sequence of the open reading frame yfiD in E. coli K12 MG1655 (Accession Number AE000344, Blattner et al. (Science 277: 1453-1474 (1997)), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany). The primers contain sequences for restriction enzymes, which are marked by underlining in the nucleotide sequence represented below. The primer yfiD1 contains the restriction site for XbaI; the primer yfiD2 contains the restriction site for HindIII.

yfiD1:

5' - GAACAAATCTAGAAATTAAGCCGGGGAGGC -3' (SEQ ID No. 1)

yfiD2:

5' - GCTACTTAAGCTTTACAGGCTTTC - 3' (SEQ ID No. 2)

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated in accordance with the manufacturer's directions using "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment with a size of about 431 bp can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A guide to methods and applications, Academic Press) with Vent-DNA-Polymerase (New England Biolabs, Frankfurt, Germany) (SEQ ID No. 3).

The PCR product is restricted with the restriction enzymes HindIII and XbaI and examined in a 0.8 % agarose gel after being cleaned up (Purification Kit, QIAGEN, Hilden, Germany). The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzymes HindIII and XbaI, and ligated with the restricted yfiD fragment. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation batch, and plasmid-bearing cells are selected on LB agar to which 50 µg/ml ampicillin have been added. The successful cloning can be demonstrated after the isolation of plasmid DNA by control cleavage with the enzymes HindIII/XbaI and HpaI. The plasmid is designated as pTrc99AyfiD (Figure 1).

1.2 Construction of the expression plasmid pTrc99ApflB

The pflB gene from E. coli K12 is amplified by using the polymerase chain reaction (PCR) and also synthetic oligonucleotides. Starting from the nucleotide sequence of the pflB gene in E. coli K12 MG1655 (Accession Number AE000192, Blattner et al. (Science 277: 1453-1474 (1997)), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany). The primers contain sequences for restriction enzymes, which are marked by underlining in the nucleotide sequence represented below. The primer pflB1 contains the

restriction site for XbaI; the primer pflB2 contains the restriction site for HindIII.

pflB1:
5' - CCACTCTAGAAGGTAGGTGTTACATGTC -3' (SEQ ID No. 5)

pflB2:
5' - CGATTTCAGTCAAAGCTTATTACATAG - 3' (SEQ ID No. 6)

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated in accordance with the manufacturer's directions using "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment with a size of about 2325 bp can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A guide to methods and applications, Academic Press) with Vent-DNA-Polymerase (New England Biolabs, Frankfurt, Germany) (SEQ ID No. 7).

The PCR product is restricted with the restriction enzymes HindIII and XbaI and examined in a 0.8 % agarose gel after being cleaned up (Purification Kit, QIAGEN, Hilden, Germany). The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzymes HindIII and XbaI, and ligated with the restricted pflB fragment. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation batch, and plasmid-bearing cells are selected on LB agar to which 50 µg/ml ampicillin have been added. The successful cloning can be demonstrated after the isolation of plasmid DNA by control cleavage with the enzymes HindIII/XbaI and PauI. The plasmid is designated as pTrc99ApflB (Figure 2).

Example 2

2.1 Production of L-threonine with the strain MG442/pTrc99AyfiD

The L-threonine-producing E. coli strain MG442 is described in patent specification US-A-4,278,765 and deposited at the Russian National Collection of Industrial Microorganisms (VKPM, Moscow, Russia) as CMIM B-1628.

The strain MG442 is transformed with the expression plasmid pTrc99AyfiD described in Example 1.1 and with the vector pTrc99A, and plasmid-bearing cells are selected on LB agar with 50 µg/ml ampicillin. In this way, the strains MG442/pTrc99AyfiD and MG442/pTrc99A arise. Selected single colonies are subsequently multiplied further on minimal medium having the following composition: 3.5 g/l $Na_2HPO_4*2H_2O$, 1.5 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.1 g/l $MgSO_4*7H_2O$, 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is examined in batch cultures of 10 ml, which are contained in 100 ml Erlenmeyer flasks. To this end, 10 ml of preculture medium having the following composition: 2 g/l yeast extract, 10 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4*7H_2O$, 15 g/l $CaCO_3$, 20 g/l glucose, 50 mg/l ampicillin, are inoculated and incubated for 16 hours at 37 °C and at 180 rpm in an ESR incubator manufactured by Kühner AG (Birsfelden, Switzerland). 250 µl at a time of this preculture are inoculated into 10 ml of production medium (25 g/l $(NH_4)_2SO_4$, 2 g/l KH_2PO_4, 1 g/l $MgSO_4*7H_2O$, 0.03 g/l $FeSO_4*7H_2O$, 0.018 g/l $MnSO_4*1H_2O$, 30 g/l $CaCO_3$, 20 g/l glucose, 50 mg/l ampicillin) and incubated for 48 hours at 37 °C. The formation of L-threonine by the initial strain MG442 is examined in the same way, there being, however, no addition of ampicillin to the medium. After the incubation, the optical density (OD) of the culture suspension is determined at a measuring wavelength of

660 nm with an LP2W photometer manufactured by Dr. Lange (Düsseldorf, Germany).

Subsequently the concentration of L-threonine which has formed is determined in the sterile-filtered culture supernatant with an amino-acid analyzer manufactured by Eppendorf-BioTronik (Hamburg, Germany), by ion-exchange chromatography and post-column reaction with detection of ninhydrin.

The result of the experiment is presented in Table 1.

Table 1

Strain	OD (660 nm)	L-threonine g/l
MG442	5.6	1.4
MG442/pTrc99A	3.8	1.3
MG442/pTrc99AyfiD	5.5	2.5

2.1 Production of L-threonine with the strain MG442/pTrc99ApflB

The L-threonine-producing E. coli strain MG442 is described in patent specification US-A-4,278,765 and deposited at the Russian National Collection of Industrial Microorganisms (VKPM, Moscow, Russia) as CMIM B-1628.

The strain MG442 is transformed with the expression plasmid pTrc99ApflB described in Example 1.2 and with the vector pTrc99A, and plasmid-bearing cells are selected on LB agar with 50 µg/ml ampicillin. In this way, the strains MG442/pTrc99ApflB and MG442/pTrc99A arise. Selected single colonies are subsequently multiplied further on minimal medium having the following composition: 3.5 g/l

$Na_2HPO_4*2H_2O$, 1.5 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.1 g/l $MgSO_4*7H_2O$, 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is examined in batch cultures of 10 ml, which are contained in 100 ml Erlenmeyer flasks. To this end, 10 ml of preculture medium having the following composition: 2 g/l yeast extract, 10 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4*7H_2O$, 15 g/l $CaCO_3$, 20 g/l glucose, 50 mg/l ampicillin, are inoculated and incubated for 16 hours at 37 °C and 180 rpm in an ESR incubator manufactured by Kühner AG (Birsfelden, Switzerland). 250 µl at a time of this preculture are inoculated into 10 ml of production medium (25 g/l $(NH_4)_2SO_4$, 2 g/l KH_2PO_4, 1 g/l $MgSO_4*7H_2O$, 0.03 g/l $FeSO_4*7H_2O$, 0.018 g/l $MnSO_4*1H_2O$, 30 g/l $CaCO_3$, 20 g/l glucose, 50 mg/l ampicillin) and incubated for 48 hours at 37 °C. With a view to complete induction of the expression of the pflB gene, 100 mg/l isopropyl-β-D-thiogalactopyranoside (IPTG) are added in parallel batches. The formation of L-threonine by the initial strain MG442 is examined in the same way, there being, however, no addition of ampicillin to the medium. After the incubation, the optical density (OD) of the culture suspension is determined at a measuring wavelength of 660 nm with an LP2W photometer manufactured by Dr. Lange (Düsseldorf, Germany).

Subsequently the concentration of L-threonine which has formed is determined in the sterile-filtered culture supernatant with an amino-acid analyzer manufactured by Eppendorf-BioTronik (Hamburg, Germany), by ion-exchange chromatography and post-column reaction with detection of ninhydrin.

The result of the experiment is presented in Table 2.

Table 2

Strain	Additives	OD (660 nm)	L-threonine g/l
MG442	-	5.6	1.4
MG442/pTrc99A	-	3.8	1.3
MG442/pTrc99ApflB	-	5.6	1.9
MG442/pTrc99ApflB	IPTG	5.2	2.2

Brief Description of the Figures:

Length data are to be interpreted as approximate data. The abbreviations and designations that are used have the following significance:

- Amp: ampicillin-resistance gene
- lacI: gene for the repressor protein of the trc promoter
- Ptrc: trc promoter region, IPTG-inducible
- yfiD: coding region of the open reading frame yfiD
- pflB: coding region of the pflB gene
- 5S: 5S rRNA region
- rrnBT: rRNA terminator region

The abbreviations for the restriction enzymes have the following significance:

- HindIII: restriction endonuclease from Haemophilus influenze R_c

- HpaI: restriction endonuclease from Haemophilus parainfluenzae
- PauI: restriction endonuclease from Paracoccus alcaliphilus
- XbaI: restriction endonuclease from Xanthomonas campestris

What is claimed is:

1. A process for the production of L-amino acids by fermentation of recombinant microorganisms of the Enterobacteriaceae family, wherein

   a) the microorganisms producing the desired L-amino acid, in which the yfiD ORF and/or the pflB gene or nucleotide sequences coding for the gene products are overexpressed, are cultured in a medium under conditions in which the desired L-amino acid in the medium or in the cells is enriched, and

   b) the desired L-amino acid is isolated, whereby optionally constituents of the fermentation broth and/or the biomass in its entirety or in portions (> 0 to 100 %) remain in the isolated product or are completely removed.

2. Process according to Claim 1, wherein recombinant microorganisms are employed that are generated by the transformation of a microorganism of the Enterobacteriaceae family with a vector, said vector containing the yfiD ORF and/or the plfB gene.

3. Process according to Claim 1, wherein the number(s) of copies of the gene/genes and/or ORF in the recombinant microorganisms is/are increased by at least 1.

4. Process according to Claim 3, wherein the increase in the number of copies of the yfiD ORF and/or of the pflB gene by at least 1 is achieved by integration of the gene into the chromosome of the microorganism.

5. Process according to Claim 3, wherein the increase in the number of copies of the yfiD ORF and/or of the pflB gene by at least 1 is achieved by means of an extrachromosomally replicating vector.

6. Process according to Claim 1, wherein with a view to achieving the overexpression

   a) the promoter and regulation region or the ribosome binding site upstream of the yfiD ORF and/or of the pflB gene is mutated, or

   b) expression cassettes or promoters are incorporated upstream of the yfiD ORF and/or of the pflB gene.

7. Process according to Claim 1, wherein use is made of a yfiD ORF and/or a pflB gene that is under the control of a promoter.

8. Process according to Claim 1, wherein through the enhancement of the yfiD ORF and/or pflB gene the concentration or activity of the YfiD gene product and/or of the PflB gene product (protein) is increased by at least 10 %, relative to the activity or concentration of the gene product in the initial strain.

9. Process according to Claim 1, wherein microorganisms selected from the genera Escherichia, Erwinia, Providencia and Serratia are employed.

10. Process according to Claim 1, wherein microorganisms are employed in which, in addition, further genes of the biosynthetic pathway of the desired L-amino acid are enhanced, in particular overexpressed.

11. Process for the production of L-threonine according to Claim 10, wherein microorganisms of the Enterobacteriaceae family are fermented in which, in addition, simultaneously one or more of the genes selected from the group comprising:

   11.1 the thrABC operon coding for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,

11.2 the pyc gene coding for pyruvate carboxylase,

11.3 the pps gene for phosphoenolpyruvate synthase,

11.4 the ppc gene coding for phosphoenolpyruvate carboxylase,

11.5 the genes pntA and pntB coding for transhydrogenase,

11.6 the gene rhtB imparting homoserine resistance,

11.7 the mqo gene coding for malate:quinone oxidoreductase,

11.8 the gene rhtC imparting threonine resistance,

11.9 the thrE gene coding for the threonine-export protein,

11.10 the gdhA gene coding for glutamate dehydrogenase,

11.11 the hns gene coding for the DNA binding protein HLP-II,

11.12 the pgm gene coding for phosphoglucomutase,

11.13 the fba gene coding for fructose biphosphate aldolase,

11.14 the ptsH gene coding for phosphohistidine protein hexose phosphotransferase,

11.15 the ptsI gene coding for enzyme I of the phosphotransferase system,

11.16 the crr gene coding for the glucose-specific IIA component,

11.17 the ptsG gene coding for the glucose-specific IIBC component,

11.18 the lrp gene coding for the regulator of the leucine regulon,

11.19 the csrA gene coding for the global regulator Csr,

11.20 the fadR gene coding for the regulator of the fad regulon,

11.21 the iclR gene coding for the regulator of central intermediary metabolism,

11.22 the mopB gene coding for the 10 kDa chaperon,

11.23 the ahpC gene coding for the small subunit of alkyl hydroperoxide reductase,

11.24 the ahpF gene coding for the large subunit of alkyl hydroperoxide reductase,

11.25 the cysK gene coding for cysteine synthase A,

11.26 the cysB gene coding for the regulator of the cys regulon,

11.27 the cysJ gene coding for the flavoprotein of NADPH sulfite reductase,

11.28 the cysI gene coding for the haemoprotein of NADPH sulfite reductase,

11.29 the cysH gene coding for adenylyl sulfate reductase,

11.30 the phoB gene coding for the positive regulator PhoB of the pho regulon,

11.31 the phoR gene coding for the sensor protein of the pho regulon,

11.32 the phoE gene coding for protein E of the outer cell membrane,

11.33 the pykF gene coding for pyruvate kinase I, which is stimulated by fructose,

11.34 the pfkB gene coding for 6-phosphofructokinase II,

11.35 the malE gene coding for the periplasmic binding protein of maltose transport,

11.36 the sodA gene coding for superoxide dismutase,

11.37 the rseA gene coding for a membrane protein with anti-sigmaE activity,

11.38 the rseC gene coding for a global regulator of the sigmaE factor,

11.39 the sucA gene coding for the decarboxylase subunit of 2-ketoglutarate dehydrogenase,

11.40 the sucB gene coding for the dihydrolipoyl transsuccinase E2 subunit of 2-ketoglutarate dehydrogenase,

11.41 the sucC gene coding for the β-subunit of succinyl-CoA synthetase,

11.42 the sucD gene coding for the α-subunit of succinyl-CoA synthetase,

11.43 the adk gene coding for adenylate kinase,

11.44 the hdeA gene coding for a periplasmic protein with chaperonin-type function,

11.45 the hdeB gene coding for a periplasmic protein with chaperonin-type function,

11.46 the icd gene coding for isocitrate dehydrogenase,

11.47 the mglB gene coding for the periplasmic, galactose-binding transport protein,

11.48 the lpd gene coding for dihydrolipoamide dehydrogenase,

11.49 the aceE gene coding for the E1 component of the pyruvate-dehydrogenase complex,

11.50 the aceF gene coding for the E2 component of the pyruvate-dehydrogenase complex,

11.51 the pepB gene coding for aminopeptidase B,

11.52 the aldH gene coding for aldehyde dehydrogenase,

11.53 the bfr gene coding for the iron-storage homoprotein,

11.54 the udp gene coding for uridine phosphorylase and

11.55 the rseB gene coding for the regulator of sigmaE-factor activity

are enhanced, in particular overexpressed.

12. Process according to Claim 1, wherein microorganisms are employed in which the metabolic pathways that diminish the formation of the desired L-amino acid are at least partially eliminated.

13. Process for the production of L-threonine according to Claim 1, wherein microorganisms of the Enterobacteriaceae family are fermented in which, in addition, simultaneously one or more of the genes selected from the group comprising:

13.1 the tdh gene coding for threonine dehydrogenase,

13.2 the mdh gene coding for malate dehydrogenase,

13.3 the gene product of the open reading frame (ORF) yjfA,

13.4 the gene product of the open reading frame (ORF) ytfP,

13.5 the pckA gene coding for phosphoenolpyruvate carboxykinase,

13.6 the poxB gene coding for pyruvate oxidase,

13.7 the aceA gene coding for isocitrate lyase,

13.8 the dgsA gene coding for the DgsA regulator of the phosphotransferase system,

13.9 the fruR gene coding for the fructose repressor,

13.10 the rpoS gene coding for the sigma38 factor,

13.11 the aspA gene coding for aspartate ammonium lyase

13.12 the aceB gene coding for malate synthase A

is/are attenuated, in particular eliminated, or the expression thereof is diminished.

14. Microorganisms of the Enterobacteriaceae family, in particular of the genus Escherichia, in which the yfiD ORF and/or the pflB gene or nucleotide sequences coding for the gene product thereof are present in enhanced form, in particular in overexpressed form.

15. Process according to Claims 1 and 12, wherein L-amino acids selected from the group comprising L-asparagine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine are produced.

16. Process according to Claims 1 to 10 and 12, wherein L-amino acids selected from the group comprising L-isoleucine, L-valine, L-methionine, L-homoserine and L-lysine are produced.

17. Process according to Claims 1 to 13, wherein L-threonine is produced.

Figure 1: Map of the vector pTrc99AyfiD

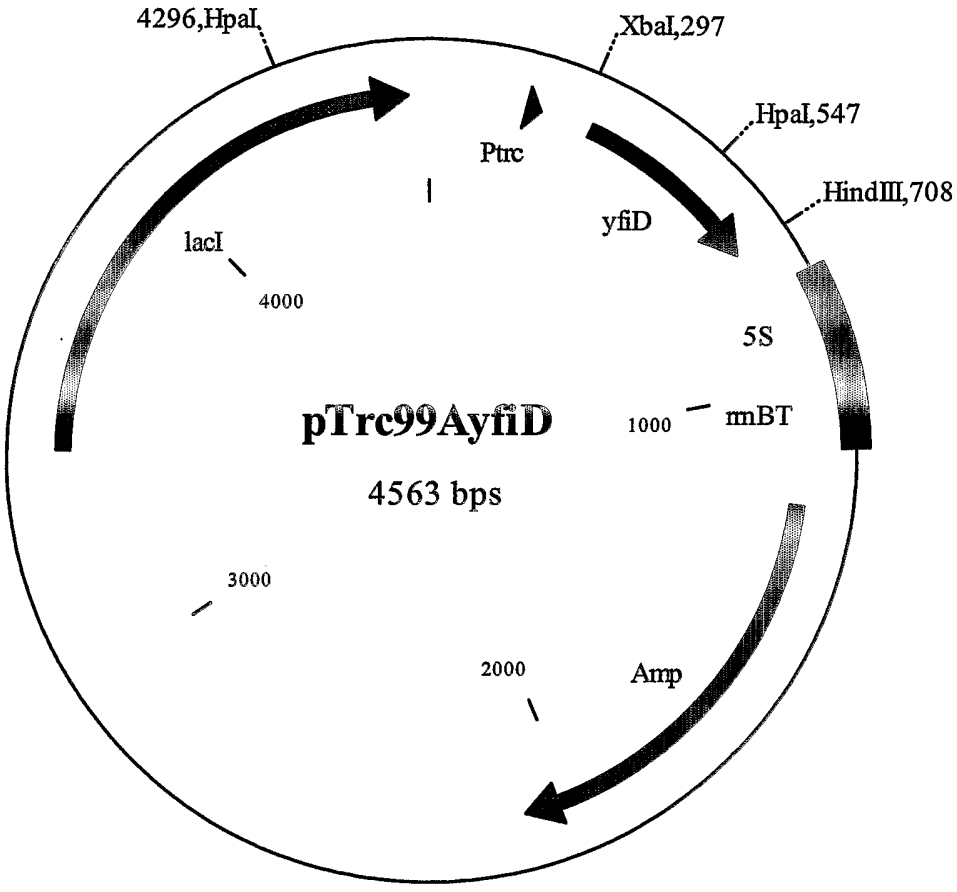
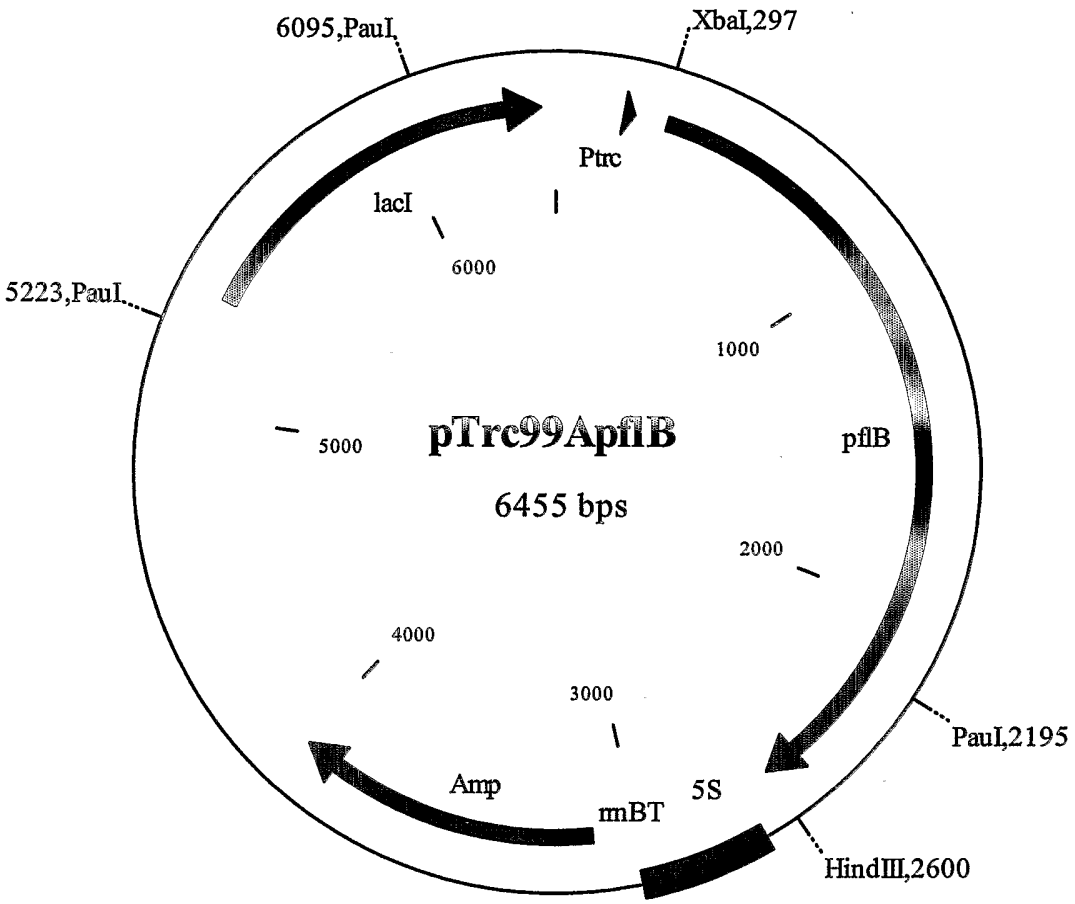


Figure 2: Map of the vector pTrc99AflB



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Glu Thr Leu Glu Asp Ala Val Lys His Pro Glu Lys Tyr Pro Gln Leu
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Ala Trp Glu Gly Phe Thr Lys Gly Asp Trp Gln Asn Glu Val Asn Val
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cgt gac ttc att cag aaa aac tac act ccg tac gag ggt gac gag tcc      149
Arg Asp Phe Ile Gln Lys Asn Tyr Thr Pro Tyr Glu Gly Asp Glu Ser
            30                  35                  40

ttc ctg gct ggc gct act gaa gcg acc acc acc ctg tgg gac aaa gta      197
Phe Leu Ala Gly Ala Thr Glu Ala Thr Thr Thr Leu Trp Asp Lys Val
        45                  50                  55

atg gaa ggc gtt aaa ctg gaa aac cgc act cac gcg cca gtt gac ttt      245
Met Glu Gly Val Lys Leu Glu Asn Arg Thr His Ala Pro Val Asp Phe
    60                  65                  70

gac acc gct gtt gct tcc acc atc acc tct cac gac gct ggc tac atc      293
Asp Thr Ala Val Ala Ser Thr Ile Thr Ser His Asp Ala Gly Tyr Ile
75                  80                  85                  90

aac aag cag ctt gag aaa atc gtt ggt ctg cag act gaa gct ccg ctg      341
Asn Lys Gln Leu Glu Lys Ile Val Gly Leu Gln Thr Glu Ala Pro Leu
                95                  100                 105

aaa cgt gct ctt atc ccg ttc ggt ggt atc aaa atg atc gaa ggt tcc      389
Lys Arg Ala Leu Ile Pro Phe Gly Gly Ile Lys Met Ile Glu Gly Ser
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tgc aaa gcg tac aac cgc gaa ctg gat ccg atg atc aaa aaa atc ttc      437
Cys Lys Ala Tyr Asn Arg Glu Leu Asp Pro Met Ile Lys Lys Ile Phe
        125                 130                 135

act gaa tac cgt aaa act cac aac cag ggc gtg ttc gac gtt tac act      485
Thr Glu Tyr Arg Lys Thr His Asn Gln Gly Val Phe Asp Val Tyr Thr
    140                 145                 150

ccg gac atc ctg cgt tgc cgt aaa tct ggt gtt ctg acc ggt ctg cca      533
Pro Asp Ile Leu Arg Cys Arg Lys Ser Gly Val Leu Thr Gly Leu Pro
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Asp Ala Tyr Gly Arg Gly Arg Ile Ile Gly Asp Tyr Arg Arg Val Ala
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ctg tac ggt atc gac tac ctg atg aaa gac aaa ctg gca cag ttc act      629
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tct ctg cag gct gat ctg gaa aac ggc gta aac ctg gaa cag act atc      677
Ser Leu Gln Ala Asp Leu Glu Asn Gly Val Asn Leu Glu Gln Thr Ile
        205                 210                 215

cgt ctg cgc gaa gaa atc gct gaa cag cac cgc gct ctg ggt cag atg      725
Arg Leu Arg Glu Glu Ile Ala Glu Gln His Arg Ala Leu Gly Gln Met
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aaa gaa atg gct gcg aaa tac ggc tac gac atc tct ggt ccg gct acc      773
Lys Glu Met Ala Ala Lys Tyr Gly Tyr Asp Ile Ser Gly Pro Ala Thr
235                 240                 245                 250

aac gct cag gaa gct atc cag tgg act tac ttc ggc tac ctg gct gct      821
Asn Ala Gln Glu Ala Ile Gln Trp Thr Tyr Phe Gly Tyr Leu Ala Ala
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gtt aag tct cag aac ggt gct gca atg tcc ttc ggt cgt acc tcc acc      869
Val Lys Ser Gln Asn Gly Ala Ala Met Ser Phe Gly Arg Thr Ser Thr
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ttc ctg gat gtg tac atc gaa cgt gac ctg aaa gct ggc aag atc acc      917
Phe Leu Asp Val Tyr Ile Glu Arg Asp Leu Lys Ala Gly Lys Ile Thr
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gaa caa gaa gcg cag gaa atg gtt gac cac ctg gtc atg aaa ctg cgt      965
Glu Gln Glu Ala Gln Glu Met Val Asp His Leu Val Met Lys Leu Arg
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atg gtt cgc ttc ctg cgt act ccg gaa tac gat gaa ctg ttc tct ggc     1013
Met Val Arg Phe Leu Arg Thr Pro Glu Tyr Asp Glu Leu Phe Ser Gly
315                 320                 325                 330

gac ccg atc tgg gca acc gaa tct atc ggt ggt atg ggc ctc gac ggt     1061
Asp Pro Ile Trp Ala Thr Glu Ser Ile Gly Gly Met Gly Leu Asp Gly
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cgt acc ctg gtt acc aaa aac agc ttc cgt ttc ctg aac acc ctg tac     1109
Arg Thr Leu Val Thr Lys Asn Ser Phe Arg Phe Leu Asn Thr Leu Tyr
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acc atg ggt ccg tct ccg gaa ccg aac atg acc att ctg tgg tct gaa     1157
Thr Met Gly Pro Ser Pro Glu Pro Asn Met Thr Ile Leu Trp Ser Glu
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aaa ctg ccg ctg aac ttc aag aaa ttc gcc gct aaa gtg tcc atc gac     1205
Lys Leu Pro Leu Asn Phe Lys Lys Phe Ala Ala Lys Val Ser Ile Asp
    380                 385                 390

acc tct tct ctg cag tat gag aac gat gac ctg atg cgt ccg gac ttc     1253
Thr Ser Ser Leu Gln Tyr Glu Asn Asp Asp Leu Met Arg Pro Asp Phe
395                 400                 405                 410

aac aac gat gac tac gct att gct tgc tgc gta agc ccg atg atc gtt     1301
Asn Asn Asp Asp Tyr Ala Ile Ala Cys Cys Val Ser Pro Met Ile Val
                415                 420                 425

ggt aaa caa atg cag ttc ttc ggt gcg cgt gca aac ctg gcg aaa acc     1349
Gly Lys Gln Met Gln Phe Phe Gly Ala Arg Ala Asn Leu Ala Lys Thr
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atg ctg tac gca atc aac ggc ggc gtt gac gaa aaa ctg aaa atg cag     1397
Met Leu Tyr Ala Ile Asn Gly Gly Val Asp Glu Lys Leu Lys Met Gln
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gtt ggt ccg aag tct gaa ccg atc aaa ggc gat gtc ctg aac tat gat     1445
Val Gly Pro Lys Ser Glu Pro Ile Lys Gly Asp Val Leu Asn Tyr Asp
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gaa gtg atg gag cgc atg gat cac ttc atg gac tgg ctg gct aaa cag     1493
Glu Val Met Glu Arg Met Asp His Phe Met Asp Trp Leu Ala Lys Gln
475                 480                 485                 490

tac atc act gca ctg aac atc atc cac tac atg cac gac aag tac agc     1541
Tyr Ile Thr Ala Leu Asn Ile Ile His Tyr Met His Asp Lys Tyr Ser
                495                 500                 505

tac gaa gcc tct ctg atg gcg ctg cac gac cgt gac gtt atc cgc acc     1589
Tyr Glu Ala Ser Leu Met Ala Leu His Asp Arg Asp Val Ile Arg Thr
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Met Ala Cys Gly Ile Ala Gly Leu Ser Val Ala Ala Asp Ser Leu Ser
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gca atc aaa tat gcg aaa gtt aaa ccg att cgt gac gaa gac ggt ctg     1685
Ala Ile Lys Tyr Ala Lys Val Lys Pro Ile Arg Asp Glu Asp Gly Leu
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gct atc gac ttc gaa atc gaa ggc gaa tac ccg cag ttt ggt aac aat     1733
Ala Ile Asp Phe Glu Ile Glu Gly Glu Tyr Pro Gln Phe Gly Asn Asn
555                 560                 565                 570

gat ccg cgt gta gat gac ctg gct gtt gac ctg gta gaa cgt ttc atg     1781
Asp Pro Arg Val Asp Asp Leu Ala Val Asp Leu Val Glu Arg Phe Met
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aag aaa att cag aaa ctg cac acc tac cgt gac gct atc ccg act cag     1829
Lys Lys Ile Gln Lys Leu His Thr Tyr Arg Asp Ala Ile Pro Thr Gln
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tct gtt ctg acc atc act tct aac gtt gtg tat ggt aag aaa acg ggt     1877
Ser Val Leu Thr Ile Thr Ser Asn Val Val Tyr Gly Lys Lys Thr Gly
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Asn Thr Pro Asp Gly Arg Arg Ala Gly Ala Pro Phe Gly Pro Gly Ala
    620                 625                 630

aac ccg atg cac ggt cgt gac cag aaa ggt gca gta gcc tct ctg act     1973
Asn Pro Met His Gly Arg Asp Gln Lys Gly Ala Val Ala Ser Leu Thr
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Ser Val Ala Lys Leu Pro Phe Ala Tyr Ala Lys Asp Gly Ile Ser Tyr
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aag acc aac ctg gct ggt ctg atg gat ggt tac ttc cac cac gaa gca     2117
Lys Thr Asn Leu Ala Gly Leu Met Asp Gly Tyr Phe His His Glu Ala
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700 705 710

atg ctg ctc gac gcg atg gaa aac ccg gaa aaa tat ccg cag ctg acc 2213
Met Leu Leu Asp Ala Met Glu Asn Pro Glu Lys Tyr Pro Gln Leu Thr
715 720 725 730

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735 740 745

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Glu Asn Arg Thr His Ala Pro Val Asp Phe Asp Thr Ala Val Ala Ser
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Thr Ile Thr Ser His Asp Ala Gly Tyr Ile Asn Lys Gln Leu Glu Lys
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Ile Val Gly Leu Gln Thr Glu Ala Pro Leu Lys Arg Ala Leu Ile Pro
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Phe Gly Gly Ile Lys Met Ile Glu Gly Ser Cys Lys Ala Tyr Asn Arg
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His Asn Gln Gly Val Phe Asp Val Tyr Thr Pro Asp Ile Leu Arg Cys
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Gln Trp Thr Tyr Phe Gly Tyr Leu Ala Ala Val Lys Ser Gln Asn Gly
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Ala Ala Met Ser Phe Gly Arg Thr Ser Thr Phe Leu Asp Val Tyr Ile
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Glu Arg Asp Leu Lys Ala Gly Lys Ile Thr Glu Gln Glu Ala Gln Glu
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Met Val Asp His Leu Val Met Lys Leu Arg Met Val Arg Phe Leu Arg
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Glu Ser Ile Gly Gly Met Gly Leu Asp Gly Arg Thr Leu Val Thr Lys
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Glu Pro Asn Met Thr Ile Leu Trp Ser Glu Lys Leu Pro Leu Asn Phe
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Lys Lys Phe Ala Ala Lys Val Ser Ile Asp Thr Ser Ser Leu Gln Tyr
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Glu Asn Asp Asp Leu Met Arg Pro Asp Phe Asn Asn Asp Asp Tyr Ala
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Gly Gly Val Asp Glu Lys Leu Lys Met Gln Val Gly Pro Lys Ser Glu
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Leu Met Asp Gly Tyr Phe His His Glu Ala Ser Ile Glu Gly Gly Gln
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705                 710                 715                 720

Glu Asn Pro Glu Lys Tyr Pro Gln Leu Thr Ile Arg Val Ser Gly Tyr
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Ala Val Arg Phe Asn Ser Leu Thr Lys Glu Gln Gln Gln Asp Val Ile
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/003207

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P13/08 C12N15/54 C12N1/21

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C12N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, MEDLINE, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WYBORN NEIL R ET AL: "Expression of the Escherichia coli yfiD gene responds to intracellular pH and reduces the accumulation of acidic metabolic end products" MICROBIOLOGY (READING), vol. 148, no. 4, April 2002 (2002-04), pages 1015-1026, XP002284817 ISSN: 1350-0872 cited in the application Abstract; TAble 3; Fig. 2 --- -/--	1-17

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 June 2004	05/07/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Lopez García, F

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/003207

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KNAPPE J ET AL: "A RADICAL-CHEMICAL ROUTE TO ACETYL-COA: THE ANAEROBICALLY INDUCED PYRUVATE FORMATE-LYASE SYSTEM OF ESCHERICHIA COLI" FEMS MICROBIOLOGY REVIEWS, ELSEVIER, AMSTERDAM, NL, vol. 75, no. 4, 1 August 1990 (1990-08-01), pages 383-398, XP002045432 ISSN: 0168-6445 Abstract, Table 4. ---	1-17
Y	DE 101 35 053 A (DEGUSSA) 6 February 2003 (2003-02-06) Abstract; col. 5, l. 31-37. ---	1-13, 15-17
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X,P	LEE J-H ET AL: "GLOBAL ANALYSES OF TRANSCRIPTOMES AND PROTEOMES OF A PARENT STRAIN AND AN L-THREONINE-OVERPRODUCING MUTANT STRAIN" JOURNAL OF BACTERIOLOGY, WASHINGTON, DC, US, vol. 185, no. 18, September 2003 (2003-09), pages 5442-5451, XP009022136 ISSN: 0021-9193 Abstract; Table 1. -----	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP2004/003207

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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