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ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: PROCESS FOR THE FERMENTATIVE PREPARATION OF L-AMINO ACIDS WITH AMPLIFICATION OF THE ZWF GENE

(57) Abstract: The invention relates to a process for the preparation of L-amino acids by fermentation of coryneform bacteria, which comprises carrying out the following steps: a) fermentation of the desired L-amino acid-producing bacteria in which at least the zwf gene is amplified, b) concentration of the L-amino acid in the medium or in the cells of the bacteria and c) isolation of the L-amino acid produced.

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Process for the fermentative preparation of L-amino acids
with amplification of the zwf gene

The invention relates to a process for the fermentative preparation of L-amino acids, in particular L-lysine, L-threonine and L-tryptophan, using coryneform bacteria in which at least the zwf gene is amplified.

Prior art

L-Amino acids are used in animal nutrition, in human medicine and in the pharmaceuticals industry.

10 It is known that amino acids are prepared by fermentation of strains of coryneform bacteria, in particular *Corynebacterium glutamicum*. Because of its great importance, work is constantly being undertaken to improve the preparation process. Improvements to the process can
15 relate to fermentation measures, such as e. g. stirring and supply of oxygen, or the composition of the nutrient media, such as e. g. the sugar concentration during the fermentation, or the working up to the product form by e. g. ion exchange chromatography, or the intrinsic output
20 properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e. g. the threonine analogue α -amino- β -hydroxyvaleric acid (AHV), or are auxotrophic for
25 metabolites of regulatory importance and produce L-amino acids such as e. g. threonine are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of
30 *Corynebacterium glutamicum* strains which produce L-amino acids.

Object of the invention

The inventors had the object of providing new improved processes for the fermentative preparation of L-amino acids with coryneform bacteria.

5 Description of the invention

L-Amino acids are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and especially in animal nutrition. There is therefore a general interest in providing new improved processes for
10 the preparation of amino acids.

The invention provides a process for the fermentative preparation of L-amino acids, in particular L-lysine, L-threonine and L-tryptophan, using coryneform bacteria in which the nucleotide sequence which codes for the Zwf
15 protein (zwf gene) is amplified, in particular over-expressed.

The strains employed preferably already produce L-amino acids before amplification of the zwf gene.

Preferred embodiments are to be found in the claims.

20 The term "amplification" in this connection describes the increase in the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or
25 using a gene which codes for a corresponding enzyme (protein) having a high activity, and optionally combining these measures.

The microorganisms which the present invention provides can prepare L-amino acids from glucose, sucrose, lactose,
30 fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They are representatives of

coryneform bacteria, in particular of the genus *Corynebacterium*. Of the genus *Corynebacterium*, there may be mentioned in particular the species *Corynebacterium glutamicum*, which is known among specialists for its ability to produce L-amino acids.

Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum*, are, for example, the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869
Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants prepared therefrom,

such as, for example, the L-threonine-producing strains

Corynebacterium glutamicum ATCC21649
Brevibacterium flavum BB69
Brevibacterium flavum DSM5399
Brevibacterium lactofermentum FERM-BP 269
Brevibacterium lactofermentum TBB-10

and such as, for example, the L-isoleucine-producing strains

Corynebacterium glutamicum ATCC 14309
Corynebacterium glutamicum ATCC 14310
Corynebacterium glutamicum ATCC 14311
Corynebacterium glutamicum ATCC 15168
Corynebacterium ammoniagenes ATCC 6871

and such as, for example, the L-tryptophan-producing strains

Corynebacterium glutamicum ATCC21850 and
Corynebacterium glutamicum KY9218 (pKW9901)

and such as, for example, the L-lysine-producing strains

Corynebacterium glutamicum FERM-P 1709

Brevibacterium flavum FERM-P 1708

Brevibacterium lactofermentum FERM-P 1712

5 Corynebacterium glutamicum FERM-P 6463

Corynebacterium glutamicum FERM-P 6464

Corynebacterium glutamicum ATCC13032

Corynebacterium glutamicum DM58-1

Corynebacterium glutamicum DSM12866.

10 It has been found that coryneform bacteria produce L-amino acids, in particular L-lysine, L-threonine and L-tryptophan, in an improved manner after over-expression of the zwf gene which codes for the Zwfp protein.

Alleles of the zwf gene which result from the degeneracy of
15 the genetic code or due to sense mutations of neutral function can furthermore be used.

JP-A-09224661 discloses the nucleotide sequence of the glucose 6-phosphate dehydrogenase gene, called zwf, of Brevibacterium flavum MJ-223 (FERM BP-1497). JP-A-09224661
20 describes the N-terminal amino acid sequence of the Zwfp polypeptide as Met Val Ile Phe Gly Val Thr Gly Asp Leu Ala Arg Lys Lys Leu.

However, it has not been possible to confirm this. Instead, the following N-terminal amino acid sequence has been
25 found: Val Ser Thr Asn Thr Thr Pro Ser Ser Trp Thr Asn Pro Leu Arg Asp. The valyl radical in the N-position can be split off in the context of post-translational modification, and Ser Thr Asn Thr Thr Pro Ser Ser Trp Thr Asn Pro Leu Arg Asp is then obtained as the N-terminal
30 amino acid sequence.

To achieve an amplification (e. g. over-expression), the number of copies of the corresponding genes is increased, or the promoter and regulation region or the ribosome

binding site upstream of the structural gene is mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative L-amino acid formation. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes or gene constructs are either present here in plasmids with a varying number of copies, or are integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 (1991)), in European Patent Specification EPS 0 472 869, in US Patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)), in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in Patent Application WO 96/15246, in Malumbres et al. (Gene 134, 15-24 (1993)), in Japanese Laid-Open Specification JP-A-10-229891, in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)) and in known textbooks of genetics and molecular biology.

By way of example, the Zwf protein was over-expressed with the aid of a plasmid. The E. coli - C. glutamicum shuttle vector pEC-T18mob2 shown in Figure 1 was used for this. After incorporation of the zwf gene into the KpnI/SalI cleavage site of pEC-T18mob2, the plasmid pEC-T18mob2zwf shown in Figure 2 was formed.

Other plasmid vectors which are capable of replication in *C. glutamicum*, such as e.g. pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991)) or pZ8-1 (EP-B- 0 375 889), can be used in the same way.

- 5 In addition, it may be advantageous for the production of L-amino acids to amplify one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the pentose phosphate pathway or of amino acid export, in addition to amplification of the *zwf* gene.
- 10 Thus, for example, in particular for the preparation of L-threonine, one or more genes chosen from the group consisting of:
- the *hom* gene which codes for homoserine dehydrogenase (Peoples et al., Molecular Microbiology 2, 63-72 (1988))
15 or the *hom^{dr}* allele which codes for a "feed back resistant" homoserine dehydrogenase (Archer et al., Gene 107, 53-59 (1991)),
 - the *gap* gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns et al., Journal of Bacteriology
20 174: 6076-6086 (1992)),
 - the *pyc* gene which codes for pyruvate carboxylase (Peters-Wendisch et al., Microbiology 144: 915-927 (1998)),
 - the *mgo* gene which codes for malate:quinone
25 oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
 - the *tkt* gene which codes for transketolase (accession number AB023377 of the European Molecular Biologies Laboratories databank (EMBL, Heidelberg, Germany)),

- the gnd gene which codes for 6-phosphogluconate dehydrogenase (JP-A-9-224662),
- the thrE gene which codes for threonine export (DE 199 41 478.5; DSM 12840),

5 • the zwf gene (DE 199 59 328.0; DSM 13115),

- the eno gene which codes for enolase (DE: 199 41 478.5)

can be amplified, in particular over-expressed, at the same time.

Thus, for example, in particular for the preparation of L-lysine, one or more genes chosen from the group consisting of

- the dapA gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335),
- the lysC gene which codes for a feed back resistant aspartate kinase (Kalinowski et al. (1990), Molecular and General Genetics 224: 317-324),
- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609),
- the tkt gene which codes for transketolase (accession number AB023377 of the European Molecular Biologies Laboratories databank (EMBL, Heidelberg, Germany)), <<
- the gnd gene which codes for 6-phosphogluconate dehydrogenase (JP-A-9-224662),

- the lysE gene which codes for lysine export (DE-A-195 48 222),
- the zwal gene (DE 199 59 328.0; DSM 13115),
- the eno gene which codes for enolase (DE 199 47 791.4)

5 can be amplified, in particular over-expressed, at the same time.

It may furthermore be advantageous for the production of L-amino acids at the same time to attenuate one of the genes chosen from the group consisting of

- 10 • the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),
- the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478, DSM 12969),
- the poxB gene which codes for pyruvate oxidase
- 15 (DE 199 51 975.7; DSM 13114),
- the zwa2 gene (DE: 199 59 327.2; DSM 13113)

in addition to the amplification of the zwf gene.

In addition to over-expression of the Zwf protein, it may furthermore be advantageous for the production of L-amino acids to eliminate undesirable side reactions (Nakayama: 20 "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms prepared according to the invention can 25 be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of L-amino acid production. A summary of known culture methods is described in the textbook by Chmiel

(Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular microorganisms in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981). Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e. g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e. g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e. g. glycerol and ethanol, and organic acids, such as e. g. acetic acid, can be used as the source of carbon. These substance can be used individually or as a mixture. Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulphate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture. Potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e. g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances

mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of L-amino acid has formed. This target is usually reached within 10 hours to 160 hours.

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

The following microorganism has been deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:

Escherichia coli K-12 DH5 α /pEC-T18mob2 as DSM 13244

The following figures are attached:

- Figure 1: Map of the plasmid pEC-T18mob2
- Figure 2: Map of the plasmid pEC-T18mob2zwf
- Figure 3: Map of the plasmid pAMC1
- 5 • Figure 4: Map of the plasmid pMC1
- Figure 5: Map of the plasmid pCR2.1poxBint

The base pair numbers stated are approx. values obtained in the context of reproducibility.

Re Figure 1 and 2:

10 The abbreviations used have the following meaning:

	Tet:	Resistance gene for tetracycline
	oriV:	Plasmid-coded replication origin of E. coli
	RP4mob:	mob region for mobilizing the plasmid
	rep:	Plasmid-coded replication origin from
15		C. glutamicum plasmid pGA1
	per:	Gene for controlling the number of copies from pGA1
	lacZ-alpha:	lacZ α gene fragment (N-terminus) of the β -galactosidase gene
20	lacZalpha':	5'-Terminus of the lacZ α gene fragment
	'lacZalpha:	3'-Terminus of the lacZ α gene fragment

Re Figure 3 and 4:

The abbreviations used have the following meaning:

	Neo r :	Neomycin/kanamycin resistance
25	ColE1 ori:	Replication origin of the plasmid ColE1
	CMV:	Cytomegalovirus promoter
	lacP:	Lactose promoter
	pgi:	Phosphoglucose isomerase gene
	lacZ:	Part of the β -galactosidase gene
30	SV40 3' splice	3' splice site of Simian virus 40

- SV40 polyA: Polyadenylation site of Simian virus
40
- fl(-)ori: Replication origin of the filamentous
phage fl
- 5 SV40 ori: Replication origin of Simian virus
40
- kan r: Kanamycin resistance
- pgi insert: Internal fragment of the pgi gene
- ori: Replication origin of the plasmid pBGS8
- 10 Re Figure 5:
The abbreviations used have the following meaning:
- ColE1 ori: Replication origin of the plasmid ColE1
- lacZ: Cloning relict of the lacZ α gene fragment
- fl ori: Replication origin of phage fl
- 15 KmR: Kanamycin resistance
- ApR: Ampicillin resistance
- poxBint: Internal fragment of the poxB gene

The meaning of the abbreviations for the various
restriction enzymes (e. g. BamHI, EcoRI etc.) are known from
20 the prior art and are summarized, for example, by Kessler
and Höltnke (Gene 47, 1-153 (1986)) or Roberts et al.
(Nucleic Acids Research 27, 312-313 (1999)).

Examples

The following examples will further illustrate this invention. The molecular biology techniques, e.g. plasmid DNA isolation, restriction enzyme treatment, ligations, standard transformations of *Escherichia coli* etc. used are, (unless stated otherwise), described by Sambrook et al., (Molecular Cloning. A Laboratory Manual (1989) Cold Spring Harbour Laboratories, USA).

Example 1

10 Expression of the Zwf protein

1.1 Preparation of the plasmid pEC-T18mob2

The *E. coli* - *C. glutamicum* shuttle vector pEC-T18mob2 was constructed according to the prior art. The vector contains the replication region rep of the plasmid pGA1 including the replication effector per (US-A- 5,175,108; Nesvera et al., Journal of Bacteriology 179, 1525-1532 (1997)), the tetracycline resistance-imparting tetA(Z) gene of the plasmid pAG1 (US-A- 5,158,891; gene library entry at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) with accession number AF121000), the replication region oriV of the plasmid pMB1 (Sutcliffe, Cold Spring Harbor Symposium on Quantitative Biology 43, 77-90 (1979)), the lacZ α gene fragment including the lac promoter and a multiple cloning site (mcs) (Norrander et al. Gene 26, 101-106 (1983)) and the mob region of the plasmid RP4 (Simon et al., (1983) Bio/Technology 1:784-791). The vector constructed was transformed in the *E. coli* strain DH5 α (Brown (ed.) Molecular Biology Labfax, BIOS Scientific Publishers, Oxford, UK, 1991). Selection for plasmid-carrying cells was made by plating out the transformation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which had been supplemented with 5 mg/l tetracycline. Plasmid DNA was

isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction with the restriction enzyme EcoRI and HindIII and subsequent agarose gel electrophoresis (0.8%).

- 5 The plasmid was called pEC-T18mob2 and is shown in Figure 1. It is deposited in the form of the strain Escherichia coli K-12 strain DH5 α pEC-T18mob2 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, 10 Braunschweig, Germany) as DSM 13244.

1.2 Preparation of the plasmid pEC-T18mob2zwf

The gene from Corynebacterium glutamicum ATCC13032 was first amplified by a polymerase chain reaction (PCR) by means of the following oligonucleotide primer:

15 zwf-forward:

5'-TCG ACG CGG TTC TGG AGC AG-3'

zwf-reverse:

5'-CTA AAT TAT GGC CTG CGC CAG-3'

- The PCR reaction was carried out in 30 cycles in the 20 presence of 200 μ M deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP), in each case 1 μ M of the corresponding oligonucleotide, 100 ng chromosomal DNA from Corynebacterium glutamicum ATCC13032, 1/10 volume 10-fold reaction buffer and 2.6 units of a heat-stable Taq-/Pwo-DNA 25 polymerase mixture (Expand High Fidelity PCR System from Roche Diagnostics, Mannheim, Germany) in a Thermocycler (PTC-100, MJ Research, Inc., Watertown, USA) under the following conditions: 94°C for 30 seconds, 64°C for 1 minute and 68°C for 3 minutes.

- 30 The amplified fragment about 1.8 kb in size was subsequently ligated with the aid of the SureClone Ligation Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) into the SmaI cleavage site of the vector pUC18 in accordance with

the manufacturer's instructions. The E. coli strain DH5 α mcr (Grant et al., Proceedings of the National Academy of Sciences of the United States of America USA (1990) 87: 4645-4649) was transformed with the entire ligation batch.

- 5 Transformants were identified with the aid of their carbenicillin resistance on LB-agar plates containing 50 μ g/mL carbenicillin. The plasmids were prepared from 7 of the transformants and checked for the presence of the 1.8 kb PCR fragment as an insert by restriction analysis.
- 10 The recombinant plasmid formed in this way is called pUC18zwf in the following.

- For construction of pEC-T18mob2zwf, pUC18zwf was digested with KpnI and SalI, and the product was isolated with the aid of the NucleoSpin Extraction Kit from Macherey-Nagel
- 15 (Düren, Germany) in accordance with the manufacturer's instructions and then ligated with the vector pEC-T18mob2, which had also been cleaved with KpnI and SalI and dephosphorylated. The E. coli strain DH5 α mcr (Grant et al., Proceedings of the National Academy of Sciences of the
- 20 United States of America USA (1990) 87: 4645-4649) was transformed with the entire ligation batch. Transformants were identified with the aid of their tetracycline resistance on LB-agar plates containing 5 μ g/mL
- 25 tetracycline. The plasmids were prepared from 12 of the transformants and checked for the presence of the 1.8 kb PCR fragment as an insert by restriction analysis. One of the recombinant plasmids isolated in this manner was called pEC-T18mob2zwf (Figure 2).

Example 2

- 30 Preparation of amino acid producers with an amplified zwf gene

The L-lysine-producing strain *Corynebacterium glutamicum* DSM5715 is described in EP-B-0435132 and the L-threonine-producing strain *Brevibacterium flavum* DSM5399 is described

in EP-B-0385940. Both strains are deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen [German Collection of Microorganisms and Cell Cultures] in Braunschweig (Germany) in accordance with the Budapest Treaty.

2.1 Preparation of the strains DSM5715/pEC-T18mob2zwf and DSM5399/pEC-T18mob2zwf

The strains DSM5715 and DSM5399 were transformed with the plasmid pEC-T18mob2zwf using the electroporation method described by Liebl et al., (FEMS Microbiology Letters, 53:299-303 (1989)) Selection of the transformants took place on LBHIS agar comprising 18.5 g/l brain-heart infusion broth, 0.5 M sorbitol, 5 g/l Bacto-tryptone, 2.5 g/l Bacto-yeast extract, 5 g/l NaCl and 18 g/l Bacto-agar, which had been supplemented with 5 mg/l tetracycline. Incubation was carried out for 2 days at 33°C.

Plasmid DNA was isolated in each case from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology 144, 915 -927), cleaved with the restriction endonucleases XbaI and KpnI, and the plasmid was checked by subsequent agarose gel electrophoresis. The strains obtained in this way were called DSM5715/pEC-T18mob2zwf and DSM5399/pEC-T18mob2zwf.

2.2 Preparation of L-threonine

The *C. glutamicum* strain DSM5399/pEC-T18mob2zwf obtained in Example 2.1 was cultured in a nutrient medium suitable for the production of threonine and the threonine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with tetracycline (5 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, a preculture was seeded (10 ml

medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the preculture.

Medium Cg III

NaCl 2.5 g/l

Bacto-Peptone 10 g/l

Bacto-Yeast extract 10 g/l

Glucose (autoclaved separately) 2% (w/v)

The pH was brought to pH 7.4

5 Tetracycline (5 mg/l) was added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660nm) of the main culture was 0.1. Medium MM was used for the main culture.

Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50 g/l
$(\text{NH}_4)_2\text{SO}_4$	25 g/l
KH_2PO_4	0.1 g/l
$\text{MgSO}_4 * 7 \text{ H}_2\text{O}$	1.0 g/l
$\text{CaCl}_2 * 2 \text{ H}_2\text{O}$	10 mg/l
$\text{FeSO}_4 * 7 \text{ H}_2\text{O}$	10 mg/l
$\text{MnSO}_4 * \text{H}_2\text{O}$	5.0 mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine (sterile-filtered)	0.1 g/l
CaCO_3	25 g/l

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the CaCO_3 autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Tetracycline (5 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of threonine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

Strain	OD	L-Threonin g/l
DSM5399	12.3	0.74
DSM5399/pEC-T18mob2zwf	10.2	1.0

2.3 Preparation of L-lysine

The *C. glutamicum* strain DSM5715/pEC-T18mob2zwf obtained in Example 2.1 was cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant was determined.

- For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with tetracycline (5 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the preculture.

Medium Cg III

NaCl 2.5 g/l

Bacto-Peptone 10 g/l

Bacto-Yeast extract 10 g/l

Glucose (autoclaved separately) 2% (w/v)

The pH was brought to pH 7.4

Tetracycline (5 mg/l) was added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660nm) of the main culture was

5 0.1. Medium MM was used for the main culture.

Medium MM

CSL (corn steep liquor) 5 g/l

MOPS (morpholinopropanesulfonic acid) 20 g/l

Glucose (autoclaved separately) 58 g/l

$(\text{NH}_4)_2\text{SO}_4$ 25 g/l

KH_2PO_4 0.1 g/l

$\text{MgSO}_4 \cdot 7 \text{ H}_2\text{O}$ 1.0 g/l

$\text{CaCl}_2 \cdot 2 \text{ H}_2\text{O}$ 10 mg/l

$\text{FeSO}_4 \cdot 7 \text{ H}_2\text{O}$ 10 mg/l

$\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 5.0mg/l

Biotin (sterile-filtered) 0.3 mg/l

Thiamine * HCl (sterile-filtered) 0.2 mg/l

L-Leucine (sterile-filtered) 0.1 g/l

CaCO_3 25 g/l

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the CaCO_3 autoclaved in the dry state.

- 5 Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Tetracycline (5 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

- 10 After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

- 15 The result of the experiment is shown in Table 2.

Table 2

Strain	OD	L-Lysine HCl g/l
DSM5715	10.8	16.0
DSM5715/pEC-T18mob2zwf	7.2	17.1

Example 3

- 20 Construction of a gene library of *Corynebacterium glutamicum* strain AS019

- A DNA library of *Corynebacterium glutamicum* strain AS019 (Yoshihama et al., Journal of Bacteriology 162, 591-597 (1985)) was constructed using λ Zap Express™ system, (Short et al., (1988) Nucleic Acids Research, 16: 7583-25 7600), as described by O'Donohue (O'Donohue, M. (1997). The

Cloning and Molecular Analysis of Four Common Aromatic Amino Acid Biosynthetic Genes from *Corynebacterium glutamicum*. Ph.D. Thesis, National University of Ireland, Galway). λ Zap ExpressTM kit was purchased from Stratagene (Stratagene, 11011 North Torrey Pines Rd., La Jolla, California 92037) and used according to the manufacturers instructions. AS019-DNA was digested with restriction enzyme *Sau*3A and ligated to *Bam*HI treated and dephosphorylated λ Zap ExpressTM arms.

10 Example 4

Cloning and sequencing of the *pgi* gene

1. Cloning

Escherichia coli strain DF1311, carrying mutations in the *pgi* and *pgl* genes as described by Kupor and Fraenkel, (Journal of Bacteriology 100: 1296-1301 (1969)), was transformed with approx. 500 ng of the AS019 λ Zap ExpressTM plasmid library described in Example 3. Selection for transformants was made on M9 minimal media, (Sambrook et al., (1989). Molecular Cloning. A Laboratory Manual Cold Spring Harbour Laboratories, USA), containing kanamycin at a concentration of 50 mg/l and incubation at 37°C for 48 hours. Plasmid DNA was isolated from one transformant according to Birnboim and Doly (Nucleic Acids Research 7: 1513-1523 (1979)) and designated pAMC1 (Figure 3).

25 2. Sequencing

For sequence analysis of the cloned insert of pAMC1 the method of Sanger et al. (Proceedings of the National Academy of Sciences USA 74, 5463-5467 (1977)) was applied using primers differentially labelled with a coloured fluorescent tag. It was carried out using the ABI prism 310 genetic analyzer from Perkin Elmer Applied Biosystems, (Perkin Elmer Corporation, Norwalk, Connecticut, U.S.A), and the ABI prism Big DyeTM Terminator Cycle Sequencing Ready Reaction kit also from Perkin Elmer.

Initial sequence analysis was carried out using the universal forward and M13 reverse primers obtained from Pharmacia Biotech (St. Albans, Herts, AL1 3AW, UK):

Universal forward primer: GTA ATA CGA CTC ACT ATA GGG C

5 M13 reverse primer: GGA AAC AGC TAT GAC CAT G

Internal primers were subsequently designed from the sequence obtained which allowed the entire *pgi* gene to be deduced. The sequence of the internal primers is as follows:

10 Internal primer 1: GGA AAC AGG GGA GCC GTC

Internal primer 2: TGC TGA GAT ACC AGC GGT

Sequence obtained was then analyzed using the DNA Strider programme, (Marck, (1988). Nucleic Acids Research 16: 1829-1836), version 1.0 on an Apple Macintosh computer. This
15 program allowed for analyses such as restriction site usage, open reading frame analysis and codon usage determination. Searches between DNA sequence obtained and those in EMBL and Genbank databases were achieved using the BLAST programme, (Altschul et al., (1997). Nucleic Acids
20 Research, 25: 3389-3402). DNA and protein sequences were aligned using the Clustal V and Clustal W programs (Higgins and Sharp, 1988 Gene 73: 237-244).

The sequence thus obtained is shown in SEQ ID NO 1. The analysis of the nucleotide sequence obtained revealed an
25 open reading frame of 1650 base pairs which was designated as *pgi* gene. It codes for a protein of 550 amino acids shown in SEQ ID NO 2.

Example 5

Preparation of an integration vector for integration
30 mutagenesis of the *pgi* gene

An internal segment of the *pgi* gene was amplified by polymerase chain reaction (PCR) using genomic DNA isolated from *Corynebacterium glutamicum* AS019, (Heery and Dunican,

(1993) Applied and Environmental Microbiology 59: 791-799), as template. The *pgi* primers used were:

fwd. Primer: ATG GAR WCC AAY GGH AA

rev. Primer: YTC CAC GCC CCA YTG RTC

5 with R=A+G; Y=C+T; W=A+T; H=A+T+C.

PCR Parameters were as follows: 35 cycles
94°C for 1 min.
47°C for 1 min.
72°C for 30 sec.
10 1.5 mM MgCl₂
approx. 150-200 ng DNA template.

The PCR product obtained was cloned into the commercially available pGEM-T vector received from Promega Corp., (Promega UK, Southampton.) using strain *E. coli* JM109,
15 (Yanisch-Perron et al., 1985. Gene, 33: 103-119), as a host. The sequence of the PCR product is shown as SEQ ID NO 3. The cloned insert was then excised as an *EcoRI* fragment and ligated to plasmid pBGS8 (Spratt et al., Gene 41: 337-342 (1986)) pretreated with *EcoRI*. The restriction enzymes
20 used were obtained from Boehringer Mannheim UK Ltd., (Bell Lane, Lewes East Sussex BN7 1LG, UK.) and used according to manufacturers instructions. *E. coli* JM109 was then transformed with this ligation mixture and
electrotransformants were selected on Luria agar
25 supplemented with IPTG (isopropyl- β -D-thiogalactopyranoside), XGAL (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) and kanamycin at a concentration of 1 mM, 0.02% and 50 mg/l respectively. Agar plates were incubated for twelve hours at 37°C. Plasmid DNA was
30 isolated from one transformant, characterized by restriction enzyme analysis using *EcoRI*, *BamHI* and *SalI* designated pMC1 (Figure 4).

Plasmid pMC1 was deposited in the form of *Escherichia coli* strain DH5a/pMC1 at the Deutsche Sammlung für

Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) as DSM 12969 according to the Budapest treaty.

Example 6

Integration mutagenesis of the *pgi* gene in the lysine
5 producer DSM 5715

The vector pMC1 mentioned in Example 5 was electroporated by the electroporation method of Tauch et al. (FEMS Microbiological Letters, 123:343-347 (1994)) in Corynebacterium glutamicum DSM 5715. Strain DSM 5715 is an
10 AEC-resistant lysine producer. The vector pMC1 cannot replicate independently in DSM5715 and is retained in the cell only if it has integrated into the chromosome of DSM 5715. Selection of clones with pMC1 integrated into the chromosome was carried out by plating out the
15 electroporation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which had been supplemented with 15 mg/l kanamycin. For detection of the integration, the internal *pgi* fragment (Example 5)
20 was labelled with the Dig hybridization kit from Boehringer Mannheim by the method of "The DIG System Users Guide for Filter Hybridization" of Boehringer Mannheim GmbH (Mannheim, Germany, 1993). Chromosomal DNA of a transformant was isolated by the method of Eikmanns et al.
25 (Microbiology 140: 1817 - 1828 (1994)) and in each case cleaved with the restriction enzymes SalI, SacI and HindIII. The fragments formed were separated by agarose gel electrophoresis and hybridized at 68°C with the Dig hybridization kit from Boehringer. It was found in this way
30 that the plasmid pMC1 was inserted within the chromosomal *pgi* gene of strain DSM5715. The strain was called DSM5715::pMC1.

Example 7

Effect of over-expression of the zwf gene with simultaneous elimination of the pgi gene on the preparation of lysine

7.1 Preparation of the strain DSM5715::pMC1/pEC-T18mob2zwf

5 The vector pEC-T18mob2zwf mentioned in Example 1.2 was electroporated by the electroporation method of Tauch et al. (1994, FEMS Microbiological Letters, 123:343-347) in *Corynebacterium glutamicum* DSM 5715::pMC1. Selection for plasmid-carrying cells was made by plating out the
10 electroporation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been supplemented with 15 mg/l kanamycin and with
15 transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology 144, 915-927) and checked by treatment with the restriction enzymes KpnI and SalI with subsequent agarose gel electrophoresis. The strain was called DSM5715::pMC1/pEC-T18mob2zwf.

20 7.2 Preparation of lysine

The *C. glutamicum* strain DSM5715::pMC1/pEC-T18mob2zwf obtained in Example 7.1 was cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant was determined.

25 For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with tetracycline (5 mg/l) and kanamycin (25 mg/l)) for 24 hours at 33°C. The cultures of the comparison strains were supplemented according to their resistance to antibiotics.
30 Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the preculture.

Medium Cg III

NaCl 2.5 g/l

Bacto-Peptone 10 g/l

Bacto-Yeast extract 10 g/l

Glucose (autoclaved separately) 2% (w/v)

The pH was brought to pH 7.4

Tetracycline (5 mg/l) and kanamycin (5 mg/l) was added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660 nm) of the main culture was 0.1. Medium MM was used for the main culture.

Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50 g/l
 (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	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine (sterile-filtered)	0.1 g/l
CaCO ₃	25 g/l

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the CaCO₃
5 autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Tetracycline (5 mg/l) and kanamycin (25 mg/l) were added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-
5 BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in Table 3.

Table 3

Strain	OD	L-Lysine HCl g/l
DSM5715	7.3	14.3
DSM5715/pEC-T18mob2zwf	7.1	14.6
DSM5715::pMC1/ pECTmob2zwf	10.4	15.2

10 Example 8

Preparation of a genomic cosmid gene library from
Corynebacterium glutamicum ATCC 13032

Chromosomal DNA from Corynebacterium glutamicum ATCC 13032 was isolated as described by Tauch et al., (1995, Plasmid
15 33:168-179), and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product
20 Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vektor Kit, Code no. 251301) was cleaved

with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase. The cosmid DNA was then cleaved with the
5 restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this manner was mixed with the treated ATCC13032 DNA and the batch was treated with T4 DNA
10 ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing Extracts (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code
15 no. 200217). For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Res. 16:1563-1575) the cells were taken up in 10 mM MgSO₄ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by
20 Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) + 100 µg/ml ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected.

Example 9

25 Isolation and sequencing of the poxB gene

The cosmid DNA of an individual colony (Example 7) was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the
30 restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Product No.
35 1758250). After separation by gel electrophoresis, the

cosmid fragments in the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany). The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, Holland, Product Description Zero Background Cloning Kit, Product No. K2500-01), was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) into the E. coli strain DH5 α MCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 μ g/ml zeocin. The plasmid preparation of the recombinant clones was carried out with Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out by the dideoxy chain-stopping method of Sanger et al. (1977, Proceedings of the National Academies of Sciences U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the

pZerol derivatives were assembled to a continuous contig. The computer-assisted coding region analysis were prepared with the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231). Further analyses were carried out
5 with the "BLAST search program" (Altschul et al., 1997, Nucleic Acids Research, 25:3389-3402), against the non-redundant databank of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA).

The resulting nucleotide sequence is shown in SEQ ID No. 4.
10 Analysis of the nucleotide sequence showed an open reading frame of 1737 base pairs, which was called the poxB gene. The poxB gene codes for a polypeptide of 579 amino acids (SEQ ID NO. 5).

Example 10

15 Preparation of an integration vector for integration mutagenesis of the poxB gene

From the strain ATCC 13032, chromosomal DNA was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)). On the basis of the sequence of the poxB gene
20 known for *C. glutamicum* from Example 8, the following oligonucleotides were chosen for the polymerase chain reaction:

poxBint1:

5' TGC GAG ATG GTG AAT GGT GG 3'

25 poxBint2:

5' GCA TGA GGC AAC GCA TTA GC 3'

The primers shown 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.
30 A guide to methods and applications, 1990, Academic Press) with Pwo-Polymerase from Boehringer. With the aid of the polymerase chain reaction, a DNA fragment approx. 0.9 kb in

size was isolated, this carrying an internal fragment of the poxB gene and being shown in SEQ ID No. 6.

- The amplified DNA fragment was ligated with the TOPO TA Cloning Kit from Invitrogen Corporation (Carlsbad, CA, USA; Catalogue Number K4500-01) in the vector pCR2.1-TOPO (Mead at al. (1991) Bio/Technology 9:657-663). The E. coli strain DH5 α was then electroporated with the ligation batch (Hanahan, In: DNA cloning. A practical approach. Vol.I. IRL-Press, Oxford, Washington DC, USA, 1985). Selection for plasmid-carrying cells was made by plating out the transformation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been supplemented with 25 mg/l kanamycin. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction with the restriction enzyme EcoRI and subsequent agarose gel electrophoresis (0.8%). The plasmid was called pCR2.1poxBint (Figure 5).
- Plasmid pCR2.1poxBint has been deposited in the form of the strain Escherichia coli DH5 α /pCR2.1poxBint as DSM 13114 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

Example 11

Integration mutagenesis of the poxB gene in the lysine producer DSM 5715

- The vector pCR2.1poxBint mentioned in Example 10 was electroporated by the electroporation method of Tauch et al. (FEMS Microbiological Letters, 123:343-347 (1994)) in Corynebacterium glutamicum DSM 5715. Strain DSM 5715 is an AEC-resistant lysine producer. The vector pCR2.1poxBint

cannot replicate independently in DSM5715 and is retained in the cell only if it has integrated into the chromosome of DSM 5715. Selection of clones with pCR2.1poxBint integrated into the chromosome was carried out by plating out the electroporation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which had been supplemented with 15 mg/l kanamycin. For detection of the integration, the poxBint fragment was labelled with the Dig hybridization kit from Boehringer by the method of "The DIG System Users Guide for Filter Hybridization" of Boehringer Mannheim GmbH (Mannheim, Germany, 1993). Chromosomal DNA of a potential integrant was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)) and in each case cleaved with the restriction enzymes SalI, SacI and HindIII. The fragments formed were separated by agarose gel electrophoresis and hybridized at 68°C with the Dig hybridization kit from Boehringer. The plasmid pCR2.1poxBint mentioned in Example 9 had been inserted into the chromosome of DSM5715 within the chromosomal poxB gene. The strain was called DSM5715::pCR2.1poxBint.

Example 12

Effect of over-expression of the zwf gene with simultaneous elimination of the poxB gene on the preparation of lysine

12.1 Preparation of the strain DSM5715::pCR2.1poxBint/pEC-T18mob2zwf

The strain DSM5715::pCR2.1poxBint was transformed with the plasmid pEC-T18mob2zwf using the electroporation method described by Liebl et al., (FEMS Microbiology Letters, 53:299-303 (1989)). Selection of the transformants took place on LBHIS agar comprising 18.5 g/l brain-heart infusion broth, 0.5 M sorbitol, 5 g/l Bacto-tryptone, 2.5 g/l Bacto-yeast extract, 5 g/l NaCl and 18 g/l Bacto-

agar, which had been supplemented with 5 mg/l tetracycline and 25 mg/l kanamycin. Incubation was carried out for 2 days at 33°C.

Plasmid DNA was isolated in each case from a transformant
5 by conventional methods (Peters-Wendisch et al., 1998, Microbiology 144, 915-927), cleaved with the restriction endonucleases XbaI and KpnI, and the plasmid was checked by subsequent agarose gel electrophoresis. The strain obtained in this way was called DSM5715:pCR2.1poxBint/pEC-
10 T18mob2zwf.

12.2 Preparation of L-lysine

The *C. glutamicum* strain DSM5715::pCR2.1poxBint/pEC-T18mob2zwf obtained in Example 12.1 was cultured in a nutrient medium suitable for the production of lysine and
15 the lysine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with tetracycline (5 mg/l) and kanamycin (25 mg/l)) for 24 hours
20 at 33°C. The comparison strains were supplemented according to their resistance to antibiotics. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the preculture.

Medium Cg III

NaCl	2.5 g/l
Bacto-Peptone	10 g/l
Bacto-Yeast extract	10 g/l
Glucose (autoclaved separately)	2% (w/v)

The pH was brought to pH 7.4

Tetracycline (5 mg/l) and kanamycin (25 mg/l) were added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660nm) of the main culture was 0.1. Medium MM was used for the main culture.

Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	58 g/l
$(\text{NH}_4)_2\text{SO}_4$	25 g/l
KH_2PO_4	0.1 g/l
$\text{MgSO}_4 \cdot 7 \text{ H}_2\text{O}$	1.0 g/l
$\text{CaCl}_2 \cdot 2 \text{ H}_2\text{O}$	10 mg/l
$\text{FeSO}_4 \cdot 7 \text{ H}_2\text{O}$	10 mg/l
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine (sterile-filtered)	0.1 g/l
CaCO_3	25 g/l

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the CaCO_3 autoclaved in the dry state.

- 5 Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Tetracycline (5 mg/l) and kanamycin (25 mg/l) were added. Culturing was carried out at 33°C and 80% atmospheric humidity.

- 10 After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

- 15 The result of the experiment is shown in Table 4.

Table 4

Strain	OD	L-Lysine HCl g/l
DSM5715	10.8	16.0
DSM5715/pEC-T18mob2zwf	8.3	17.1
DSM5715::pCR2.1poxBint	7.1	16.7
DSM5715::pCR2.1poxBint/ pEC-Tmob2zwf	7.8	17.7

PCT

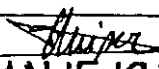
990239 BT

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1-1	page	10
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1-3	Identification of Deposit	
1-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	20 January 2000 (20.01.2000)
1-3-4	Accession Number	DSMZ 13244
1-4	Additional Indications	NONE
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Patent claims

1. A process for the preparation of L-amino acids by
fermentation of coryneform bacteria
5 w h i c h c o m p r i s e s
carrying out the following steps:
 - a) fermentation of the desired L-amino acid-
producing bacteria in which at least the zwf gene
is amplified,
 - 10 b) concentration of the L-amino acid in the medium
or in the cells of the bacteria and
 - c) isolation of the L-amino acid produced.
2. The process as claimed in claim 1,
w h e r e i n
15 bacteria in which further genes of the biosynthesis
pathway of the desired L-amino acid are additionally
amplified, in particular over-expressed, are employed.
3. The process as claimed in claim 1,
w h e r e i n
20 coryneform bacteria which prepare L-threonine, L-lysine
or L-tryptophan are used.
4. The process as claimed in claim 3,
w h e r e i n
coryneform bacteria which prepare L-lysine are used.
- 25 5. A process for the fermentative preparation of L-lysine
as claimed in claim 2,
w h e r e i n
in the coryneform microorganisms which in particular
already produce L-lysine, one or more genes chosen
30 from the group consisting of

- 5.1 the dapA gene which codes for dihydrodipicolinate synthase,
- 5.2 the lysC gene which codes for a feed back resistant aspartate kinase,
- 5 5.3 the gap gene which codes for glycerolaldehyde 3-phosphate dehydrogenase,
- 5.4 the pyc gene which codes for pyruvate carboxylase,
- 5.5 the tkt gene which codes for transketolase,
- 10 5.6 the gnd gene which codes for glucose 6-phosphate dehydrogenase,
- 5.7 the lysE gene which codes for lysine export,
- 5.8 the zwf gene,
- 5.9 the eno gene which codes for enolase
- 15 is or are amplified or over-expressed at the same time.
6. A process for the fermentative preparation of L-threonine as claimed in claim 2,
w h e r e i n
- 20 in the coryneform microorganisms which in particular already produce L-threonine, one or more genes chosen from the group consisting of
- 6.1 the hom gene which codes for homoserine dehydrogenase or the hom^{dr} allele which codes for
- 25 a "feed back resistant" homoserine dehydrogenase,
- 6.2 the gap gene which codes for glycerolaldehyde 3-phosphate dehydrogenase,

6.3 the pyc gene which codes for pyruvate carboxylase,

6.4 the mgo gene which codes for malate:quinone oxidoreductase,

5 6.5 the tkt gene which codes for transketolase,

6.6 the zwf gene which codes for glucose 6-phosphate dehydrogenase,

6.7 the thrE gene which codes for threonine export,

6.8 the zwal gene,

10 6.9 the eno gene which codes for enolase

is or are amplified, in particular over-expressed, at the same time.

7. The process as claimed in claim 2,
w h e r e i n

15 for the preparation of L-amino acids, in particular L-lysine or L-threonine, bacteria in which one or more genes chosen from the group consisting of,

7.1 the pck gene which codes for phosphoenol pyruvate carboxykinase,

20 7.2 the pgi gene which codes for glucose 6-phosphate isomerase

7.3 the poxB gene which codes for pyruvate oxidase or

7.4 the zwa2 gene

is or are attenuated at the same time, are fermented.

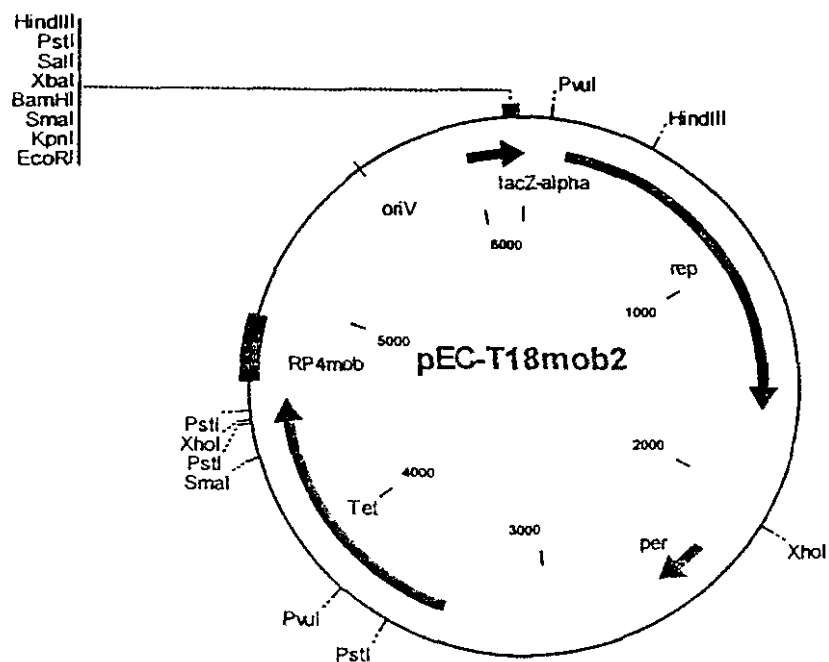
25 8. The process as claimed in claims 2 to 6,
w h e r e i n
to achieve the amplification, the number of copies of

the genes or nucleotide sequences is increased by transformation of the microorganisms with plasmid vectors which carry these genes or nucleotide sequences.

- 5 9. The plasmid vector pEC-T18mob2 deposited under the designation DSM13244 in E.coli K-12 DH5 α , shown in Figure 2.
- 10 10. A coryneform microorganism, in particular of the genus Corynebacterium, transformed by the introduction of
10 the plasmid vector as claimed in claim 9, which additionally contains the zwf gene.

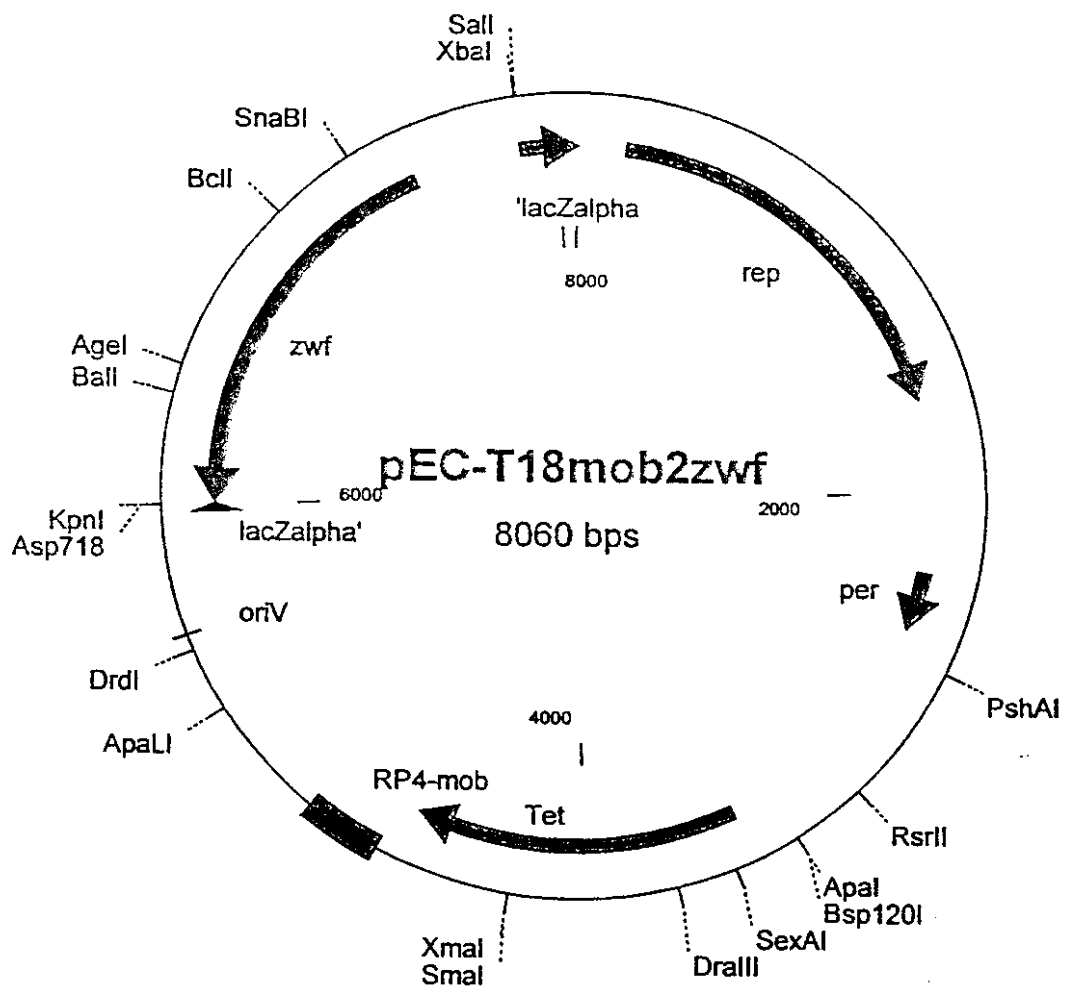
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Figure 1: Map of the plasmid pEC-T18mob2



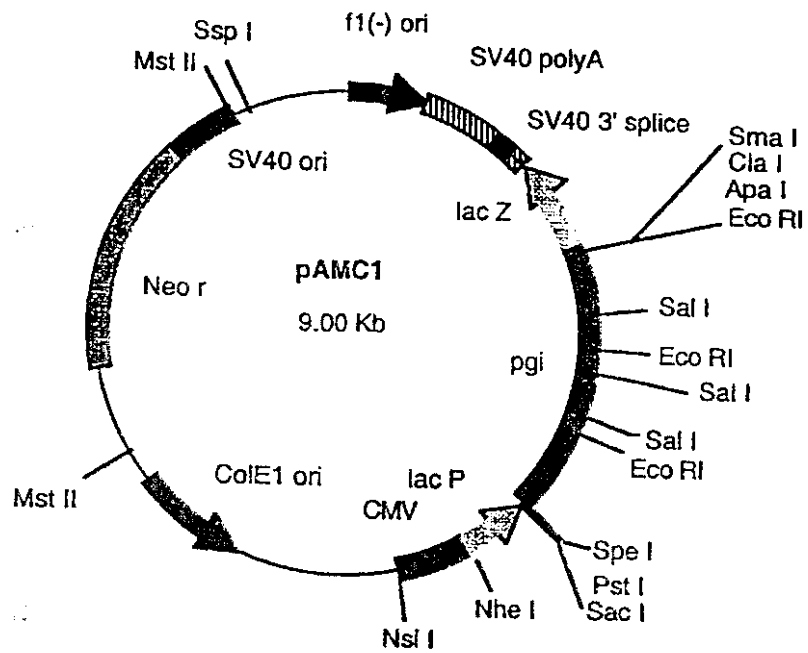
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Figure 2: Map of the plasmid pEC-T18mob2zwf



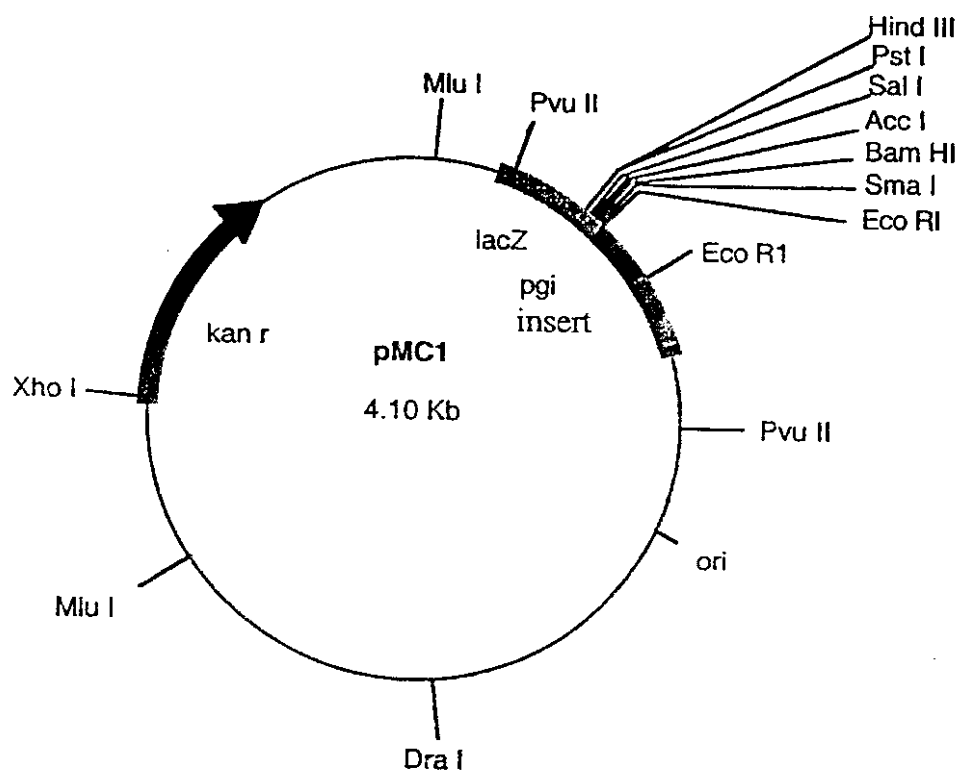
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Figure 3



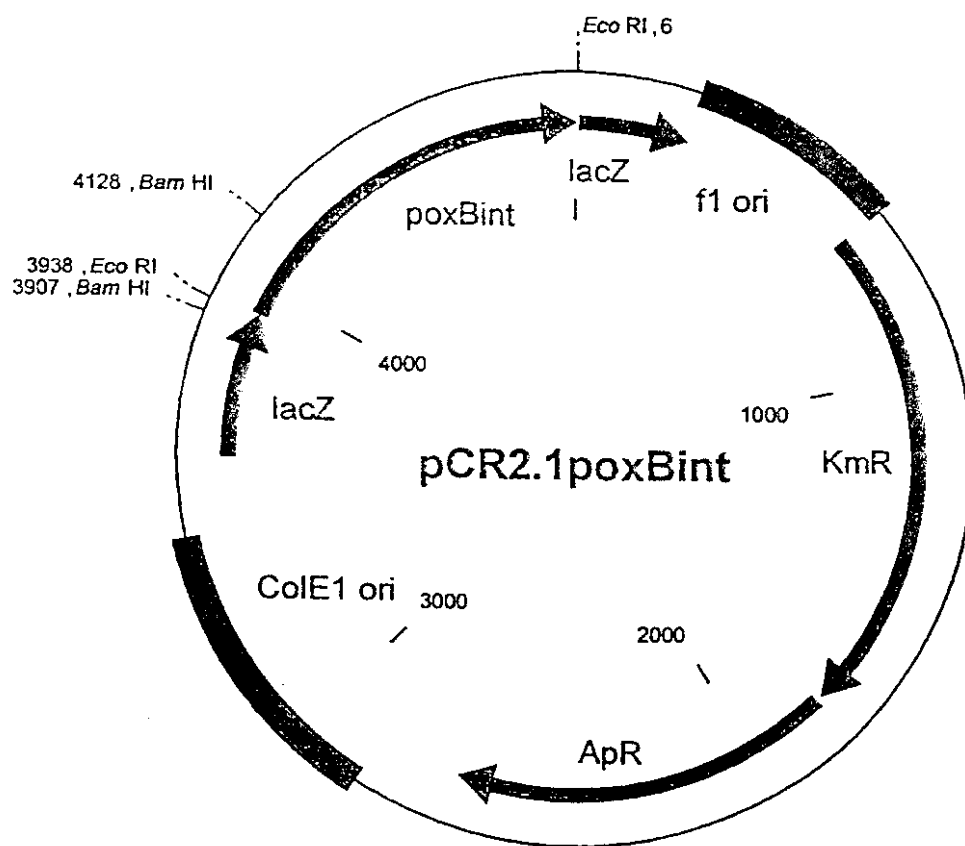
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Figure 4:



5/5

Figure 5:



SEQUENCE PROTOCOL

<110> Degussa-Hüls AG

National University of Ireland, Galway

Forschungszentrum Jülich GmbH

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L-amino acids using the zwf gene.

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10 ctc gtg gag gga cag cca gaa ttt ggt act gac cat gag gaa gtg aat 1793
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15 ttc gca gag att gcg gcg gct gcg ggt atc aaa tcg gta cgc atc acc 1841
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gat ccg aag aaa gtt cgc gag cag cta gct gag gca ttg gca tat cct 1889
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20 gga cct gta ctg atc gat atc gtc acg gat cct aat gcg ctg tcg atc 1937
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25 cca cca acc atc acg tgg gaa cag gtc atg gga ttc agc aag gcg gcc 1985
 Pro Pro Thr Ile Thr Trp Glu Gln Val Met Gly Phe Ser Lys Ala Ala
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30 acc cga acc gtc ttt ggt gga gga gta gga gcg atg atc gat ctg gcc 2033
 Thr Arg Thr Val Phe Gly Gly Gly Val Gly Ala Met Ile Asp Leu Ala
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35 cgt tcg aac ata agg aat att cct act cca tgatgattga tacacctgct 2083
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 35 40 45

55 Glu Glu Ala Ala Ala Phe Ala Ala Gly Ala Glu Ser Leu Ile Thr Gly
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10

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11

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 5 Asp Phe Val Gly Ser Phe Arg His Gly Thr Met Ala Asn Ala Leu Pro
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 aaatcaccca tcgggcatgc gctgggtggt aagcagtaca tccagcatga gaatccggtt 360
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12

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Information on patent family members

PCT/EP 00/06303

Form PCT/ISA/210 (patent family annex) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/06303

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/53 C12N15/77 C12P13/08

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, WPI Data, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP 09 224661 A (MITSUBISHI CHEM CORP) 2 September 1997 (1997-09-02) cited in the application the whole document & DATABASE WPI Section Ch, Week 199745 Derwent Publications Ltd., London, GB; Class B04, AN 1997-484096 HATAKEYAMA KAZUHISA ET AL.: "Glucose-6-phosphate dehydrogenase " & JP 09 224661 A (MITSUBISHI CHEM CORP), 2 September 1997 (1997-09-02) abstract — -/-	9,10

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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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
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search

13 February 2001

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/06303

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	L. EGGELING ET AL.: "L-Glutamate and L-Lysine: traditional products with impetuous developments" APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 52, August 1999 (1999-08), pages 146-153, XP000979507 the whole document	1-10

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[51] Int. Cl.⁷
C12N 15/53
C12N 15/77 C12P 13/08

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[86] 国际申请 PCT/EP00/06303 2000.7.5
[87] 国际公布 WO01/70995 英 2001.9.27
[85] 进入国家阶段日期 2001.11.12
[71] 申请人 德古萨股份公司
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权利要求书 3 页 说明书 38 页 附图页数 5 页

[54] 发明名称 经扩增 zwf 基因发酵制备 L-氨基酸的方法

[57] 摘要

本发明涉及一种通过发酵棒状细菌制备 L-氨基酸的方法,其包括进行以下步骤:a) 发酵产生所需 L-氨基酸的细菌,该细菌中至少 zwf 基因是扩增的,b) 浓缩培养基或细菌细胞中的 L-氨基酸,及 c) 分离产生的 L-氨基酸。

I S S N 1 0 0 0 - 4 2 7 4

权 利 要 求 书

1. 一种通过发酵棒状细菌制备 L-氨基酸的方法, 包括进行以下步骤:

a) 发酵产生所需 L-氨基酸的细菌, 该细菌中至少 zwf 基因是扩增的,

b) 浓缩培养基或细菌细胞中的 L-氨基酸, 及

c) 分离产生的 L-氨基酸。

2. 权利要求 1 的方法, 其中使用的细菌中, 所需 L-氨基酸的生物合成途径的其它基因也被扩增, 尤其是过表达。

3. 权利要求 1 的方法, 其中使用生产 L-苏氨酸, L-赖氨酸, 或 L-色氨酸的棒状细菌。

4. 权利要求 3 的方法, 其中使用生产 L-赖氨酸的棒状细菌。

5. 如权利要求 2 发酵制备 L-赖氨酸的方法, 其中在尤其已经产生 L-赖氨酸的棒状微生物中, 选自以下一组的一或多个基因被同时扩增或过表达:

5.1 编码二氢-2, 6-吡啶二羧酸合酶的 dapA 基因,

5.2 编码反馈抗性天冬氨酸激酶的 lysC 基因,

5.3 编码甘油醛 3-磷酸脱氢酶的 gap 基因,

5.4 编码丙酮酸羧化酶的 pyc 基因,

5.5 编码转酮酶的 tkt 基因,

5.6 编码 6-磷酸葡萄糖酸脱氢酶的 gnd 基因,

5.7 编码赖氨酸输出的 lysE 基因,

5.8 zwf 基因,

5.9 编码烯醇化酶的 eno 基因。

6. 如权利要求 2 发酵制备 L-苏氨酸的方法, 其中在尤其已经产生 L-苏氨酸的棒状微生物中, 选自以下一组的一或多个基因被同时扩增或过表达:

6.1 编码高丝氨酸脱氢酶的 *hom* 基因或编码“反馈抗性”高丝氨酸脱氢酶的 *hom^{dr}* 等位基因，

6.2 编码甘油醛-3-磷酸脱氢酶的 *gap* 基因，

6.3 编码丙酮酸羧化酶的 *pyc* 基因，

6.4 编码苹果酸:醌氧化还原酶的 *mqo* 基因，

6.5 编码转酮酶的 *tkt* 基因

6.6 编码葡萄糖-6-磷酸脱氢酶的 *zwf* 基因，

6.7 编码苏氨酸输出的 *thrE* 基因，

6.8 *zwa1* 基因，

6.9 编码烯醇化酶的 *eno* 基因。

7. 权利要求 2 的方法，其中为制备 L-氨基酸尤其 L-赖氨酸或 L-苏氨酸，发酵这样的细菌，该细菌中选自以下一组的一或多个基因同时被弱化：

7.1 编码烯醇丙酮酸磷酸羧激酶的 *pck* 基因，

7.2 编码葡萄糖-6-磷酸异构酶的 *pgi* 基因，

7.3 编码丙酮酸氧化酶的 *poxB* 基因，

7.4 *zwa2* 基因。

8. 权利要求 2-6 任一项的方法，其中为实现扩增，所述基因或核苷酸序列的拷贝数，通过用携带这些基因或核苷酸序列的质粒载体转化微生物而提高。

9. 图 2 所示的质粒载体 pEC-T18mob2，其以在大肠杆菌 K-12 DH5 α 中的形式保藏，保藏号 DSM 13244。

10. 一种通过导入权利要求 9 的质粒载体转化的棒状微生物，尤其棒杆菌属，该质粒载体另外还含有 *zwf* 基因。

说明书

经扩增 zwf 基因发酵制备 L-氨基酸的方法

本发明涉及用棒状细菌经发酵制备 L-氨基酸，特别是 L-赖氨酸，L-苏氨酸和 L-色氨酸的方法，其中棒状细菌中至少 zwf 基因是扩增的。

现有技术

L-赖氨酸，L-苏氨酸和 L-异亮氨酸用于用于动物营养，人用药物及制药工业。

已知氨基酸可通过棒状细菌菌株，尤其谷氨酸棒杆菌的发酵而生产。由于 L-氨基酸的极其重要性，已持续进行改良生产方法的尝试。生产方法的改良可涉及发酵措施，如搅拌和供氧，或营养培养基的组分如发酵期间的糖浓度，或产物形式的加工方法，例如通过离子交换层析，或微生物本身的固有生产性质。

为改良这些微生物的生产性质，可使用诱变，选择及突变体选择等方法，以此方法可获得对抗代谢物如苏氨酸类似物 α -氨基- β -羟戊酸（AHV）有抗性或重要的调节氨基酸缺陷的并产生 L-氨基酸如苏氨酸的菌株。

一段时间以来，重组 DNA 技术的方法也用于改良生产 L-氨基酸的谷氨酸棒杆菌菌株。

发明目的

本发明目的是提供新的用棒状细菌经发酵制备 L-氨基酸的改良方法。

发明描述

L-氨基酸用于人用药物及制药工业，食品工业，特别是动物营养。因此提供生产这些氨基酸的新改良方法总是令人感兴趣的。

本发明提供了用棒状细菌经发酵生产 L-氨基酸,尤其 L-赖氨酸, L-苏氨酸, 和 L-色氨酸的方法, 在该棒状细菌中编码 Zwf 蛋白的核苷酸序列 (zwf 基因) 是扩增的, 尤其是过表达的。

使用的菌株优选在 zwf 基因扩增之前已生产 L-氨基酸。

优选的实施方案见于权利要求。

文中术语“扩增”是指微生物中由相应的 DNA 编码的一或多种酶 (蛋白质) 的胞内活性的提高, 例如通过提高基因的拷贝数, 或使用强启动子或编码高活性相应酶 (蛋白质) 的基因, 及任选地组合使用这些方法。

本发明的微生物可从葡萄糖、蔗糖、乳糖、果糖、麦芽糖、糖蜜、淀粉, 纤维素或从甘油和乙醇中生产 L-氨基酸。所述微生物可以是棒状细菌的代表菌, 尤其是棒杆菌属, 在棒杆菌属中尤其应提及的是谷氨酸棒杆菌, 本领域技术人员已知其生产 L-氨基酸的能力。

以下已知的野生型菌株:

谷氨酸棒杆菌 ATCC 13032

醋谷棒杆菌 ATCC 15806

嗜乙酰乙酸棒杆菌 ATCC 13870

嗜热产氨棒杆菌 FERM BP-1539

黄色短杆菌 ATCC 14067

乳发酵短杆菌 ATCC 13869 和

扩展短杆菌 ATCC 14020

和从中获得的生产 L-氨基酸的突变株,

如生产 L-苏氨酸的菌株

谷氨酸棒杆菌 ATCC21649

黄色短杆菌 BB69

黄色短杆菌 DSM5399

乳发酵短杆菌 FERM-BP 269

乳发酵短杆菌 TBB-10

以及如生产 L-异亮氨酸的菌株

谷氨酸棒杆菌 ATCC14309

谷氨酸棒杆菌 ATCC14310

谷氨酸棒杆菌 ATCC14311

谷氨酸棒杆菌 ATCC15168

产氨棒杆菌 ATCC6871

以及如生产 L-色氨酸的菌株

谷氨酸棒杆菌 ATCC21850

谷氨酸棒杆菌 KY9218 (pKW9901)

以及如生产 L-赖氨酸的菌株

谷氨酸棒杆菌 FERM-P 1709

黄色短杆菌 FERM-P 1708

乳发酵短杆菌 FERM-P 1712

谷氨酸棒杆菌 FERM-P 6463

谷氨酸棒杆菌 FERM-P6464

谷氨酸棒杆菌 ATCC13032

谷氨酸棒杆菌 DSM58-1

谷氨酸棒杆菌 DSM12866

是棒杆菌属，尤其谷氨酸棒杆菌合适菌株的实例。

已发现在编码 Zwf 蛋白的 zwf 基因过表达之后，棒状细菌以改良方式生产 L-氨基酸，尤其 L-赖氨酸，L-苏氨酸，和 L-色氨酸。

得自遗传密码简并或由于中性功能的有义突变所导致的 zwf 基因的等位基因也可使用。

JP-A-09224661 揭示了黄色短杆菌 MJ-223 (FERM BP-1497) 的葡萄糖-6-磷酸脱氢酶基因的核苷酸序列，称为 zwf 基因。JP-A-09224661 阐述了 Zwf 多肽的 N 末端氨基酸序列为 Met Val Ile Phe Gly Val Thr Gly Asp Leu Ala Arg Lys Lys Leu。

然而，还不能证实这些序列，相反却发现以下 N 末端氨基酸序列：

Val Ser Thr Asn Thr Thr Pro Ser Ser Trp Thr Asn Pro Leu Arg Asp. N 位的缬氨酰基在翻译后修饰中可脱落，而后获得 Ser Thr Asn Thr Thr Pro Ser Ser Trp Thr Asn Pro Leu Arg Asp 作为 N 末端的氨基酸序列。

为获得扩增（如过表达），可提高相应基因的拷贝数，或可使位于结构基因上游的启动子和调节区或核糖体结合位点突变。掺入结构基因上游的表达盒以同样方式工作。通过可诱导启动子，在 L-氨基酸发酵生产期间增加表达也是可能的。延长 mRNA 寿命的措施也可改善表达。另外，通过防止酶蛋白的分解也可提高酶活性。基因或基因构建体可以不同拷贝数存在于质粒中，或可在染色体中整合与扩增。或者，通过改变培养基的组分和培养条件，也可获得相关基因的过表达。

本领域熟练技术人员从以下文献中可发现对此的详述，参见 Martin 等（生物/技术 5，137—146(1987)），Guerrero 等（基因 138，35—41(1994)），Tsuchiya 和 Morinaga（生物/技术 6,428-430(1988)），Eikmanns 等（基因 102，93—98(1991)），欧洲专利说明书 EPS0472869，美国专利 4,601,893，Schwarzer 和 Puhler（生物/技术 9，84—87(1991)），Reischeid 等（应用及环境微生物学 60，126—132(1994)），LaBarre 等（细菌学杂志 175，1001—1007(1993)），专利申请 WO96/15246，Malumbres 等（基因 134，15—24(1993)），日本特许公开 JP—A—10—229891，Jensen 和 Hammer（生物技术及生物工程 58,191-195(1998)），及已知关于遗传及分子生物学的教材。

例如，Zwf 蛋白已借助于质粒被过表达。为此使用图 1 所示的大肠杆菌—谷氨酸棒杆菌穿梭载体 pEC-T18mob2。在将 zwf 基因掺入 pEC-T18mob2 的 KpnI/SalI 切割位点之后，形成图 2 所示的质粒 pEC-T18mob2zwf。

能在谷氨酸棒杆菌中复制的其它质粒载体如 pEKEx1（Eikmanns 等，基因 102：93—98（1991）），或 pZ8-1（EP-B-0375889）也可以同样方式使用。

另外，除扩增 zwf 基因之外，特定生物合成途径，糖酵解，添补反应，戊糖磷酸途径或氨基酸输出的一或多种酶的扩增，对 L-氨基酸的生产可以是有益的。

因此，例如选自以下一组的一或多个基因可被同时扩增、尤其是过表达是以生产 L-苏氨酸：

- 编码高丝氨酸脱氢酶的 hom 基因 (Peoples 等, 分子微生物学 2, 63-72 (1988)) , 或编码“反馈抗性”高丝氨酸脱氢酶的 hom^{dr} 等位基因 (Archer 等, 基因 107, 53-59 (1991)) ,

- 编码甘油醛-3-磷酸脱氢酶的 gap 基因 (Eikamanns(1992), 细菌学杂志 174, 6076-6086) ,

- 编码丙酮酸羧化酶的 pyc 基因 (Peters-Wendisch 等, 微生物学 144: 915-927 (1998)) ,

- 编码苹果酸:醌氧化还原酶的 mqo 基因 (Molenaar 等(1998), 欧洲生物化学杂志 254, 395-403) ,

- 编码转酮酶的 tkt 基因 (欧洲分子生物学实验室 (EMBL, Heidelberg, 德国) 的数据库, 登记号 AB023377) ,

- 编码 6-磷酸葡萄糖酸脱氢酶的 gnd 基因 (JP-A-09224661) ,

- 编码苏氨酸输出的 thrE 基因 (DE 199 41 478.5; DSM12840) ,

- zwal 基因 (DE 199 59 328.0; DSM13115) ,

- 编码烯醇酶的 eno 基因 (DE:19941478.5) 。

因此，例如选自以下一组的一或多个基因可被同时扩增、尤其是过表达以生产 L-赖氨酸：

- 编码二氢-2, 6-吡啶二羧酸合酶的 dapA 基因 (EP-B-0197335) ,

- 编码反馈抗性天冬氨酸激酶的 lysC 基因 (Kalinowski 等, (1990), 分子及普通遗传学 224: 317-324) ,

- 编码甘油醛-3-磷酸脱氢酶的 gap 基因 (Eikmanns(1992), 细菌学杂志 174, 6076-6086) ,

• 编码丙酮酸羧化酶的 *pyc* 基因 (Eikmanns(1992), 细菌学杂志 174, 6076—6086),

• 编码转酮酶的 *tkt* 基因 (欧洲分子生物学实验室 (EMBL, Heidelberg, 德国) 的数据库, 登记号 AB023377),

• 编码 6-磷酸葡萄糖酸脱氢酶的 *gnd* 基因 (JP-A-09224661),

• 编码赖氨酸输出蛋白的 *lysE* 基因 (DE-A-195 48 222),

• *zwa1* 基因 (DE 199 59 328.0, DSM13115),

• 编码烯醇酶的 *eno* 基因 (DE:19947791.4)。

除扩增 *zwf* 基因之外, 同时弱化以下基因对 L-氨基酸的生产也可以是有益的:

• 编码烯醇丙酮酸磷酸羧激酶的 *pck* 基因 (DE 199 50 409.1, DSM13047),

• 编码葡萄糖-6-磷酸异构酶的 *pgi* 基因 (US 09/396478, DSM12969),

• 编码丙酮酸氧化酶的 *poxB* 基因 (DE19951975.7, DSM13114),

• *zwa 2* 基因 (DE19959327.2, DSM13113)。

除过表达 *Zwf* 蛋白之外, 消除非所需的副反应对 L-氨基酸的生产也是有益的 (Nakayama: “生产氨基酸的微生物的育种”, 微生物产物的过量产生, Krumphanzl, Sikyta, Vanek(编辑), 学术出版社, 伦敦, 英国, 1982)。

根据本发明产生的微生物可连续培养或非连续地通过分批法, 补料分批法或重复补料发批法培养, 以生产 L-氨基酸。已知培养法见于由 Chmiel(生物方法技术学 1, 生物工程入门, Gustav Fischer Verlag, Stuttgart, 1991)所著教材, 或由 Storhas(生物反应器及外周设备, Vieweg Verlag, Brunswick/Wiesbaden, 1994)所著教材中所述。

所用培养基必须以适当方式符合特定菌株的需求。关于各种微生物培养基的阐述见于美国细菌学会的“细菌学通用方法手册”(华盛顿 D.C..USA, 1981)。可使用的碳源包括糖及碳水化合物, 例如葡萄

糖，蔗糖，乳糖，果糖，麦芽糖，糖蜜，淀粉和纤维素，油和脂肪如豆油，葵花油，落花生油和椰子油，脂肪酸如棕榈酸，硬脂酸和亚油酸，醇如甘油和乙醇，及有机酸如乙酸。这些物质可单独或混合使用。可使用的氮源包括含氮的有机化合物如胨，酵母提取物，肉膏，麦芽提取物，玉米浸液，大豆粉和尿素，或无机化合物如硫酸铵，氯化铵，磷酸铵，碳酸铵和硝酸铵。氮源可单独或混合使用。可使用的磷源包括磷酸，磷酸二氢钾或磷酸氢二钾，或相应钠盐。培养基另外还必须含有为生长所需的金属盐如硫酸镁或硫酸铁。最后，除了上述物质之外，可使用生长必需物质如氨基酸和维生素。此外，可将适当前体加入培养基中，上述物质可以单批形式或在培养期间适当补加。

可以适当方式加入碱性化合物如 NaOH, KOH, 氨或氨水，或酸性化合物如磷酸或硫酸，以调节培养物的 PH 值。抗泡沫剂例如脂肪酸聚乙二醇可用于控制泡沫产生。适当的选择性作用物质例如抗生素，可加入培养基中以保持质粒的稳定性，氧气或含氧混合气，例如空气，可充入培养物中以保持有氧条件。培养温度通常在 20℃~45℃，优选 25℃—40℃。持续培养直至 L-氨基酸形成最大量。此目的通常在 10~160 小时范围内达到。

L-氨基酸的分析可通过阴离子交换层析，随后经过如 Spackman 等（分析化学 30(1958)1190）所述茚三酮衍生化作用进行，或通过如 Lindroth 等（分析化学（1979）51: 1167—1174）所述反相 HPLC 进行。

以下微生物根据布达佩斯条约，已保藏在德意志微生物保藏中心（DSMZ，不伦瑞克，德国）：

大肠杆菌 K-12 DH5 α /pEC-T18mob2，保藏号 DSM13244。

本发明包括以下附图：

图 1：质粒 pEC-T18mob2 图。

图 2：质粒 pEC-T18mob2zwf 图。

图 3：质粒 pAMC1 图。

图 4: 质粒 pMC1 图。

图 5: 质粒 pCR2.1poxBint 图。

所述碱基对数目是根据再现性所得的大约值。

图中所采用的缩写和符号有如下含义:

图 1 和图 2 所用缩写有如下含义:

Tet: 四环素抗性基因

oriV: 大肠杆菌的质粒编码的复制源点

RP4mob: 转移质粒的 mob 区域

rep: 谷氨酸棒杆菌质粒 pGA1 的质粒编码的复制源点

per: pGA1 的控制拷贝数的基因

lacZ α : β 一半乳糖苷酶基因的 lacZ α 基因片段 (N 末端)

lacZ α ': lacZ α 基因片段的 5' 末端

'lacZ α : lacZ α 基因片段的 3' 末端

图 3 和图 4 所用缩写有如下含义:

Neo^r: 新霉素/卡那霉素抗性

ColE1 ori: 质粒 ColE1 的复制源点

CMV: 巨细胞病毒的启动子

lacP: 乳糖启动子

pgi: 磷酸葡萄糖异构酶基因

lacZ: β 一半乳糖苷酶基因的一部分

SV40 3' 剪接: 猴病毒 40 的 3' 剪接位点

SV40 polyA: 猴病毒 40 的聚腺苷酸化位点

f1 (–) ori: 丝状噬菌体 f1 的复制源点

SV40ori: 猴病毒 40 的复制源点

Kan^r: 卡那霉素抗性基因

pgi 插入: pgi 基因的内部片段

ori: 质粒 pBGS8 的复制源点

图 5 所用缩写有如下含义:

ColE1 ori: 质粒 ColE1 的复制源点

lacZ: lacZ α 基因片段的克隆残存片段

f1 ori: 噬菌体 f1 的复制源点

KmR: 卡那霉素抗性

ApR: 氨苄青霉素抗性

poxBint: poxB 基因的内部片段

本领域已知各种限制酶缩写（如 BamHI, EcoRI 等）的含义，并例如 Kessler 和 Holtke（基因 47, 1—153（1986））或 Poberts 等（核酸研究 27, 312—313（1999））所综述。

实施例

以下实施例进一步举例说明了本发明。所使用的分子生物学技术如质粒 DNA 分离，限制酶处理，连接，大肠杆菌的标准转化等方法，除非特别说明，均如 Sambrook 等所述进行（分子克隆实验手册（1989），冷泉港实验室，美国）。

实施例 1: Zwf 蛋白的表达

1.1 制备质粒 pEC-T18mob2

根据现有技术构建大肠杆菌—谷氨酸棒杆菌穿梭载体 pEC-T18mob2。此载体含有质粒 pGA1 的包括复制效应子 per 的复制区 rep（US—A—5, 175, 108; Nesvera 等，细菌学杂志 179, 1525—1532（1997）），质粒 pAG1 的四环素抗性基因 tetA(Z) (USA-5158891; 国家生物技术信息中心（NCBI, Bethesda, MD, USA）的基因文库，登记号 AF121000），质粒 pMB1（Sutcliffe, 关于定量生物学的冷泉港讨论会 43, 77—90（1979））的复制起点 oriV，包括 lac 启动子及多克隆位点(mcs)(Norlander, J.M.等, 基因 26, 101—106(1983))的 lacZ α 基因片段，和质粒 RP4（Simon 等，（1983）生物/技术 1: 784—791）

的 mob 区域。将此构建的载体转化入大肠杆菌菌株 DH5 α 中 (Brown 编辑, 分子生物学实验指导, 生物科学出版社, 英国牛津, 1991)。通过将转化混合物铺板于补加 5mg/l 四环素的 LB 平板 (Sambrook 等, 分子克隆实验手册, 第二版, 冷泉港实验室出版社, 冷泉港, N.Y.) 上, 选择携带质粒的细胞。借助于 Qiagen 公司的 QIAprep Spin 微量制备试剂盒从转化体中分离质粒 DNA, 并用限制酶 EcoRI 和 HindIII 限制, 及随后经琼脂糖凝胶电泳 (0.8%) 检测。

此质粒称为 pEC-T18mob2 并示于图 1。其以大肠杆菌 K-12 菌株 DH5 α /pEC-T18mob2 形式保藏在德意志微生物保藏中心 (DSMZ) (Braunschweig, 德国), 保藏号 DSM 13244。

1.2 制备质粒 pEC-T18mob2zwf

谷氨酸棒杆菌 ATCC13032 的基因, 首先利用以下寡核苷酸引物, 通过聚合酶链反应 (PCR) 扩增:

zwf 正向引物: 5' -TCG ACG CGG TTC TGG AGC AG-3'

zwf 反向引物: 5' -CTA AAT TAT GGC CTG CGC CAG-3'

PCR 反应在存在 200 μ M 脱氧核苷三磷酸 (dATP, dCTP, dGTP, dTTP) 的情况下进行 30 次循环, 用 1 μ M 相应的寡核苷酸, 100ng 谷氨酸棒杆菌 ATCC13032 的染色体 DNA, 1/10 体积的 10 倍反应缓冲液和 2.6 单位的热稳定的 Taq-/Pwo-DNA 聚合酶混合物 (Roche Diagnostics 的膨胀高保真 PCR 系统, 德国曼海姆), 在热循环仪 (PTC-100, MJ 研究公司, 美国 Watertown) 中, 在以下条件下进行反应: 94 $^{\circ}$ C 30 秒, 64 $^{\circ}$ C 1 分钟, 68 $^{\circ}$ C 3 分钟。

接着将大约 1.8kb 的扩增的片段, 借助于 SureClone 连接试剂盒 (Amersham Pharmacia Biotech, Uppsala, Sweden), 根据说明书连接于载体 pUC18 的 SmaI 切割位点中。用全部连接混合物转化大肠杆菌 DH5 α mcr (Grant 等, 美国科学院院报 (1990) 87: 4645-4649)。在含有 50 μ g/ml 羧苄青霉素的 LB 琼脂平板上, 借助于其羧苄青霉素

抗性鉴别转化体。从 7 个转化体中制备质粒，并通过限制分析检测作为插入体的 1.8kb 的 PCR 片段的存在情况。以此方式形成的重组质粒在下文称为 pUC18zwf。

为构建 pEC-T18mob2zwf，将 pUC18zwf 用 KpnI 和 SalI 消化，并借助于 Macherey-Nagel (Duren, 德国) 的 NucleoSpin 提取试剂盒，根据说明书分离产物，然后与已用 KpnI 和 SalI 酶切并去磷酸化的载体 pEC-T18mob 连接。用全部连接混合物转化大肠杆菌 DH5 α mcr (Grant 等, 美国科学院院报 (1990) 87: 4645—4649)。在含有 5 μ g/ml 四环素的 LB 琼脂平板上，借助于其四环素抗性鉴别转化体。从 12 个转化体中制备质粒，并通过限制分析检测作为插入体的 1.8kb 的 PCR 片段的存在情况。以此方式分离的重组质粒之一称为 pEC-T18mob2zwf (图 2)。

实施例 2: 制备具有扩增的 zwf 基因的氨基酸生产菌株

生产 L-赖氨酸的菌株谷氨酸棒杆菌 DSM5715 见于 EP-B-0435132 所述，生产 L-苏氨酸的菌株黄色短杆菌 DSM5399 见于 EP-B-0385940 所述。这两个菌株根据布达佩斯条约均保藏在德国微生物和细胞培养中心 (Braunschweig, 德国)。

2.1 制备菌株 DSM5715/pEC-T18mob2zwf 和 DSM5399/pEC-T18mob2zwf

通过 Liebl 等所述的电穿孔方法 (FEMS 微生物通信, 53: 299—303 (1989)) 用质粒 pEC-T18mob2zwf 转化菌株 DSM5715 和 DSM5399。在含有 18.5g/l 脑心浸液, 0.5M 山梨糖醇, 5g/l Bacto-tryptone, 2.5g/l Bacto-酵母膏, 5g/l NaCl 和 18g/l Bacto-琼脂, 并已补加 5mg/l 四环素的 LBHIS 琼脂上, 选择转化体。在 33°C 温育 2 天。

用常规方法 (Peters-Wendish 等, 1998, 微生物学 144, 915—927) 从转化子中分离质粒 DNA, 用限制性内切酶 XbaI 和 KpnI 酶切, 并

随后经琼脂糖凝胶电泳检查质粒。以此方式获得的菌株称为 DSM5715/pEC-T18mob2zwf 和 DSM5399/pEC-T18mob2zwf。

2.2 制备 L-苏氨酸

将实施例 2.1 中所得的谷氨酸棒杆菌菌株 DSM5399/ pEC-T18mob2zwf, 在适于生产苏氨酸的营养培养基中培养, 并确定培养物上清中苏氨酸含量。

首先, 在 33℃ 在具有适当抗生素的琼脂板 (含 5mg/l 四环素的脑心琼脂) 上培养菌株 24 小时。此琼脂板培养物用于接种预培养物 (10ml 培养基于 100ml 锥形瓶)。用于预培养的培养基是完全培养基 CgIII。

培养基 CgIII

NaCl	2.5g/l
Bacto-肽胨	10g/l
Bacto-酵母膏	10g/l
葡萄糖 (单独高压灭菌)	2%(w/v)

pH 调节为 7.4。

加入四环素 (5mg/l)。在摇床上于 33℃ 以 240rpm 振荡培养预培养物 16 小时。此预培养物接种主培养物, 从而主培养物的起始 OD (波长 660nm) 为 0.1。培养基 MM 用作主培养物。

培养基 MM:

CSL (玉米浆)	5g/l
MOPS(吗啉代丙磺酸)	20g/l
葡萄糖 (单独高压灭菌)	50g/l
(NH ₄) ₂ SO ₄	25g/l
KH ₂ PO ₄	0.1g/l
MgSO ₄ · 7H ₂ O	1.0g/l
CaCl ₂ · 2H ₂ O	10mg/l
FeSO ₄ · 7H ₂ O	10mg/l

MnSO ₄ · H ₂ O	5.0mg/l
生物素（过滤灭菌）	0.3mg/l
硫胺素 · HCl（过滤灭菌）	0.2mg/l
L-亮氨酸（过滤灭菌）	0.1g/l
CaCO ₃	25g/l

用氨水将 CSL, MOPS 和盐溶液调至 PH7 并高压灭菌。然后加入无菌的底物和维生素溶液，并加入干态高压灭菌的 CaCO₃。

以在具有档板的 100ml 锥形瓶中的 10ml 体积进行培养。加入四环素（5mg/l）。在 33℃ 和 80% 大气湿度下进行培养。

72 小时后，用 Biomek 1000(Beckmann Instruments GmbH, Munich) 测定在 660nm 波长的 OD。用 Eppendorf-BioTronik 公司的氨基酸分析仪（汉堡，德国）经离子交换层析和用茚三酮检测柱后衍生化而确定苏氨酸生成量。

实验结果示于表 1。

表 1

菌株	OD	L-苏氨酸 (g/l)
DSM5399	12.3	0.74
DSM5399/pEC-T18mob2zwf	10.2	1.0

2.3 制备 L-赖氨酸

将实施例 2.1 中所得的谷氨酸棒杆菌菌株 DSM5715/pEC-T18mob2zwf，在适于生产赖氨酸的营养培养基中培养，并确定培养物上清中赖氨酸含量。

首先，在 33℃ 在具有适当抗生素的琼脂板（含 5mg/l 四环素的脑心琼脂）上培养菌株 24 小时。此琼脂板培养物用于接种预培养物（10ml 培养基于 100ml 锥形瓶）。用于预培养的培养基是完全培养基 CgIII。

培养基 CgIII

NaCl	2.5g/l
Bacto-肽胨	10g/l
Bacto-酵母膏	10g/l
葡萄糖（单独高压灭菌）	2%(w/v)

pH 调节为 7.4。

加入四环素（5mg/l）。在摇床上于 33℃ 以 240rpm 振荡培养预培养物 16 小时。此预培养物接种主培养物，从而主培养物的起始 OD（波长 660nm）为 0.1。培养基 MM 用作主培养物。

培养基 MM:

CSL（玉米浆）	5g/l
MOPS(吗啉代丙磺酸)	20g/l
葡萄糖（单独高压灭菌）	58g/l
(NH ₄) ₂ SO ₄	25g/l
KH ₂ PO ₄	0.1g/l
MgSO ₄ · 7H ₂ O	1.0g/l
CaCl ₂ · 2H ₂ O	10mg/l
FeSO ₄ · 7H ₂ O	10mg/l
MnSO ₄ · H ₂ O	5.0mg/l
生物素（过滤灭菌）	0.3mg/l
硫胺素 · HCl（过滤灭菌）	0.2mg/l
L-亮氨酸（过滤灭菌）	0.1g/l
CaCO ₃	25g/l

用氨水将 CSL, MOPS 和盐溶液调至 PH7 并高压灭菌。然后加入无菌的底物和维生素溶液，并加入干态高压灭菌的 CaCO₃。

以在具有档板的 100ml 锥形瓶中的 10ml 体积进行培养。加入四环素（5mg/l）。在 33℃ 和 80% 大气湿度下进行培养。

72 小时后，用 Biomek 1000(Beckmann Instruments GmbH, Munich) 测定在 660nm 波长的 OD。用 Eppendorf-BioTronik 公司的氨基酸分析

仪（汉堡，德国）经离子交换层析和用茚三酮检测柱后衍生化而确定赖氨酸生成量。

实验结果示于表 2。

菌株	OD (660)	赖氨酸 HCl (g/l)
DSM5715	10.8	16.0
DSM5715/pEC-T18mob2zwf	7.1	13.7

实施例 3：谷氨酸棒杆菌菌株 AS019 基因文库的构建

用 λ Zap Express™ 系统(Short 等, (1988)核酸研究, 16: 7583-7600)构建了谷氨酸棒杆菌菌株 AS019 (Yoshihama 等, 细菌生物学杂志 162, 591-597 (1985)) 的 DNA 文库, 如 O'Donohue 所述 (O'Donohue, M. (1997), 谷氨酸棒杆菌四种常见芳香族氨基酸生物合成基因的克隆与分子分析, 博士论文, 国立爱尔兰大学, Galway)。 λ Zap Express™ 系统试剂盒购自 Stratagene(Stratagene, 11011 North Torrey Pines Rd., La Jolla, California 92037) 并按说明书使用。AS019-DNA 用限制性内切酶 Sau3A 消化后连接到用 BamHI 消化并去磷酸化的 λ Zap Express™ 臂上。

实施例 4：pgi 基因的克隆和测序

1. 克隆

将携带如 Kupor 和 Fraenkel 所述的 pgi 和 pgl 基因中的突变的大肠杆菌菌株 DF1311 (细菌学杂志 100: 1296-1301 (1969)), 用实施例 3 所述的大约 500ng 的 AS019 λ Zap Express™ 质粒文库转化。在含有 50mg/l 卡那霉素的 M9 基本培养基 (Sambrook 等, 分子克隆实验手册, 冷泉港实验室, 美国) 上, 在 37℃ 温育 48 小时选择转化体。根据 Birnboim 和 Doly 所述 (核酸研究 7: 1513-1523 (1979)) 从

一个转化体中分离质粒 DNA，并称为 pAMC1（图 3）。

2. 测序

通过 Sanger 等的方法（美国科学院院报 74, 5463—5467（1977）），用经有色荧光标记而差示标记的引物，对 pAMC1 中克隆的插入体进行序列分析，使用的是 Perkin Elmer 应用生物系统的 ABI prism 310 遗传分析仪（Perkin Elmer 公司，Norwalk, Connecticut, 美国），和也是 Perkin Elmer 的 ABI prism Big Dye™ 终止循环测序反应试剂盒。

用得自 Pharmacia Biotech（St.Albans, Herts, AL1 3AW, 英国）的通用正向和 M13 反向引物，进行初始序列分析：

通用正向引物：GTA ATA CGA CTC ACT ATA GGG C

M13 反向引物：GGA AAC AGC TAT GAC CAT G

接着从所得序列中设计内部引物，以推导完整 *pgi* 基因。内部引物的序列如下：

内部引物 1：GGA AAC AGG GGA GCC GTC

内部引物 2：TGC TGA GAT ACC AGC GGT

所得的序列在苹果麦金托什计算机上用 DNA Strider 程序 1.0 版 (Marck, (1998). 核酸研究 16: 1829-1836) 进行分析。这一程序可以进行限制性位点使用、开放读框分析和密码子使用分析。使用 BLAST 程序 (Altschul 等, (1997) 核酸研究, 25: 3389-3402) 在所得 DNA 序列和 EMBL 和 Genbank 中的序列之间进行查询。DNA 和蛋白质序列比较使用 Clustal V 和 Clustal W 程序 (Higgins and Sharp, 1988 基因 73: 237-244)。

所得的序列如 SEQ ID NO: 1 所示。对所得核苷酸序列分析揭示一 1650 个碱基对的开放读框，命名为 *pgi* 基因，其编码一个 550 个氨基酸的蛋白质，如 SEQ ID NO: 2 所示。

实施例 5：制备整合载体以整合诱变 *pgi* 基因

用分离自谷氨酸棒杆菌 AS019 (Heery 和 Dunican, (1993), 应用和环境微生物学 59: 791-799) 的基因组 DNA 作模板, 通过聚合酶链反应 (PCR) 扩增 *pgi* 基因的内部区段。使用的 *pgi* 引物是:

正向引物: ATG GAR WCC AAY GGH AA

反向引物: YTC CAC GCC CCA YTG RTC

其中 R=A+G; Y=C+T; W=A+T; H=A+T+C。

PCR 参数如下: 35 次循环 94°C 1 分钟

47°C 1 分钟

72°C 30 秒

1.5mM MgCl₂

大约 150-200ng DNA 模板

将所得 PCR 产物克隆入商购自 Promega 公司 (Promega UK, Southampton) 的 pGEM-T 载体中, 用大肠杆菌菌株 JM109 (Yanisch-Perron 等, 1985, 基因 33: 103-119) 作宿主。PCR 产物的序列示于 SEQ ID NO: 3。然后将克隆的插入体用 EcoRI 酶切为片段, 并连接于用 EcoRI 预处理的质粒 pBGS8 (Spratt 等, 基因 41: 337-342 (1986))。所用限制酶得自英国 Boehringer Mannheim 公司 (Bell Lane, Lews East Sussex BN7 1LG, 英国) 并根据说明书使用。然后用此连接混合物转化大肠杆菌 JM109, 在补加 1mM IPTG (异丙基-β-D-硫代吡喃半乳糖苷), 0.02% XGAL (5-溴-4-氯-3-吡啶-D-吡喃半乳糖苷) 和 50mg/l 卡那霉素的 Luria 琼脂上选择电转化体。将琼脂板在 37°C 温育 12 小时。从一个转化体中分离质粒 DNA, 通过用 EcoRI, BamHI 和 SalI 进行限制酶分析鉴定, 称为 pMC1 (图 4)。

质粒 pMC1 以大肠杆菌菌株 DH5 α / pMC1 形式, 根据布达佩斯条约保藏在德意志微生物保藏中心, DSMZ; Braunschweig, 德国), 保藏号 DSM12969。

实施例 6: 在生产赖氨酸的 DSM5715 中整合诱变 *pgi* 基因

将实施例 5 中所述的载体 pMC1, 通过 Tauch 等的电穿孔方法 (FEMS 微生物学通信, 123: 343—347 (1994)), 电穿孔入谷氨酸棒杆菌 DSM5715 中。菌株 DSM5715 是一种 AEC 抗性赖氨酸生产菌。载体 pMC1 在 DSM5715 中不能独立复制, 并只有已整合入 DSM5715 的染色体中时, 才在细胞中保留。通过将电穿孔混合物铺板于 LB 琼脂 (Sambrook 等, 分子克隆实验手册, 第二版, 冷泉港实验室出版社, 冷泉港, N.Y., 1989) 上, 选择具有整合入染色体的 pCR2.1poxBint 的克隆, 所用 LB 琼脂已补加 15mg/l 卡那霉素。为检测整合情况, 将内部 pgi 片段 (实施例 5) 用 Boehringer 公司的 Dig 杂交试剂盒, 通过 Boehringer Mannheim GmbH 的“进行滤膜杂交的 DIG 系统使用指导” (Mannheim, 德国, 1993) 进行标记。转化体的染色体 DNA 是通过 Eikmanns 等的方法 (微生物学 140: 1817—1828 (1994)) 分离的, 并分别用限制酶 SalI, SacI 和 HindIII 酶切。形成的片段通过琼脂糖凝胶电泳分离, 并在 68℃ 用 Boehringer 公司的 Dig 杂交试剂盒杂交。以此方式发现质粒 pMC1 插入菌株 DSM5715 的染色体 pgi 基因中。此菌株称为 DSM5715::pMC1。

实施例 7: 过表达 zwf 基因同时消除 pgi 基因对赖氨酸制备的作用

7.1 制备菌株 DSM5715::pMC1/pEC-T18mob2zwf

将实施例 1.2 所述载体 pEC-T18mob2zwf, 通过 Tauch 等的电穿孔方法 (1994, FEMS 微生物学通信 123: 343—347), 电穿孔入谷氨酸棒杆菌 DSM5715::pMC1 中。将电穿孔混合物铺板于已补加 15mg/l 卡那霉素和 5mg/l 四环素的 LB 琼脂 (Sambrook 等, 分子克隆实验手册, 第二版, 冷泉港实验室出版社, 冷泉港, N.Y., 1989) 上, 选择携带质粒的细胞, 并通过限制酶 KpnI 和 SalI 处理及随后的琼脂糖凝胶电泳检测。此菌株称为 DSM5715::pMC1/pEC-T18mob2zwf。

7.2 制备赖氨酸

将实施例 7.1 中所得的谷氨酸棒杆菌菌株 DSM5715::pMC1/pEC-T18mob2zwf, 在适于生产赖氨酸的营养培养基中培养, 并确定培养物上清中赖氨酸含量。

首先, 在 33℃ 在具有适当抗生素的琼脂板 (含 5mg/l 四环素和 25mg/l 卡那霉素的脑心琼脂) 上培养菌株 24 小时。此琼脂板培养物用于接种预培养物 (10ml 培养基于 100ml 锥形瓶)。用于预培养的培养基是完全培养基 CgIII。

培养基 CgIII

NaCl	2.5g/l
Bacto-肽胨	10g/l
Bacto-酵母膏	10g/l
葡萄糖 (单独高压灭菌)	2%(w/v)

pH 调节为 7.4。

加入四环素 (5mg/l) 和卡那霉素 (5mg/l)。在摇床上于 33℃ 以 240rpm 振荡培养预培养物 16 小时。此预培养物接种主培养物, 从而主培养物的起始 OD (波长 660nm) 为 0.1。培养基 MM 用作主培养物。

培养基 MM:

CSL (玉米浆)	5g/l
MOPS(吗啉代丙磺酸)	20g/l
葡萄糖 (单独高压灭菌)	50g/l
(NH ₄) ₂ SO ₄	25g/l
KH ₂ PO ₄	0.1g/l
MgSO ₄ · 7H ₂ O	1.0g/l
CaCl ₂ · 2H ₂ O	10mg/l
FeSO ₄ · 7H ₂ O	10mg/l
MnSO ₄ · H ₂ O	5.0mg/l

生物素（过滤灭菌）	0.3mg/l
硫胺素·HCl（过滤灭菌）	0.2mg/l
L-亮氨酸（过滤灭菌）	0.1g/l
CaCO ₃	25g/l

用氨水将 CSL, MOPS 和盐溶液调至 PH7 并高压灭菌。然后加入无菌的底物和维生素溶液，并加入干态高压灭菌的 CaCO₃。

以在具有档板的 100ml 锥形瓶中的 10ml 体积进行培养。加入四环素（5mg/l）和卡那霉素（25mg/l）。在 33℃ 和 80% 大气湿度下进行培养。

72 小时后，用 Biomek 1000(Beckmann Instruments GmbH, Munich) 测定在 660nm 波长的 OD。用 Eppendorf-BioTronik 公司的氨基酸分析仪（汉堡，德国）经离子交换层析和用茚三酮检测柱后衍生化而确定赖氨酸生成量。

实验结果示于表 3。

表 3

菌株	OD	赖氨酸 HCl(g/l)
DSM5715	7.3	14.3
DSM5715/pEC-T18mob2zwf	7.1	14.6
DSM5715::pMC1/pEC-T18mob2zwf	10.4	15.2

实施例 8：制备谷氨酸棒杆菌 ATCC13032 的基因组粘粒文库

如 Tauch 等(1995,质粒 33:168-179)所述分离谷氨酸棒杆菌 ATCC 13032 的染色体 DNA,并用限制性内切酶 Sau3AI(Amersham Pharmacia, Freiburg,德国, 产品描述 Sau3AI,编码 27-0913-02) 部分酶切。用虾碱性磷酸酶 (Roche Molecular Biochemicals, 德国曼海姆, 产品描述 SAP, 编码 1758250) 将 DNA 片段去磷酸化。将得自 Stratagene 公司 (La Jolla, USA, 产品描述 SuperCos1 粘粒载体试剂盒, 编码 251301)

的粘粒载体 Super Cos1 (Wahl 等, (1987)美国科学院院报 84: 2160—2164) 的 DNA, 用限制性内切酶 XbaI (Amersham Pharmacia, Freiburg, 德国, 产品描述 XbaI 编码 27—0948—02) 酶切, 并也用虾碱性磷酸酶去磷酸化。粘粒 DNA 然后用限制性的内切酶 BamHI (Amersham Pharmacia, Freiburg, 德国, 产品描述 BamHI, 编码 27—0868—04) 酶切。将以此方式处理的粘粒 DNA 与处理的 ATCC 13032 DNA 混合, 并用 T4 DNA 连接酶 (Amersham Pharmacia, Freiburg, 德国, 产品描述 T4—DNA—连接酶, 编码 27—0870—04) 处理。连接混合物然后用 Gigapack II XL 包装提取物 (Stratagene, La Jolla, USA, 产品描述 Gigapack II XL 包装提取物, 编码 200217) 包装进噬菌体中。为感染大肠杆菌菌株 NM554 (Raleigh 等, 1988, 核酸研究 16: 1563—1575), 将细菌置于 10mM MgSO₄ 中并与噬菌体悬浮液混合。如 Sambrook 等 (1989, 分子克隆实验手册, 冷泉港) 所述进行粘粒文库的感染和滴定, 细胞在含 100mg/l 氨苄青霉素的 LB 琼脂 (Lennox, 1955, 病毒学, 1: 190) 上铺板。在 37℃ 保温过夜后, 选择重组克隆。

实施例 9: poxB 基因的分离与测序

用 Qiaprep Spin 微量制备试剂盒 (产品号 27106, Qiagen, Hilden, 德国) 根据厂商指导分离单个菌落的粘粒 DNA (实施例 7), 并用限制性内切酶 Sau 3AI (Amersham Pharmacia, Freiburg, 德国, 产品描述 Sau 3AI, 编码 27—0913—02) 部分酶切。用虾碱性磷酸酶 (Roche 分子生物化学, 德国曼海姆, 产品描述 SAP, 编码 1758250) 将 DNA 片段去磷酸化。经凝胶电泳分离后, 用 QiaExII 凝胶提取试剂盒 (产品号 20021, Qiagen, Hilden, 德国) 分离大小范围为 1500—2000bp 的粘粒片段。将得自 Invitrogen 公司 (Groningen, 荷兰, 产品描述 Zero 背景克隆试剂盒, 产品号 K2500-01) 的测序载体 pZero-1 的 DNA, 用限制性内切酶 BamHI (Amersham Pharmacia, Freiburg, 德国, 产品描述 BamHI, 产

品号 27-0868-04) 酶切。如 Sambrook 等 (1989, 分子克隆实验手册, 冷泉港) 所述进行粘粒片段在测序载体 pZero-1 中的连接, 将 DNA 混合物与 T4 连接酶 (Pharmacia Biotech, Freiburg, 德国) 保温过夜。然后将连接混合物电穿孔 (Tauch 等, 1994, FEMS 微生物学通信, 123343-7) 进大肠杆菌菌株 DH5 α MCR (Grant, 1990, 美国科学院院报 87: 4645-4649), 并在含有 50mg/l zeocin 的 LB 琼脂 (Lennox, 1955, 病毒学, 1: 190) 上铺板。重组克隆的质粒制备用 Biorobot 9600 (产品号 900200, Qiagen, Hilden, 德国) 进行。测序用 Zimmermann 等 (1990, 核酸研究 18: 1067) 改良的 Sanger 等 (1977, 美国科学院院报 74: 5463-5467) 的双脱氧链终止法进行。使用得自 PE 应用生物系统公司的“RR 罗丹明终止循环测序试剂盒” (产品号 403044, Weiterstadt, 德国), 在带有购自 PE 应用生物系统公司 (Weiterstadt, 德国) 的“ABI Prism 377”测序仪的“Rotiphoresis NF 丙烯酰胺/双丙烯酰胺”凝胶 (29: 1) (产品号 A124.1, Roth, Karlsruhe, 德国) 中, 进行凝胶电泳分离和序列分析。

所得原始序列数据资料然后用 Staden 程序包 (1986, 核酸研究, 14: 217-231) 版本 97-0 处理。pZero-1 衍生物的各个序列组装成连续重叠群。用 XNIP 程序 (Staden, 1986, 核酸研究, 14: 217-231) 进行计算机辅助编码区分析。用“BLAST 搜索程序” (Altschul 等, 1997, 核酸研究, 25: 3389-3402) 对“国家生物技术信息中心” (NCBI, Bethesda, MD, USA) 的非冗余数据库进行进一步分析。

获得的核苷酸序列如 SEQ ID NO: 4 所示, 对该核苷酸序列的分析显示一 1737 碱基对的开放读框, 其被称为 poxB 基因, poxB 基因编码 579 个氨基酸的多肽 (SEQ ID NO: 5)。

实施例 10: 制备整合载体以整合诱变 poxB 基因

从菌株 ATCC13032 中, 通过 Eikmanns 等所述方法 (微生物学 140: 1817-1828 (1994)) 分离染色体 DNA。基于从实施例 8 中已知的

谷氨酸棒杆菌的 *poxB* 基因序列，选择以下寡核苷酸进行聚合酶链反应：

*poxB*int1: 5' TGC GAG ATG GTG AAT GGT GG 3'

*poxB*int2: 5' GCA TGA GGC AAC GCA TTA GC 3'

所示引物是通过 MWG Biotech (Ebersberg, 德国) 合成的，通过 Innis 等的标准 PCR 方法 (PCR 方案，方法指导和应用，1990, Academic 出版社)，用 Boehringer 的 Pwo-聚合酶进行 PCR 反应。借助于聚合酶链反应，分离大约 0.9kb 的 DNA 片段，其携带 *poxB* 基因的内部片段，并如 SEQ ID NO: 6 所示。

将扩增的 DNA 片段用 Invitrogen 公司的 TOPO TA 克隆试剂盒 (Carlsbad, CA, 美国; Catalogue Number K4500-01)，连接在载体 pCR2.1-TOPO (Mead 等, (1991), 生物/技术 9: 657-663) 中。然后将连接混合物电穿孔入大肠杆菌菌株 DH5 α (Hanahan, DNA 克隆实用方法，第一卷，IRL 出版社，牛津，华盛顿 DC，美国，1985)。将转化混合物铺板于 LB 琼脂 (Sambrook 等，分子克隆实验手册，第二版，冷泉港实验室出版社，冷泉港，N.Y., 1989) 上，选择携带质粒的细胞，所用 LB 琼脂已补加 25mg/ml 卡那霉素。借助于 Qiagen 公司的 QIAprep Spin 微量制备试剂盒从转化体中分离质粒 DNA，并通过限制酶 *EcoRI* 限制及随后的琼脂糖凝胶电泳 (0.8%) 检测。此质粒称为 pCR2.1*poxB*int (图 5)。

质粒 pCR2.1*poxB*int 以大肠杆菌菌株 DH5 α / pCR2.1*poxB*int 形式，根据布达佩斯条约保藏在德意志微生物保藏中心 (DSMZ; Braunschweig, 德国)，保藏号 DSM13114。

实施例 11：在赖氨酸生产菌 DSM5715 中整合诱变 *poxB* 基因

将实施例 10 中所述的载体 pCR2.1*poxB*int，通过 Tauch 等的电穿孔方法 (FEMS 微生物学通信，123: 343-347 (1994))，电穿孔入谷氨酸棒杆菌 DSM5715 中。菌株 DSM5715 是一种 AEC 抗性赖氨酸

生产菌。载体 pCR2.1poxBint 在 DSM5715 中不能独立复制，并只有已整合入 DSM5715 的染色体中时，才在细胞中保留。通过将电穿孔混合物铺板于 LB 琼脂（Sambrook 等，分子克隆实验手册，第二版，冷泉港实验室出版社，冷泉港，N.Y.，1989）上，选择具有整合入染色体的 pCR2.1poxBint 的克隆，所用 LB 琼脂已补加 15mg/l 卡那霉素。为检测整合情况，将 poxBint 片段用 Boehringer 公司的 Dig 杂交试剂盒，通过 Boehringer Mannheim GmbH 的“进行滤膜杂交的 DIG 系统使用指导”（Mannheim，德国，1993）进行标记。潜在整合子的染色体 DNA 是通过 Eikmanns 等的方法（微生物学 140:1817—1828(1994)）分离的，并分别用限制酶 SallI, SacI 和 HindIII 酶切。形成的片段通过琼脂糖凝胶电泳分离，并在 68℃用 Boehringer 公司的 Dig 杂交试剂盒杂交。实施例 9 中所述的质粒 pCR2.1poxBint 已插入 DSM5715 染色体的染色体 poxB 基因内。此菌株称为 DSM5715::pCR2.1poxBint。

实施例 12：过表达 zwf 基因同时消除 poxB 基因对赖氨酸制备的作用

12.1 制备菌株 DSM5715::pCR2.1poxBint/pEC-T18mob2zwf

通过 Libel 等所述电穿孔方法（FEMS 微生物学通信，53: 299—303（1989）），将菌株 DSM5715::pCR2.1poxBint 用质粒 pEC-T18mob2zwf 转化。在含有 18.5g/l 脑心浸液，0.5M 山梨糖醇，5g/l Bacto-胰化蛋白胨，2.5g/l Bacto-酵母膏，5g/l NaCl，和 18g/l Bacto-琼脂，并补加 5mg/l 四环素和 25mg/l 卡那霉素的 LBHIS 琼脂上，选择转化体。在 33℃温育 2 天。

通过常规方法（Peters-Wendisch 等，1998，微生物学 144，915—927）从转化体中分离质粒 DNA，用限制性内切酶 XbaI 和 KpnI 酶切，并通过随后的琼脂糖凝胶电泳检测此质粒。以此方式获得的菌株称为 DSM5715::pCR2.1poxBint/pEC—T18mob2zwf。

12.2 制备 L-赖氨酸

将实施例 12.1 中所得的谷氨酸棒杆菌菌株 DSM5715::pCR2.1poxBint/pEC-T18mob2zwf, 在适于生产赖氨酸的营养培养基中培养, 并确定培养物上清中赖氨酸含量。

首先, 在 33℃ 在具有适当抗生素的琼脂板 (含 5mg/l 四环素和 25mg/l 卡那霉素的脑心琼脂) 上培养菌株 24 小时。此琼脂板培养物用于接种预培养物 (10ml 培养基于 100ml 锥形瓶)。用于预培养的培养基是完全培养基 CgIII。

培养基 CgIII

NaCl	2.5g/l
Bacto-肽胨	10g/l
Bacto-酵母膏	10g/l
葡萄糖 (单独高压灭菌)	2%(w/v)

pH 调节为 7.4。

加入四环素 (5mg/l) 和卡那霉素 (25mg/l)。在摇床上于 33℃ 以 240rpm 振荡培养预培养物 16 小时。此预培养物接种主培养物, 从而主培养物的起始 OD (波长 660nm) 为 0.1。培养基 MM 用作主培养物。

培养基 MM:

CSL (玉米浆)	5g/l
MOPS(吗啉代丙磺酸)	20g/l
葡萄糖 (单独高压灭菌)	58g/l
(NH ₄) ₂ SO ₄	25g/l
KH ₂ PO ₄	0.1g/l
MgSO ₄ · 7H ₂ O	1.0g/l
CaCl ₂ · 2H ₂ O	10mg/l
FeSO ₄ · 7H ₂ O	10mg/l
MnSO ₄ · H ₂ O	5.0mg/l

生物素（过滤灭菌）	0.3mg/l
硫胺素·HCl（过滤灭菌）	0.2mg/l
L-亮氨酸（过滤灭菌）	0.1g/l
CaCO ₃	25g/l

用氨水将 CSL, MOPS 和盐溶液调至 PH7 并高压灭菌。然后加入无菌的底物和维生素溶液，并加入干态高压灭菌的 CaCO₃。

以在具有档板的 100ml 锥形瓶中的 10ml 体积进行培养。加入四环素（5mg/l）和卡那霉素（25mg/l）。在 33℃ 和 80% 大气湿度下进行培养。

72 小时后，用 Biomek 1000(Beckmann Instruments GmbH, Munich) 测定在 660nm 波长的 OD。用 Eppendorf-BioTronik 公司的氨基酸分析仪（汉堡，德国）经离子交换层析和用茚三酮检测柱后衍生化而确定赖氨酸生成量。

实验结果示于表 4。

表 4

菌株	OD	赖氨酸 HCl(g/l)
DSM5715	10.8	16.0
DSM5715/pEC-T18mob2zwf	8.3	17.1
DSM5715::pCR2.1poxBint	7.1	16.7
DSM5715::pCR2.1poxBint/pEC-T18mob2zwf	7.8	17.7

PCT

990213BT

原始表格（为递交） — 2000 年 7 月 3 日 02:54:24PM 打印

0-1	PCT/RO/134(EASY)表 关于保藏微生物或其他生物材料 的说明（PCT 细则 13 之二）	
0-1-1	使用如右栏程序制备	PCT-EASY 版本 2.90 (2000 年 3 月 8 日更新)
0-2	国际申请号	PCT/EP00/06303
0-3	申请人或代理人文档号	990239BT

1	如下说明涉及的保藏微生物或 其他生物材料见说明书	
1-1	页	7
1-2	行	倒 5
1-3	保藏事项	
1-3-1	保藏单位名称	德意志微生物保藏中心
1-3-2	保藏单位地址	德国不伦瑞克
1-3-3	保藏日期	2000 年 1 月 20 日
1-3-4	保藏编号	DSMZ 13244
1-4	补充说明	无
1-5	本说明是为下列指定国作的	所有指定国
1-6	单独提供说明	无
	下列说明将随后向国际局提供	

由受理局填写

0-4	本页已经和国际申请一起收到 (是或否)	是
0-4-1	受权官员	

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0-5	国际局收到本页日期	
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序列表

<110> 德古萨一于乐斯股份公司
国立爱尔兰大学戈尔韦分校
于利希研究中心有限公司

<120> 用zwf基因发酵制备L-氨基酸的方法

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 Pro Ser Ile Leu Gly Ala Leu Ile Ala Leu Tyr Glu His Thr Val Met
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 Val Gln Gly Val Ile Trp Asp Ile Asn Ser Phe Asp Gln Trp Gly Val
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aatagccata acgttgagga gttcag atg gca cac agc tac gca gaa caa tta	353
Met Ala His Ser Tyr Ala Glu Gln Leu	
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Ile Asp Thr Leu Glu Ala Gln Gly Val Lys Arg Ile Tyr Gly Leu Val	
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Glu Trp Val His Val Arg Asn Glu Glu Ala Ala Ala Phe Ala Ala Gly	
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gcg gaa tcg ttg atc act ggg gag ctg gca gta tgt gct gct tct tgt	545
Ala Glu Ser Leu Ile Thr Gly Glu Leu Ala Val Cys Ala Ala Ser Cys	
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Gly Pro Gly Asn Thr His Leu Ile Gln Gly Leu Tyr Asp Ser His Arg	
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Glu Cys Ser Gly Tyr Cys Glu Met Val Asn Gly Gly Glu Gln Gly Glu	
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Arg Ile Leu His His Ala Ile Gln Ser Thr Met Ala Gly Lys Gly Val	
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Ser Val Val Val Ile Pro Gly Asp Ile Ala Lys Glu Asp Ala Gly Asp	
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Gly Thr Tyr Ser Asn Ser Thr Ile Ser Ser Gly Thr Pro Val Val Phe	
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ccg gat cct act gag gct gca gcg ctg gtg gag gcg att aac aac gct	929
Pro Asp Pro Thr Glu Ala Ala Ala Leu Val Glu Ala Ile Asn Asn Ala	
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Lys Ser Val Thr Leu Phe Cys Gly Ala Gly Val Lys Asn Ala Arg Ala	

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Thr	Ile	Glu	Asn	Ile	Leu	Pro	His	Val	Lys	Glu	Lys	Thr	Asp	Arg	Ser			
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Phe	Leu	Asp	Arg	Met	Leu	Lys	Ala	His	Glu	Arg	Lys	Leu	Ser	Ser	Val			
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Glu	Tyr	Val	Ala	Ser	Ile	Leu	Asn	Glu	Leu	Ala	Asp	Lys	Asp	Ala	Val			
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Thr	Met	Ala	Asn	Ala	Leu	Pro	His	Ala	Ile	Gly	Ala	Gln	Ser	Val	Asp			
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cga	aac	cgc	cag	gtg	atc	gcg	atg	tgt	ggc	gat	ggt	ggt	ttg	ggc	atg	1649		
Arg	Asn	Arg	Gln	Val	Ile	Ala	Met	Cys	Gly	Asp	Gly	Gly	Leu	Gly	Met			
			430						435					440				
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 ctc gtg gag gga cag cca gaa ttt ggt act gac cat gag gaa gtg aat 1793
 Leu Val Glu Gly Gln Pro Glu Phe Gly Thr Asp His Glu Glu Val Asn
 475 480 485
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 Phe Ala Glu Ile Ala Ala Ala Ala Gly Ile Lys Ser Val Arg Ile Thr
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 50 55 60
 Glu Leu Ala Val Cys Ala Ala Ser Cys Gly Pro Gly Asn Thr His Leu

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Glu Thr His Pro Glu Ile Leu Phe Lys Glu Cys Ser Gly Tyr Cys Glu	115		120		125	
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Ala Leu Val Glu Ala Ile Asn Asn Ala Lys Ser Val Thr Leu Phe Cys	195		200		205	
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Lys Ile Lys Ser Pro Ile Gly His Ala Leu Gly Gly Lys Gln Tyr Ile	225		230		235	240
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Gly Ala Cys Val Asp Ala Ser Asn Glu Ala Asp Leu Leu Ile Leu Leu	260		265		270	
Gly Thr Asp Phe Pro Tyr Ser Asp Phe Leu Pro Lys Asp Asn Val Ala	275		280		285	
Gln Val Asp Ile Asn Gly Ala His Ile Gly Arg Arg Thr Thr Val Lys	290		295		300	
Tyr Pro Val Thr Gly Asp Val Ala Ala Thr Ile Glu Asn Ile Leu Pro	305		310		315	320
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Asn Glu Leu Ala Asp Lys Asp Ala Val Phe Thr Val Asp Thr Gly Met	370		375		380	

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说明书附图

质粒pEC-T18mob2的图谱

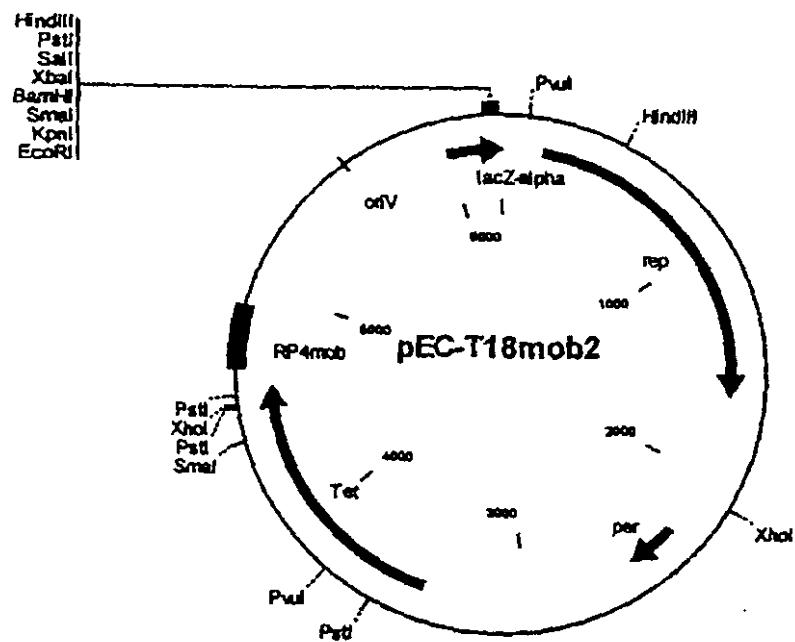


图1

质粒pEC-T18mob2zwf的图谱

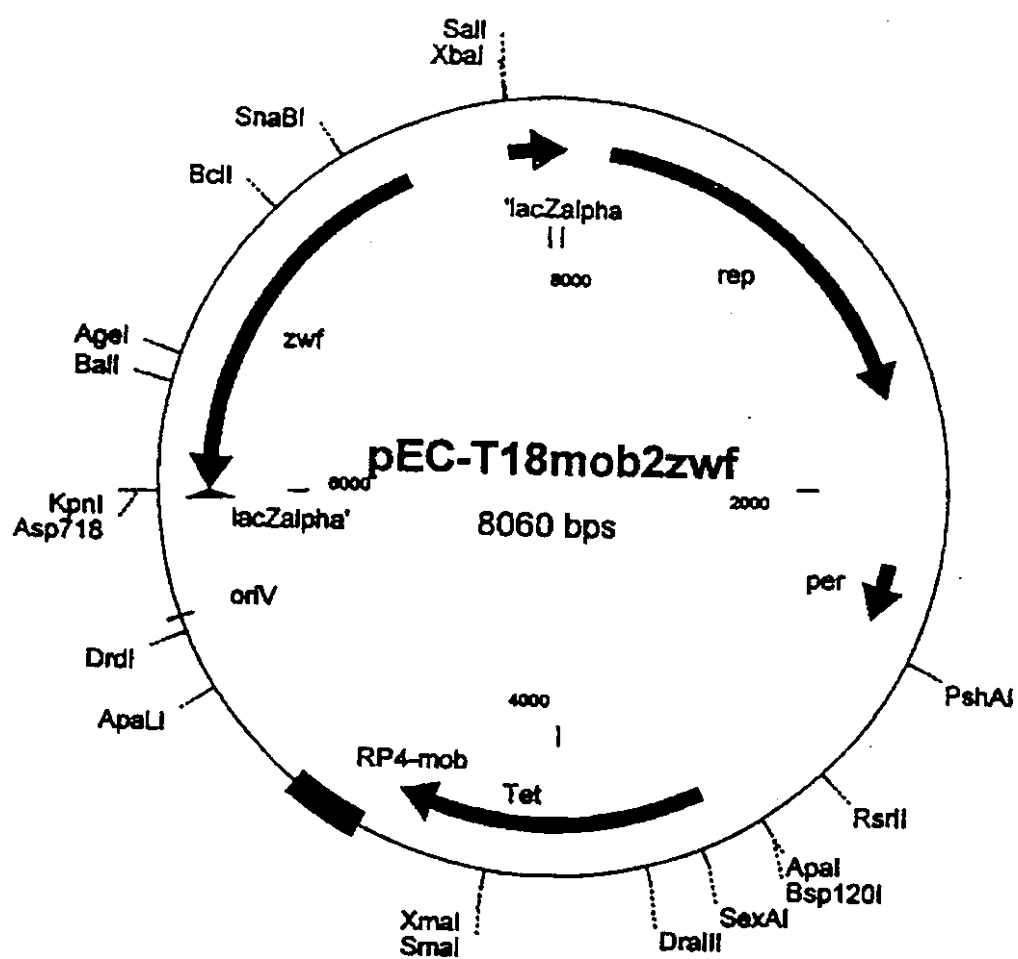


图2

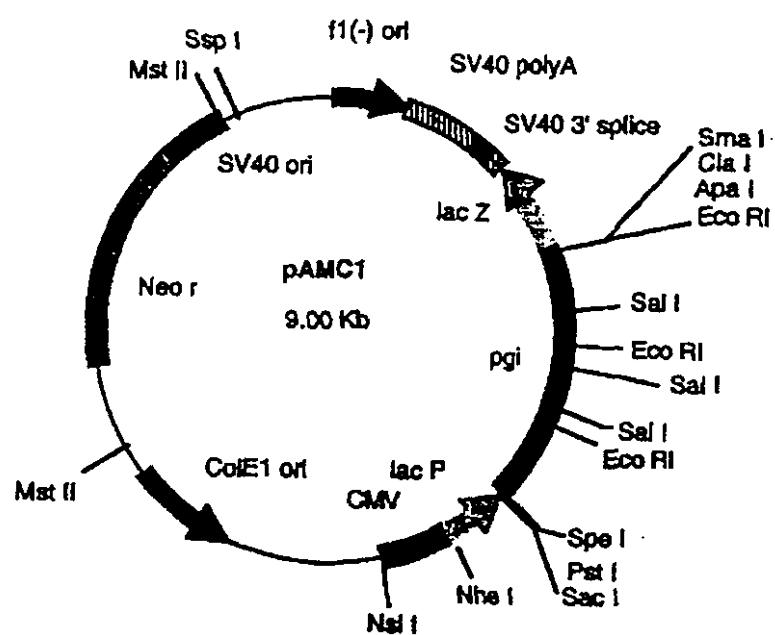


图 3

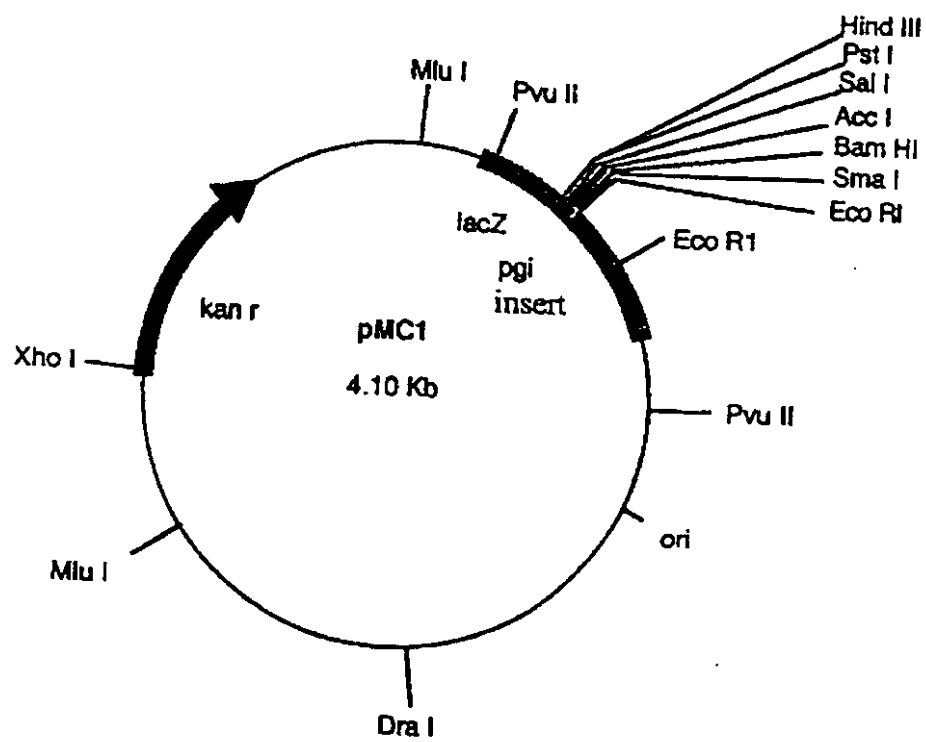


图4

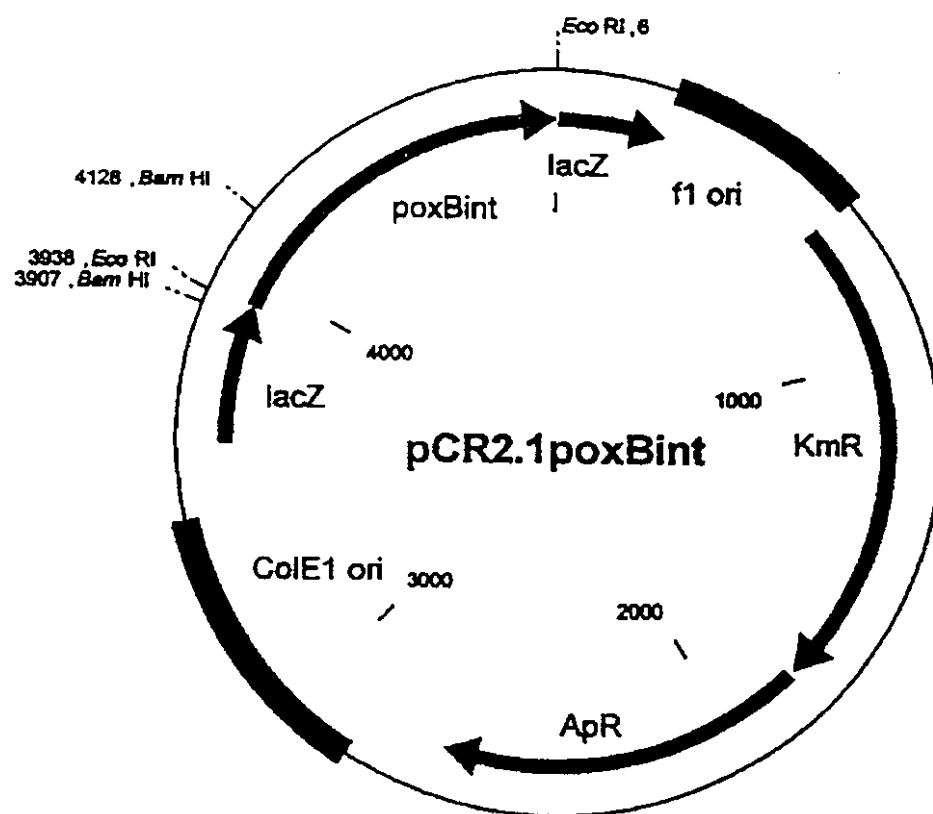


图5