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(54) Title: NUCLEOTIDE SEQUENCES WHICH CODE FOR THE LUXS GENE

(57) Abstract: The invention relates to an isolated polynucleotide comprising a polynucleotide sequence chosen from the group consisting of: a) polynucleotide which is identical to the extent of at least 70 % to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2; b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70 % to the amino acid sequence of SEQ ID No. 2; c) polynucleotide which is complementary to the polynucleotides of a) or b), and d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c), and a process for the fermentative preparation of L-amino acids using coryneform bacteria in which at least the luxS gene is present in attenuated form, and the use of polynucleotides which comprise the sequences according to the invention as hybridization probes.



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Nucleotide sequences which code for the luxS gene

Field of the Invention

The invention provides nucleotide sequences from coryneform bacteria which code for the luxS gene and a process for the fermentative preparation of amino acids using bacteria in which the luxS gene is attenuated.

Prior Art

L-Amino acids, in particular lysine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

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

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and which produce amino acids are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of *Corynebacterium* strains which produce L-amino acid, by

amplifying individual amino acid biosynthesis genes and investigating the effect on the amino acid production.

Object of the Invention

The inventors had the object of providing new measures for improved fermentative preparation of amino acids.

Summary of the Invention

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

When L-lysine or lysine are mentioned in the following, not only the bases but also the salts, such as e.g. lysine monohydrochloride or lysine sulfate, are meant by this.

The invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the luxS gene, chosen from the group consisting of

- a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
- b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,
- c) polynucleotide which is complementary to the polynucleotides of a) or b), and

d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of the histidine kinase LuxS.

The invention also provides the abovementioned polynucleotide, this preferably being a DNA which is capable of replication, comprising:

- (i) the nucleotide sequence, shown in SEQ ID No.1, or
- 10 (ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or
- (iii) at least one sequence which hybridizes with the sequences complementary to sequences (i) or (ii),
- 15 and optionally
- (iv) sense mutations of neutral function in (i).

The invention also provides:

a polynucleotide, in particular DNA, which is capable of replication and comprises the nucleotide sequence as shown in SEQ ID No.1;

a polynucleotide which codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID No. 2;

a vector containing parts of the polynucleotide according to the invention, but at least 15 successive nucleotides of the sequence claimed,

and coryneform bacteria in which the luxS gene is attenuated, in particular by an insertion or deletion.

The invention also provides polynucleotides which substantially comprise a polynucleotide sequence, which are obtainable by screening by means of hybridization of a corresponding gene library of a coryneform bacterium, which
5 comprises the complete gene or parts thereof, with a probe which comprises the sequence of the polynucleotide according to the invention according to SEQ ID No.1 or a fragment thereof, and isolation of the polynucleotide sequence mentioned.

10 Detailed description of the Invention

Polynucleotides which comprise the sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, nucleic acids or polynucleotides or genes which code for
15 the histidine kinase LuxS or to isolate those nucleic acids or polynucleotides or genes which have a high similarity with the sequence the luxS gene.

Polynucleotides which comprise the sequences according to the invention are furthermore suitable as primers with the
20 aid of which DNA of genes which code for the histidine kinase LuxS can be prepared by the polymerase chain reaction (PCR).

Such oligonucleotides which serve as probes or primers comprise at least 30, preferably at least 20, very
25 particularly preferably at least 15 successive nucleotides. Oligonucleotides which have a length of at least 40 or 50 nucleotides are also suitable.

"Isolated" means separated out of its natural environment.

"Polynucleotide" in general relates to polyribonucleotides
30 and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom and also those which are at least 70%, preferably at least 80% and in particular at least 90% to 95% identical to the polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom.

"Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of the histidine kinase LuxS, and also those which are at least 70%, preferably at least 80% and in particular at least 90% to 95% identical to the polypeptide according to SEQ ID No. 2 and have the activity mentioned.

The invention furthermore relates to a process for the fermentative preparation of amino acids chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine using coryneform bacteria which in particular already produce amino acids and in which the nucleotide sequences which code for the luxS gene are attenuated, in particular eliminated or expressed at a low level.

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

The microorganisms to which the present invention relates can prepare amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They can be 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 experts for its ability to produce L-amino acids.

Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum* (*C. glutamicum*), are in particular the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium melassecola ATCC17965
Corynebacterium thermoaminogenes FERM BP-1539
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants or strains prepared therefrom.

The new *luxS* gene from *C. glutamicum* which codes for the histidine kinase LuxS has been isolated. The histidine kinase LuxS is part of a two-component system. Two-component regulation systems are distinguished in that various response regulator proteins can be activated by sensor kinases.

To isolate the *luxS* gene or also other genes of *C. glutamicum*, a gene library of this microorganism is first set up in *Escherichia coli* (*E. coli*). The setting up of gene libraries is described in generally known textbooks and handbooks. The textbook by Winnacker: Gene und Klone,

Eine Einführung in die Gentechnologie (Verlag Chemie, Weinheim, Germany, 1990), or the handbook by Sambrook et al.: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989) may be mentioned as an
5 example. A well-known gene library is that of the E. coli K-12 strain W3110 set up in λ vectors by Kohara et al. (Cell 50, 495 -508 (1987)). Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene
10 library of C. glutamicum ATCC13032, which was set up with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the E. coli K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326) (1992)) in turn describe a gene library of C. glutamicum ATCC13032 using the cosmid pHC79 (Hohn and Collins, 1980, Gene 11, 291-298).

To prepare a gene library of C. glutamicum in E. coli it is also possible to use plasmids such as pBR322 (Bolivar,
20 1979, Life Sciences, 25, 807-818) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable hosts are, in particular, those E. coli strains which are restriction- and recombination-defective, such as, for example, the strain DH5 α mc^r, which has been described by Grant et al.
25 (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids or other λ vectors can then in turn be subcloned and subsequently sequenced in the usual vectors which are suitable for DNA sequencing, such as is described
30 e. g. by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as
35 e.g. that of Staden (Nucleic Acids Research 14, 217-

232(1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

5 The new DNA sequence of *C. glutamicum* which codes for the luxS gene and which, as SEQ ID No. 1, is a constituent of the present invention has been found. The amino acid sequence of the corresponding protein has furthermore been derived from the present DNA sequence by the methods described above. The resulting amino acid sequence of the
10 luxS gene product is shown in SEQ ID No. 2.

Coding DNA sequences which result from SEQ ID No. 1 by the degeneracy of the genetic code are also a constituent of the invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a
15 constituent of the invention. Conservative amino acid exchanges, such as e.g. exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are furthermore known among experts as "sense mutations" which do not lead to a fundamental change in the activity of the
20 protein, i.e. are of neutral function. It is furthermore known that changes on the N and/or C terminus of a protein cannot substantially impair or can even stabilize the function thereof. Information in this context can be found by the expert, inter alia, in Ben-Bassat et al. (Journal of
25 Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks of genetics and molecular biology. Amino acid sequences which result in a
30 corresponding manner from SEQ ID No. 2 are also a constituent of the invention.

In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Finally, DNA sequences which are prepared by the
35 polymerase chain reaction (PCR) using primers which result

from SEQ ID No. 1 are a constituent of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

Instructions for identifying DNA sequences by means of hybridization can be found by the expert, inter alia, in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology 41: 255-260 (1991)). The hybridization takes place under stringent conditions, that is to say only hybrids in which the probe and target sequence, i. e. the polynucleotides treated with the probe, are at least 70% identical are formed. It is known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out under a relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

A 5x SSC buffer at a temperature of approx. 50 - 68°C, for example, can be employed for the hybridization reaction. Probes can also hybridize here with polynucleotides which are less than 70% identical to the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved, for example, by lowering the salt concentration to 2x SSC and optionally subsequently 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995) a temperature of approx. 50 - 68°C being established. It is optionally possible to lower the salt concentration to 0.1x SSC. Polynucleotide fragments which are, for example, at least 70% or at least 80% or at least 90% to 95% identical to the sequence of the probe employed can be isolated by increasing the hybridization

temperature stepwise from 50 to 68°C in steps of approx. 1°C - 2°C. Further instructions on hybridization are obtainable on the market in the form of so-called kits (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalogue No. 1603558).

Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by the expert, inter alia, in the handbook by Gait: Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

It has been found that coryneform bacteria produce amino acids in an improved manner after attenuation of the luxS gene.

To achieve an attenuation, either the expression of the luxS gene or the regulatory or catalytic properties of the enzyme protein can be reduced or eliminated. The two measures can optionally be combined.

The reduction in gene expression can take place by suitable culturing or by genetic modification (mutation) of the signal structures of gene expression. Signal structures of gene expression are, for example, repressor genes, activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. The expert can find information on this e.g. in the patent application WO 96/15246, in Boyd and Murphy (Journal of Bacteriology 170: 5949 (1988)), in Voskuil and Chambliss (Nucleic Acids Research 26: 3548 (1998)), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191 (1998)), in Pátek et al. (Microbiology 142: 1297 (1996)), Vasicova et al. (Journal of Bacteriology 181: 6188 (1999)) and in known textbooks of genetics and molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or

that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations which lead to a change or reduction in the catalytic properties of enzyme proteins are known from the prior art; examples which may be mentioned are the works by Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Sugimoto et al. (Bioscience Biotechnology and Biochemistry 61: 1760-1762 (1997)) and Möckel ("Die Threonindehydratase aus Corynebacterium glutamicum: Aufhebung der allosterischen Regulation und Struktur des Enzyms", Reports from the Jülich Research Centre, Jül-2906, ISSN09442952, Jülich, Germany, 1994). Summarizing descriptions can be found in known textbooks of genetics and molecular biology, such as e.g. that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Possible mutations are transitions, transversions, insertions and deletions. Depending on the effect of the amino acid exchange on the enzyme activity, "missense mutations" or "nonsense mutations" are referred to. Insertions or deletions of at least one base pair (bp) in a gene lead to frame shift mutations, as a consequence of which incorrect amino acids are incorporated or translation is interrupted prematurely. Deletions of several codons typically lead to a complete loss of the enzyme activity. Instructions on generation of such mutations are prior art and can be found in known textbooks of genetics and molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

A common method of mutating genes of *C. glutamicum* is the method of "gene disruption" and "gene replacement"

described by Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)).

In the method of gene disruption a central part of the coding region of the gene of interest is cloned in a
5 plasmid vector which can replicate in a host (typically E. coli), but not in C. glutamicum. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pK18mobsacB or pK19mobsacB (Jäger et al.,
10 Journal of Bacteriology 174: 5462-65 (1992)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84; US Patent 5,487,993), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular
15 Biology, 234: 534-541 (1993)) or pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516). The plasmid vector which contains the central part of the coding region of the gene is then transferred into the desired strain of C. glutamicum by conjugation or transformation. The method
20 of conjugation is described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan
25 (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross-over" event, the coding region of the gene in question is interrupted by the vector sequence and two incomplete alleles are
30 obtained, one lacking the 3' end and one lacking the 5' end. This method has been used, for example, by Fitzpatrick et al. (Applied Microbiology and Biotechnology 42, 575-580 (1994)) to eliminate the recA gene of C. glutamicum.

In the method of "gene replacement", a mutation, such as
35 e.g. a deletion, insertion or base exchange, is established

in vitro in the gene of interest. The allele prepared is in turn cloned in a vector which is not replicative for *C. glutamicum* and this is then transferred into the desired host of *C. glutamicum* by transformation or conjugation.

5 After homologous recombination by means of a first "cross-over" event which effects integration and a suitable second "cross-over" event which effects excision in the target gene or in the target sequence, the incorporation of the mutation or of the allele is achieved. This method was

10 used, for example, by Peters-Wendisch et al. (Microbiology 144, 915 - 927 (1998)) to eliminate the *pyc* gene of *C. glutamicum* by a deletion.

A deletion, insertion or a base exchange can be incorporated into the *luxS* gene in this manner.

15 In addition, it may be advantageous for the production of L-amino acids to enhance, in particular over-express, one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the citric acid cycle, of the pentose phosphate cycle, of amino acid export and

20 optionally regulatory proteins, in addition to the attenuation of the *luxS* gene.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by

25 the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or using a gene or allele which codes for a corresponding enzyme (protein) having a high activity, and optionally combining these measures.

30 Thus, for the preparation of L-amino acids, in addition to the attenuation of the *luxS* gene at the same time one or more of the genes chosen from the group consisting of

- the dapA gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335),
- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174: 6076-6086),
- the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the zwf gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661),
- the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609),
- the mqo gene which codes for malate-quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998),
- the lysC gene which codes for a feed-back resistant aspartate kinase (Accession No.P26512; EP-B-0387527; EP-A-0699759; WO 00/63388),
- the lysE gene which codes for lysine export (DE-A-195 48 222)
- the hom gene which codes for homoserine dehydrogenase (EP-A 0131171),
- the ilvA gene which codes for threonine dehydratase (Möckel et al., Journal of Bacteriology (1992) 8065-8072)) or the ilvA(Fbr) allele which codes for a "feed back resistant" threonine dehydratase (Möckel et al., (1994) Molecular Microbiology 13: 833-842),

- the ilvBN gene which codes for acetohydroxy-acid synthase (EP-B 0356739),
- the ilvD gene which codes for dihydroxy-acid dehydratase (Sahm and Eggeling (1999) Applied and Environmental
5 Microbiology 65: 1973-1979),
- the zwal gene which codes for the Zwa1 protein (DE: 19959328.0, DSM 13115)

can be enhanced, in particular over-expressed.

10 It may furthermore be advantageous for the production of amino acids, in addition to the attenuation of the luxS gene at the same time for one or more of the genes chosen from the group consisting of

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1, DSM 13047),
- 15 • the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478, DSM 12969),
- the poxB gene which codes for pyruvate oxidase (DE:1995 1975.7, DSM 13114),
- the zwa2 gene which codes for the Zwa2 protein (DE:
20 19959327.2, DSM 13113)

to be attenuated, in particular for the expression thereof to be reduced.

In addition to the attenuation of the luxS gene it may furthermore be advantageous for the production of amino
25 acids to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The invention also provides the microorganisms prepared according to the invention, and these can 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 production of L-amino acids. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains 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, for example, soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols, such as, for example, glycerol and ethanol, and organic acids, such as, for example, acetic acid, can be used as the source of carbon. These substances 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 sulfate, 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.

Phosphoric acid, 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
5 metals, such as, for example, 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 abovementioned substances. Suitable precursors can moreover be added to the culture
10 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 aqueous ammonia, or acid compounds,
15 such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as, for example, fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as, for
20 example, 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, for example, air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and
25 preferably 25°C to 40°C. Culturing is continued until a maximum of the desired product has formed. This target is usually reached within 10 hours to 160 hours.

Methods for the determination of L-amino acids are known from the prior art. The analysis can thus be carried out,
30 for example, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by anion exchange chromatography with subsequent ninhydrin derivatization, or it can be carried out by reversed phase HPLC, for example as described by Lindroth et al. (Analytical Chemistry
35 (1979) 51: 1167-1174).

The process according to the invention is used for fermentative preparation of amino acids.

The following microorganism was deposited as a pure culture on 23.02.2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of
5 Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:

- *Escherichia coli* Top10/pCR2.1luxSint as DSM 14082.

The present invention is explained in more detail in the
10 following with the aid of embodiment examples.

The isolation of plasmid DNA from *Escherichia coli* and all techniques of restriction, Klenow and alkaline phosphatase treatment were carried out by the method of Sambrook et al. (Molecular Cloning. A Laboratory Manual, 1989, Cold Spring
15 Harbour Laboratory Press, Cold Spring Harbor, NY, USA). Methods for transformation of *Escherichia coli* are also described in this handbook.

The composition of the usual nutrient media, such as LB or TY medium, can also be found in the handbook by Sambrook et
20 al.

Example 1

Preparation of a genomic cosmid gene library from *C. glutamicum* ATCC 13032

Chromosomal DNA from *C. glutamicum* ATCC 13032 was isolated
25 as described by Tauch et al. (1995, Plasmid 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
30 Molecular Biochemicals, Mannheim, Germany, Product 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 Vector Kit, Code no. 251301) was cleaved
5 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 restriction enzyme
10 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 ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-
15 Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217).

For infection of the E. coli strain NM554 (Raleigh et al.
20 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 Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring
25 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 2

30 Isolation and sequencing of the luxS gene

The cosmid DNA of an individual colony 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 restriction enzyme
Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product
Description Sau3AI, Product No. 27-0913-02). The DNA
fragments were dephosphorylated with shrimp alkaline
5 phosphatase (Roche Molecular Biochemicals, Mannheim,
Germany, Product Description SAP, Product No. 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.
10 20021, Qiagen, Hilden, Germany).

The DNA of the sequencing vector pZero-1, obtained from
Invitrogen (Groningen, The Netherlands, Product Description
Zero Background Cloning Kit, Product No. K2500-01) was
cleaved with the restriction enzyme BamHI (Amersham
15 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
20 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.,
25 87:4645-4649). Letters, 123:343-7) and plated out on LB
agar (Lennox, 1955, Virology, 1:190) with 50 mg/l 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
30 dideoxy chain termination 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
35 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 analyses were prepared with the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231). Further analyses were carried out 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. 1. Analysis of the nucleotide sequence showed an open reading frame of 1272 bp, which was called the luxS gene. The luxS gene codes for a polypeptide of 423 amino acids.

Example 3

Preparation of an integration vector for integration mutagenesis of the luxS 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 luxS gene known for *C. glutamicum* from example 2, the following oligonucleotides were chosen for the polymerase chain reaction (see SEQ ID No. 3 and SEQ ID No. 4):

luxS-int1:

5' TCG TGA CCG TGG CTA TTG AT 3'

luxS-int2:

5` CTT GAG CAA TTC GCA GAA GG 3`

The primers shown were synthesized by MWG Biotech (Ebersberg, Germany) and the PCR reaction was carried out
5 by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press) with the Taq-polymerase from Boehringer Mannheim (Germany, Product Description Taq DNA polymerase, Product No. 1 146 165). With the aid of the polymerase chain reaction, the
10 primers allow amplification of an internal fragment of the luxS gene 492 bp in size. The product amplified in this way was tested electrophoretically in a 0.8% agarose gel.

The amplified DNA fragment was ligated with the TOPO TA Cloning Kit from Invitrogen Corporation (Carlsbad, CA, USA;
15 Catalogue Number K4500-01) in the vector pCR2.1-TOPO (Mead at al. (1991) Bio/Technology 9:657-663).

The E. coli strain TOP10 was then electroporated with the ligation batch (Hanahan, In: DNA cloning. A practical approach. Vol.I. IRL-Press, Oxford, Washington DC, USA,
20 1985). Selection of plasmid-carrying cells was carried out 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 50 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 pCR2.1luxSint and is shown
30 in Figure 1.

Example 4

Integration mutagenesis of the luxS gene in the strain DSM 5715

The vector pCR2.1luxSint mentioned in example 3 was
5 electroporated by the electroporation method of Tauch et al. (FEMS Microbiological Letters, 123:343-347 (1994)) in Corynebacterium glutamicum DSM 5715. The strain DSM 5715 is an AEC-resistant lysine producer. The vector pCR2.1luxSint cannot replicate independently in DSM5715 and is retained
10 in the cell only if it has integrated into the chromosome of DSM 5715. Selection of clones with pCR2.1luxSint 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
15 Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which had been supplemented with 15 mg/l kanamycin.

For detection of the integration, the luxSint fragment was labelled with the Dig hybridization kit from Boehringer by the method of "The DIG System Users Guide for Filter
20 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, EcoRI and PstI. The fragments
25 formed were separated by means of agarose gel electrophoresis and hybridized at 68°C with the Dig hybridization kit from Boehringer. The plasmid pCR2.1luxSint mentioned in example 3 had been inserted into the chromosome of DSM5715 within the chromosomal luxS gene. The
30 strain was called DSM5715::pCR2.1luxSint.

Example 5

Preparation of lysine

The *C. glutamicum* strain DSM5715::pCR2.1luxSint obtained in example 4 was cultured in a nutrient medium suitable for
5 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 kanamycin (25 mg/l) for 24 hours at 33°C. Starting from
10 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

15 Kanamycin (25 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 OD. 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) 50g/l

Salts:

$(\text{NH}_4)_2\text{SO}_4$ 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 $\cdot$ HCl (sterile-filtered) 0.2 mg/l

Leucine (sterile-filtered) 0.1 g/l

CaCO_3 25 g/l

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

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Kanamycin (25 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric
10 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 1.

Table 1

Strain	OD (660 nm)	Lysine HCl g/l
DSM5715	8.0	12.64
DSM5715::pCR2.1luxSint	9.3	14.39

10 Brief Description of the Figure:

Figure 1: Map of the plasmid pCR2.1luxSint.

The abbreviations and designations used have the following meaning.

KmR:	Kanamycin resistance gene
EcoRI:	Cleavage site of the restriction enzyme EcoRI
PstI:	Cleavage site of the restriction enzyme PstI
SalI:	Cleavage site of the restriction enzyme SalI
luxSint:	Internal fragment of the luxS gene
ColE1:	Replication origin of the plasmid ColE1

What is claimed is:

1. An isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the luxS gene, chosen from the group consisting of
 - 5 a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
 - 10 b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,
 - c) polynucleotide which is complementary to the polynucleotides of a) or b), and
 - 15 d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),the polypeptide preferably having the activity of the histidine kinase LuxS.
- 20 2. A polynucleotide as claimed in claim 1, wherein the polynucleotide is a preferably recombinant DNA which is capable of replication in coryneform bacteria.
3. A polynucleotide as claimed in claim 1, wherein the polynucleotide is an RNA.
- 25 4. A polynucleotide as claimed in claim 2, comprising the nucleic acid sequence as shown in SEQ ID No. 1.
5. A DNA as claimed in claim 2 which is capable of replication, comprising

- (i) the nucleotide sequence shown in SEQ ID No. 1,
or
 - (ii) at least one sequence which corresponds to
sequence (i) within the range of the
5 degeneration of the genetic code, or
 - (iii) at least one sequence which hybridizes with the
sequence complementary to sequence (i) or (ii),
and optionally
 - (iv) sense mutations of neutral function in (i).
- 10 6. A DNA as claimed in claim 2 which is capable of
replication, wherein the hybridization is carried out
under a stringency corresponding to at most 2x SSC.
7. A polynucleotide sequence as claimed in claim 1, which
codes for a polypeptide which comprises the amino acid
15 sequences shown in SEQ ID No. 2.
8. A coryneform bacterium in which the luxS gene is
attenuated, in particular eliminated.
9. The vector pCR2.1luxSint, which
- 9.1 carries an internal fragment of the luxS gene
20 492 bp in size,
- 9.2 the restriction map of which is reproduced in
figure 1, and
- 9.3 which is deposited in the E. coli strain
Top10/pCR2.1luxSint under no. DSM 14082 at the
25 Deutsche Sammlung für Mikroorganismen und
Zellenkulturen [German Collection of
Microorganisms and Cell Cultures].

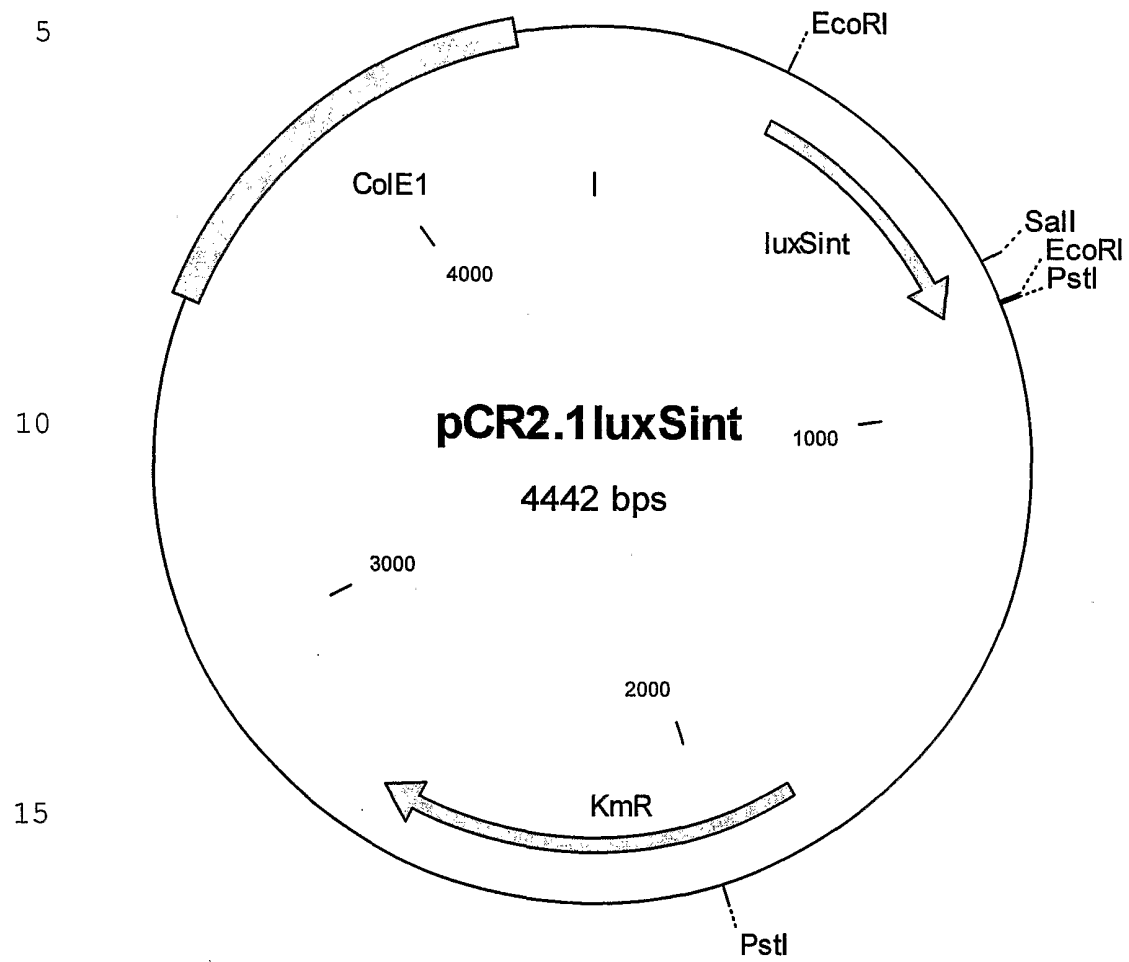
10. A process for the fermentative preparation of L-amino acids, in particular lysine, which comprises carrying out the following steps:
- 5 a) fermentation of the coryneform bacteria which produce the desired L-amino acid and in which at least the luxS gene or nucleotide sequences which code for it are attenuated, in particular eliminated;
 - 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.
11. A process as claimed in claim 10, wherein bacteria in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are
15 employed.
12. A process as claimed in claim 10, wherein bacteria in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.
- 20 13. A process as claimed in claim 10, wherein the expression of the polynucleotide(s) which code(s) for the luxS gene is attenuated, in particular eliminated.
14. A process as claimed in claim 10, wherein the regulatory or catalytic properties of the polypeptide
25 (enzyme protein) for which the polynucleotide luxS codes are reduced.
15. A process as claimed in claim 10, wherein for the preparation of L-amino acids, coryneform microorganisms in which at the same time one or more of the genes
30 chosen from the group consisting of

- 15.1 the dapA gene which codes for dihydrodipicolinate synthase,
- 15.2 the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase,
- 5 15.3 the tpi gene which codes for triose phosphate isomerase,
- 15.4 the pgk gene which codes for 3-phosphoglycerate kinase,
- 10 15.5 the zwf gene which codes for glucose 6-phosphate dehydrogenase,
- 15.6 the pyc gene which codes for pyruvate carboxylase,
- 15.7 the mqo gene which codes for malate-quinone oxidoreductase,
- 15 15.8 the lysC gene which codes for a feed-back resistant aspartate kinase,
- 15.9 the lysE gene which codes for lysine export,
- 15.10 the hom gene which codes for homoserine dehydrogenase
- 20 15.11 the ilvA gene which codes for threonine dehydratase or the ilvA(Fbr) allele which codes for a feed back resistant threonine dehydratase,
- 25 15.12 the ilvBN gene which codes for acetohydroxy-acid synthase,
- 15.13 the ilvD gene which codes for dihydroxy-acid dehydratase,
- 15.14 the zwal gene which codes for the Zwal protein

is/are enhanced or over-expressed are fermented.

16. A process as claimed in claim 10, wherein for the preparation of L-amino acids, coryneform microorganisms in which at the same time one or more of the genes
5 chosen from the group consisting of
- 16.1 the pck gene which codes for phosphoenolpyruvate carboxykinase,
 - 16.2 the pgi gene which codes for glucose 6-phosphate isomerase,
 - 10 16.3 the poxB gene which codes for pyruvate oxidase
 - 16.4 the zwa2 gene which codes for the Zwa2 protein
- is or are attenuated are fermented.
17. A coryneform bacterium which contains a vector which carries parts of the polynucleotide but at least 15
15 successive nucleotides of the sequence as claimed in claim 1.
18. A process as claimed in one or more of the claims 10-16, wherein microorganisms of the species *Corynebacterium glutamicum* are employed.
- 20 19. A process for discovering RNA, cDNA and DNA in order to isolate nucleic acids, or polynucleotides or genes which code for the histidine kinase LuxS or have a high similarity with the sequence of the luxS gene, which comprises employing the polynucleotide comprising the
25 polynucleotide sequences as claimed in claims 1, 2, 3 or 4 as hybridization probes.

Figure 1: Plasmid pCR2.1luxSint



SEQUENCE PROTOCOL

<110> Degussa AG

5 <120> New nucleotide sequences which code for the luxS gene

<130> 000457 BT

<140>

10 <141>

<160> 4

<170> PatentIn Ver. 2.1

15

<210> 1

<211> 1902

<212> DNA

<213> Corynebacterium glutamicum

20

<220>

<221> CDS

<222> (342)..(1610)

<223> luxS gene

25

<400> 1

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cgcgctggcc gttggtatcg gcgaccagc cggtgccag gtaggggcat gcggtttgtg 120

30

cggtgcgttc gaccgcgggc atcgcgtcga tgggaaggcc gtcagtaatt acttccgggg 180

ctgcctcggg gttggtctct ggggttgctt caggttccgc cggggtacaa gcggtgagca 240

35

tgatggaagc agcgaggata gtaggtaatg tacgacgcag gcagtcaagc ctagatcgtg 300

tgtcggaaac cggacgcaat gagctcgatg ttgaaaccct t gtg aag aag ggg aat 356

Met Lys Lys Gly Asn

1

5

40

caa ccg ggc gcg atg agc tat cgc aac agt atc cac att ttg aca gcc 404

Gln Pro Gly Ala Met Ser Tyr Arg Asn Ser Ile His Ile Leu Thr Ala

10

15

20

45

tcg ctg ctg gtc gtg ggg ttg gga gct tcc gcc cgc ctg acg ctg ccg 452

Ser Leu Leu Val Val Gly Leu Gly Ala Ser Ala Arg Leu Thr Leu Pro

25

30

35

50

atg ttt gcg ctg tcg tgc gtg ctg ttg ttt gtg tgg ggt ttt ctg tac 500

Met Phe Ala Leu Ser Cys Val Leu Leu Phe Val Trp Gly Phe Leu Tyr

40

45

50

55

ttc tat gga tca acc aaa cgc gta gat ttg agc cac ggc atg cag ctg 548

Phe Tyr Gly Ser Thr Lys Arg Val Asp Leu Ser His Gly Met Gln Leu

55

60

65

ggc tgg ctg ttt gtg ctg acg ctg gtg tgg att ttt atg gtg ccg atc 596

Gly Trp Leu Phe Val Leu Thr Leu Val Trp Ile Phe Met Val Pro Ile

70

75

80

85

5	gtg	ccc	gtg	tcc	att	tat	ctg	ctg	ttc	ccg	ctg	ttt	ttc	ctc	tat	cta	644
	Val	Pro	Val	Ser	Ile	Tyr	Leu	Leu	Phe	Pro	Leu	Phe	Phe	Leu	Tyr	Leu	90
10	cag	gtg	atg	cct	gac	gtg	aga	ggc	att	att	gcg	att	ttg	ggg	gca	aca	692
	Gln	Val	Met	Pro	Asp	Val	Arg	Gly	Ile	Ile	Ala	Ile	Leu	Gly	Ala	Thr	105
15	gcg	att	gcg	att	gcc	agc	cag	tat	tcc	gtg	ggg	ttg	acc	ttt	ggg	ggg	740
	Ala	Ile	Ala	Ile	Ala	Ser	Gln	Tyr	Ser	Val	Gly	Leu	Thr	Phe	Gly	Gly	120
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35	gcg	ggg	att	gct	gcg	gaa	cgt	caa	cgt	att	gcg	cat	gaa	att	cat	gac	932
	Ala	Gly	Ile	Ala	Ala	Glu	Arg	Gln	Arg	Ile	Ala	His	Glu	Ile	His	Asp	185
40	acg	gtc	gcc	cag	gga	ctc	tcc	tcc	att	caa	atg	ctg	ctg	cat	gtc	tct	980
	Thr	Val	Ala	Gln	Gly	Leu	Ser	Ser	Ile	Gln	Met	Leu	Leu	His	Val	Ser	200
45	gaa	cag	gag	att	ctc	gtt	gct	gag	atg	gaa	gag	aag	cca	aag	gag	gcg	1028
	Glu	Gln	Glu	Ile	Leu	Val	Ala	Glu	Met	Glu	Glu	Lys	Pro	Lys	Glu	Ala	215
50	atc	gtg	aag	aag	atg	cgc	ctt	gcc	cga	caa	aca	gcc	tcc	gac	aat	ctc	1076
	Ile	Val	Lys	Lys	Met	Arg	Leu	Ala	Arg	Gln	Thr	Ala	Ser	Asp	Asn	Leu	230
55	agt	gag	gct	cgc	gcg	atg	att	gcg	gcg	ttg	caa	ccg	gca	gcg	ctg	tct	1124
	Ser	Glu	Ala	Arg	Ala	Met	Ile	Ala	Ala	Leu	Gln	Pro	Ala	Ala	Leu	Ser	250
60	aaa	acc	tcc	ttg	gaa	gca	gca	ctt	cac	cgc	gtc	aca	gaa	ccg	ttg	ttg	1172
	Lys	Thr	Ser	Leu	Glu	Ala	Ala	Leu	His	Arg	Val	Thr	Glu	Pro	Leu	Leu	265
65	ggg	att	aat	ttt	gtg	att	tct	gtc	gac	ggg	gat	gtt	cgc	caa	ctg	ccc	1220
	Gly	Ile	Asn	Phe	Val	Ile	Ser	Val	Asp	Gly	Asp	Val	Arg	Gln	Leu	Pro	280
70	atg	aaa	act	gaa	gcc	acc	ctt	ctg	cga	att	gct	caa	ggg	gcg	atc	gga	1268
	Met	Lys	Thr	Glu	Ala	Thr	Leu	Leu	Arg	Ile	Ala	Gln	Gly	Ala	Ile	Gly	295

	aat	gtg	gcg	aaa	cat	tca	gag	gcg	aaa	aac	tgc	cac	gtg	aca	cta	acc	1316
	Asn	Val	Ala	Lys	His	Ser	Glu	Ala	Lys	Asn	Cys	His	Val	Thr	Leu	Thr	
	310					315					320					325	
5	tac	gaa	gac	aca	gaa	gta	cgc	ctt	gat	gtg	gtt	gat	gac	ggt	gtg	ggt	1364
	Tyr	Glu	Asp	Thr	Glu	Val	Arg	Leu	Asp	Val	Val	Asp	Asp	Gly	Val	Gly	
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10	ttt	gag	cct	tcg	gaa	gtg	tcc	agt	acc	ccc	gct	ggc	ctt	ggc	cat	atc	1412
	Phe	Glu	Pro	Ser	Glu	Val	Ser	Ser	Thr	Pro	Ala	Gly	Leu	Gly	His	Ile	
					345				350					355			
15	ggc	tta	acc	gca	ttg	cag	cag	cgt	gcg	atg	gaa	ttg	cac	ggc	gaa	gtt	1460
	Gly	Leu	Thr	Ala	Leu	Gln	Gln	Arg	Ala	Met	Glu	Leu	His	Gly	Glu	Val	
					360			365					370				
20	ata	gtg	gaa	tct	gca	tat	ggg	cag	ggt	act	gcg	gta	tct	gca	gca	ttg	1508
	Ile	Val	Glu	Ser	Ala	Tyr	Gly	Gln	Gly	Thr	Ala	Val	Ser	Ala	Ala	Leu	
					375		380					385					
25	ccg	gtg	gag	cca	cca	gag	ggg	ttt	gtc	ggg	gcg	ccg	gtt	ttg	gca	gat	1556
	Pro	Val	Glu	Pro	Pro	Glu	Gly	Phe	Val	Gly	Ala	Pro	Val	Leu	Ala	Asp	
	390					395					400					405	
30	tcg	gac	tca	agt	gct	aca	ggc	gag	gtt	gaa	cta	agt	tct	cca	act	gac	1604
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	Asp	Glu															
40	gcattttcat	tcgaaacgaa	gtgcgccatt	gggaaggacc	tagttcaaac	aatgattcgc											1720
45	gtgctgcttg	ctgatgacca	cgaaatcgtg	aggctcggac	tccgagctgt	gctggaaagc											1780
50	gccgaggaca	ttgaagtgg	gggcgaagtc	tccaccgccg	aagggtgcgg	gcaggcagcc											1840
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	1				5		</										

	His	Gly	Met	Gln	Leu	Gly	Trp	Leu	Phe	Val	Leu	Thr	Leu	Val	Trp	Ile	
	65					70					75					80	
5	Phe	Met	Val	Pro	Ile	Val	Pro	Val	Ser	Ile	Tyr	Leu	Leu	Phe	Pro	Leu	
					85					90					95		
	Phe	Phe	Leu	Tyr	Leu	Gln	Val	Met	Pro	Asp	Val	Arg	Gly	Ile	Ile	Ala	
				100					105					110			
10	Ile	Leu	Gly	Ala	Thr	Ala	Ile	Ala	Ile	Ala	Ser	Gln	Tyr	Ser	Val	Gly	
			115					120					125				
	Leu	Thr	Phe	Gly	Gly	Val	Met	Gly	Pro	Val	Val	Ser	Ala	Ile	Val	Thr	
15		130					135					140					
	Val	Ala	Ile	Asp	Tyr	Ala	Phe	Arg	Thr	Leu	Trp	Arg	Val	Asn	Asn	Glu	
	145					150					155					160	
20	Lys	Gln	Glu	Leu	Ile	Asp	Gln	Leu	Ile	Glu	Thr	Arg	Ser	Gln	Leu	Ala	
					165					170					175		
	Val	Thr	Glu	Arg	Asn	Ala	Gly	Ile	Ala	Ala	Glu	Arg	Gln	Arg	Ile	Ala	
				180					185					190			
25	His	Glu	Ile	His	Asp	Thr	Val	Ala	Gln	Gly	Leu	Ser	Ser	Ile	Gln	Met	
			195					200					205				
	Leu	Leu	His	Val	Ser	Glu	Gln	Glu	Ile	Leu	Val	Ala	Glu	Met	Glu	Glu	
30		210					215					220					
	Lys	Pro	Lys	Glu	Ala	Ile	Val	Lys	Lys	Met	Arg	Leu	Ala	Arg	Gln	Thr	
	225					230					235					240	
35	Ala	Ser	Asp	Asn	Leu	Ser	Glu	Ala	Arg	Ala	Met	Ile	Ala	Ala	Leu	Gln	
					245					250					255		
	Pro	Ala	Ala	Leu	Ser	Lys	Thr	Ser	Leu	Glu	Ala	Ala	Leu	His	Arg	Val	
				260					265					270			
40	Thr	Glu	Pro	Leu	Leu	Gly	Ile	Asn	Phe	Val	Ile	Ser	Val	Asp	Gly	Asp	
			275					280					285				
	Val	Arg	Gln	Leu	Pro	Met	Lys	Thr	Glu	Ala	Thr	Leu	Leu	Arg	Ile	Ala	
45		290					295					300					
	Gln	Gly	Ala	Ile	Gly	Asn	Val	Ala	Lys	His	Ser	Glu	Ala	Lys	Asn	Cys	
	305					310					315					320	
50	His	Val	Thr	Leu	Thr	Tyr	Glu	Asp	Thr	Glu	Val	Arg	Leu	Asp	Val	Val	
					325					330					335		
	Asp	Asp	Gly	Val	Gly	Phe	Glu	Pro	Ser	Glu	Val	Ser	Ser	Thr	Pro	Ala	
				340					345					350			
55	Gly	Leu	Gly	His	Ile	Gly	Leu	Thr	Ala	Leu	Gln	Gln	Arg	Ala	Met	Glu	
			355					360					365				

	Leu	His	Gly	Glu	Val	Ile	Val	Glu	Ser	Ala	Tyr	Gly	Gln	Gly	Thr	Ala	
	370						375					380					
5	Val	Ser	Ala	Ala	Leu	Pro	Val	Glu	Pro	Pro	Glu	Gly	Phe	Val	Gly	Ala	
	385					390					395					400	
	Pro	Val	Leu	Ala	Asp	Ser	Asp	Ser	Ser	Ala	Thr	Gly	Glu	Val	Glu	Leu	
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BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG
Kantstr. 2

33790 Halle/Künsebeck

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: Top 10/pCR2.1luxSint	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 14082
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I. above was accompanied by: ☒ a scientific description ☒ a proposed taxonomic designation (Mark with a cross where applicable).	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2001-02-23 (Date of the original deposit) ¹ .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 2001-02-26

¹ Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG
Kantstr. 2

33790 Halle/Künsebeck

VIABILITY STATEMENT

issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Degussa AG Kantstr. 2 Address: 33790 Halle/Künsebeck	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 14082 Date of the deposit or the transfer¹: 2001-02-23
III. VIABILITY STATEMENT The viability of the microorganism identified under II above was tested on 2001-02-23². On that date, the said microorganism was ☒³ viable ☐³ no longer viable	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED⁴	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): <i>V. Weis</i> Date: 2001-02-26

¹ Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.

⁴ Fill in if the information has been requested and if the results of the test were negative.