Title: NUCLEOTIDE SEQUENCES WHICH CODE FOR THE ATR43 GENE

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 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 atr43 gene is present in attenuated form, and the use of polynucleotides which comprise the sequences according to the invention as hybridization probes.
Published:
— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.
Nucleotide sequences which code for the atr43 gene

Field of the Invention

The invention provides nucleotide sequences from coryneform bacteria which code for the atr43 gene and a process for the fermentative preparation of amino acids using bacteria in which the atr43 gene is attenuated.

Prior Art

L-Amino acids, in particular L-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. L-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 atr43 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 ABC transporter Atr43.

The invention also provides the above-mentioned polynucleotide, this preferably being a DNA 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 degeneration of the genetic code, or

(iii) at least one sequence which hybridizes with the sequences complementary to sequences (i) or (ii), 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 atr43 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 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.

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 the ABC transporter Atr43 or to isolate those nucleic acids or polynucleotides or genes which have a high similarity with the sequence of the atr43 gene. They are also suitable for incorporation into so-called "arrays", "micro arrays" or "DNA chips" in order to detect and determine the corresponding polynucleotides.

Polynucleotides which comprise the sequences according to the invention are furthermore suitable as primers with the aid of which DNA of genes which code for the ABC transporter Atr43 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 particularly preferably at least 15 successive nucleotides. Oligonucleotides which have a length of at least 40 or 50 nucleotides are also suitable. Oligonucleotides with a length of at least 100, 150, 200, 250 or 300 nucleotides are optionally also suitable.

"Isolated" means separated out of its natural environment.
"Polynucleotide" in general relates to polycyribonucleotides and polydeoxyriboonucleotides, 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% to 80%, preferably at least 81% to 85%, particularly preferably at least 86% to 90%, and very particularly preferably at least 91%, 93%, 95%, 97% or 99% 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 ABC transporter Atr43, and also those which are at least 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 91%, 93%, 95%, 97% or 99% 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 atr43 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.

By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein.

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 atr43 gene from C. glutamicum which codes for the ABC transporter Atr43 has been isolated.

To isolate the atr43 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 [Genes and Clones, An Introduction to Genetic Engineering] (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 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 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).


To prepare a gene library of C. glutamicum in E. coli it is also possible to use plasmids such as pBR322 (Bolivar, 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 DH5αmcr, which has been described by Grant et al. (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 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 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)).

The new DNA sequence of C. glutamicum which codes for the atr43 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 atr43 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 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 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 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 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 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°C - 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°C - 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°C to 68°C in steps of approx. 1 - 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: OligonucleotideSynthesis: 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 atr43 gene.

To achieve an attenuation, either the expression of the atr43 gene or the 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

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 Enzym [Threonine dehydratase from Corynebacterium glutamicum: Canceling the allosteric regulation and structure of the enzyme]", Reports from the Jülich Research Center, 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 [General Genetics]", 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 [Molecular Genetics]", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone [Genes and Clones]", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik [General Genetics]", 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 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., 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 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 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)), Duncan and Shivnan (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 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 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. 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 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 atr43 gene in this manner.

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 optionally regulatory proteins, in addition to the attenuation of the atr43 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 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.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on the starting microorganism.

Thus, for the preparation of L-amino acids, in addition to attenuation of the atr43 gene, at the same time one or more of the genes chosen from the group consisting of

- the dapA gene which codes for dihydrolipicolinate 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 mgo 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.FP6512; EP-B-0387527; EP-A-0699759),

• 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 Microbiology 65: 1973-1979),

• the zwal gene which codes for the Zwal protein (DE: 19959328.0, DSM 13115)

can be enhanced, in particular over-expressed.

It may furthermore be advantageous for the production of amino acids, in addition to attenuation of the atr43 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),
• 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: 19959327.2, DSM 13113)

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

In addition to the attenuation of the atr43 gene it may furthermore be advantageous for the production of amino 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 [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 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 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 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 culturing in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, 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
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 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, for example, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by anion exchange chromatography with subsequent ninhydrin derivation, or it can be carried out by reversed phase HPLC, for example as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The process according to the invention is used for the fermentative preparation of L-amino acids, in particular of L-lysine. The amino acids are in general isolated by conventional processes or separated off together with constituents of the fermentation broth and optionally the entire biomass or portions thereof.

The following microorganism was deposited as a pure culture on 11th April 2001 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 Top10/pCR2.1atr43int as DSM 14226.

The present invention is explained in more detail in the 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.

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

Example 1

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

Chromosomal DNA from C. glutamicum ATCC 13032 was isolated 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 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 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 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-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 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 MgSO_4 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 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

Isolation and sequencing of the atr43 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 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. 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. 20021, Qiagen, Hilden, Germany).
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 DH5αmcr (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 μg/ml zeocin.

The plasmid preparation of the recombinant clones was carried out with the Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out by the 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 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 pZero1 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 programs" (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 1626 bp, which was called the atr43 gene. The atr43 gene codes for a polypeptide of 541 amino acids.

Example 3

Preparation of an integration vector for integration mutagenesis of the atr43 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 atr43 gene known for C. glutamicum from example 2, the following oligonucleotides were chosen for the polymerase chain reaction:

at43-int1:
5' GCA GCT TAA AAC CCT GTC C 3'
at43-int2:
5' GTT GTC GAT CAT TCG TTC C 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. 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 primers allow amplification of an internal fragment of the atr43 gene 460 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;

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, 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 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 pCR2.1atr43int and is shown in Figure 1.

Example 4

Integration mutagenesis of the atr43 gene in the strain DSM 5715

The vector pCR2.1atr43int mentioned in example 3 was 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 (EP 435 132). The vector pCR2.1atr43int 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 pCR2.1atr43int 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 atr43int fragment was labeled 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 KpnI, EcoRI and PstI. The fragments formed were separated by means of agarose gel electrophoresis and hybridized at 68°C with the Dig hybridization kit from Boehringer. The plasmid pCR2.1atr43int mentioned in example 3 had been inserted into the chromosome of DSM5715 within the chromosomal atr43 gene. The strain was called DSM5715::pCR2.1atr43int.

Example 5

Preparation of lysine

The C. glutamicum strain DSM5715::pCR2.1atr43int obtained in example 4 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 kanamycin (25 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

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) 50 g/l
Salts:
(NH₄)₂SO₄ 25 g/l
KH₂PO₄ 0.1 g/l
MgSO₄ * 7 H₂O 1.0 g/l
CaCl₂ * 2 H₂O 10 mg/l
FeSO₄ * 7 H₂O 10 mg/l
MnSO₄ * H₂O 5.0 mg/l
Biotin (sterile-filtered) 0.3 mg/l
Thiamine * HCl (sterile-filtered) 0.2 mg/l
Leucine (sterile-filtered) 0.1 g/l
CaCO₃ 25 g/l

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

Culturing was 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 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-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivation with ninhydrin detection.

The result of the experiment is shown in Table 1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>OD (660 nm)</th>
<th>Lysine HCl g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSM5715</td>
<td>8.7</td>
<td>12.64</td>
</tr>
<tr>
<td>DSM5715::pCR2.1atr43int</td>
<td>9.1</td>
<td>13.57</td>
</tr>
</tbody>
</table>

Brief Description of the Figure:

Figure 1: Map of the plasmid pCR2.1atr43int.

The abbreviations and designations used have the following meaning.

KmR: Kanamycin resistance gene
KpnI: Cleavage site of the restriction enzyme KpnI
EcoRI: Cleavage site of the restriction enzyme EcoRI
PstI: Cleavage site of the restriction enzyme PstI
atr43int: Internal fragment of the atr43 gene
ColEl: Replication origin of the plasmid ColEl
What is claimed is:

1. Isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the atr43 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 ABC transporter Atr43.

2. Polynucleotide according to claim 1, wherein the polynucleotide is a preferably recombinant DNA which is capable of replication in coryneform bacteria.

3. Polynucleotide according to claim 1, wherein the polynucleotide is an RNA.

4. Polynucleotide according to claim 2, comprising the nucleic acid sequence as shown in SEQ ID No. 1.

5. DNA according to 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 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).

6. DNA according to claim 5 which is capable of replication, wherein the hybridization is carried out under a stringency corresponding to at most 2x SSC.

7. Polynucleotide sequence according to claim 1, which codes for a polypeptide which comprises the amino acid sequence shown in SEQ ID No. 2.

8. Coryneform bacteria in which the atr43 gene is attenuated, in particular eliminated.

9. Coryneform bacteria in which the atr43 gene is eliminated by integration mutagenesis.

10. Integration vector pCR2.1atr43int, deposited in the strain DSM 14226 at the deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), shown in figure 1.

11. Process for the fermentative preparation of L-amino acids, in particular L-lysine, wherein the following steps are carried out:

a) fermentation of the coryneform bacteria which produce the desired L-amino acid and in which at least the atr43 gene or nucleotide sequences which
code for it are attenuated, in particular eliminated,

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, wherein optionally constituents of the fermentation broth or the entire biomass are present.

12. Process according to claim 11, wherein bacteria in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are employed.

13. Process according to claim 11, wherein bacteria in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.

14. Process according to claim 11, wherein the expression of the polynucleotide(s) which code(s) for the atr43 gene is attenuated, in particular eliminated.

15. Process according to claim 11, wherein the catalytic properties of the polypeptide (enzyme protein) for which the polynucleotide atr43 codes are reduced.

16. Process according to claim 12, wherein for the preparation of L-amino acids, coryneform microorganisms in which at the same time one or more of the genes chosen from the group consisting of

16.1 the dapA gene which codes for dihydrolipicolinate synthase,

16.2 the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase,
the tpi gene which codes for triose phosphate isomerase,

the pgk gene which codes for 3-phosphoglycerate kinase,

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

the pyc gene which codes for pyruvate carboxylase,

the mgo gene which codes for malate-quinone oxidoreductase,

the lysC gene which codes for a feed-back resistant aspartate kinase,

the lysE gene which codes for lysine export,

the hom gene which codes for homoserine dehydrogenase

the ilvA gene which codes for threonine dehydratase or the ilvA(Fbr) allele which codes for a feed back resistant threonine dehydratase,

the ilvBN gene which codes for acetohydroxy-acid synthase,

the ilvD gene which codes for dihydroxy-acid dehydratase,

the zwal gene which codes for the Zwal protein,

is or are enhanced or over-expressed are fermented.

Process according to claim 14, wherein for the preparation of L-amino acids, coryneform microorganisms
in which at the same time one or more of the genes
chosen from the group consisting of

17.1 the pck gene which codes for phosphoenol
pyruvate carboxykinase,

17.2 the pgi gene which codes for glucose 6-
phosphate isomerase,

17.3 the poxB gene which codes for pyruvate oxidase,

17.4 the zwa2 gene which codes for the Zwa2 protein,
is or are attenuated are fermented.

18. Coryneform bacteria which contain a vector which
carries parts of the polynucleotide according to claim
1, but at least 15 successive nucleotides of the
sequence claimed.

19. Process according to one or more of claims 11-17,
wherein microorganisms of the genus Corynebacterium
 glutamicum are employed.

20. Process for discovering RNA, cDNA and DNA in order to
isolate nucleic acids or polynucleotides or genes which
code for the ABC transporter Atr43 or have a high
similarity with the sequence of the atr43 gene,
characterized in that the polynucleotide comprising the
polynucleotide sequences according to claims 1, 2, 3 or
4 is employed as hybridization probes.

21. A process as claimed in claim 20, wherein arrays, micro
arrays or DNA chips are employed.
Figure 1: Plasmid pCR2.1atr43int
SEQUENCE PROTOCOL

<110> Degussa AG

5 <120> Nucleotide sequences which code for the atr43 gene

<130> 000543 BT

<140>

<141>

<160> 4

<170> PatentIn Ver. 2.1

15 <210> 1
<211> 2080
<212> DNA
<213> Corynebacterium glutamicum

20 <220>
<221> CDS
<222> (249)...(1871)
<223> atr43 gene

25 <400> 1
aagaggaacc caccagcacc gaagaacacg cgtcagaaac tgagcagcct tctgaacctg 60
aagaggaacc gacctgtgttt gcttgaagct ctaacggtgg cagttttgtc ggcgttttag 120
cgctgctggc agccgcttggt gcgcagctcg gtcagcctct cggattttgt cagtttga 180
tggctggggg cgcgtggaaag cagcttttatt gagctggcga cttaatttga tgaactgtta 240
gg tgtgta ttg tca cca atg att aga ggt ggc tgt tgg cgc acg tac ctt 290

30 Met Ser Pro Met Ile Arg Gly Ala Arg Thr Tyr Leu

35 ctc gat gcc cca ggt cag ctc ctt cgg gtc gag cca ggc gac gct att 338
Leu Asp Ala Pro Gly Gln Leu Leu Arg Val Gln Pro Gly Asp Arg Ile

40 ggt ctg gtt ggt aga aat ggt ggc ggc aaa acc acc acc atg cga atc 386
Gly Leu Val Gly Arg Asn Gly Lys Thr Thr Thr Met Arg Ile

45 ctc tcc ggc ccc acc aag ccc tac gga gga ttc gta acc aca tct ggt 434
Leu Ser Gly Glu Thr Lys Pro Tyr Gly Gly Ser Val Thr Thr Ser Gly

50 gaa atc gtt tac ctg ccc cag cac tcc cgc gaa ggc aac atc gaa cca 482
Glu Ile Gly Tyr Leu Pro Gln Asp Ser Arg Glu Gly Asp Ile Glu Gln

55 acc gcc cgc gac cga gtc ctc tcc gcc cgt ggt ctt gag cag ctc cga 530
Thr Ala Arg Asp Arg Val Ser Ala Arg Gly Leu Asp Gln Leu Arg
aac ggc aag gcc ctg acc aag atg tac ggc tcc ctc gaa gtc ttc gcc
Asp Ala Lys Gly Leu Thr Lys Met Tyr Gly Ser Leu Glu Val Phe Ala
335 340 345 350

10
ggc gtc gac cta gcc atc gac aag gcc tcc cgc gta gtc gtc ctc gga
Gly Val Asp Leu Ala Ile Asp Lys Gly Ser Arg Val Val Val Leu Gly
355 360 365

15
ttc aac ggt gca ggt aag acc acc ctg ctc aac ctc ctc ggc ggt gtg
Phe Asn Gly Ala Gly Thr Thr Leu Leu Lys Ala Gly Val
370 375 380

20
gaa cgc acc gcc gaa ggc gcc atc acc gga tac ggc ctc aag
Glu Arg Thr Asp Gly Ser Gly Ile Val Thr Gly Tyr Leu Lys
385 390 395

25
atc ggc tac ttc gcc cag gcc cgc gac acc atc gcc ccc gcc gac aag
Ile Gly Tyr Phe Ala Gln Glu His Asp Thr Ile Asp Pro Asp Lys Ser
400 405 410

30
gtc tgg caa acc acc atc gaa gcc tgc gcc gac gcc gac caa caa agc
Val Trp Gln Asn Thr Ile Gln Leu Val Asp Ala Asp Glu Gln Gln Ser
415 420 425 430

35
ctc cgc agc ctc ctc gcc gaa tcc ttc atg ttc gcc gaa cca ctc gac
Leu Arg Ser Leu Leu Gly Ser Phe Met Phe Ser Gly Glu Gln Leu Asp
435 440 445

40
caa cca gca gga aca ctc ctc gcc ggt gaa aag acc cgc ctc gca ctg
Gln Pro Ala Gly Thr Leu Ser Gly Gly Lys Thr Arg Leu Ala Leu
450 455 460

45
gcc acc ctc gtc tcc tcc cgc gca aac gtc ctg ctt ctc gcc gac gag ccc
Ala Thr Leu Val Ser Ser Arg Ala Asn Val Leu Leu Leu Asp Glu Pro
465 470 475

50
acc aac acc ctt gcc ccg atc tcc cgc gaa cag gtc ctc gac gca ctg
Thr Asn Asn Leu Asp Pro Ile Ser Ser Arg Glu Gln Val Leu Asp Ala Leu
480 485 490

55
cgc acc tac acc gcc gca gtc gtc ctg gtt acc cac gcc ccg ggt gca
Arg Thr Tyr Thr Gly Ala Val Val Leu Val Thr His Asp Pro Gly Ala
495 500 505 510

50
gtc aag gcc ctt gag cca gaa cgg gcc atc gtc gtt cct gat ggc ctc
Val Lys Ala Leu Glu Pro Glu Arg Val Ile Val Leu Pro Asp Gly Thr
515 520 525

55
gag gat ctt tgg aat gat cag tac atg gaa atc gtc gaa ttg ggc
Glu Asp Leu Trp Asp Asp Gln Tyr Met Glu Ile Val Glu Leu Ala
530 535 540

55
tagttctaa ggctgtttat gctggcaag actgtttcgt tttaaactcc tgcactttgc 1931
tgcocoaag ttggctgcat gaattctac atcgaggctta tttaagctgc ttggctgatgg 1991
ttgaggccag ggaagaaggg gacataagtg tccaaggttg tcgataccgt cgtatgccccc 2051
<210> 2
<211> 541
<212> PRT
<213> Corynebacterium glutamicum

<400> 2
Met Ser Pro Met Ile Arg Gly Ala Arg Trp Arg Thr Tyr Leu Leu Asp
  1  5  10  15
Ala Pro Gly Glu Leu Leu Arg Val Gln Pro Gly Asp Arg Ile Gly Leu
  20  25  30
Val Gly Arg Asn Gly Ala Gly Lys Thr Thr Thr Met Arg Ile Leu Ser
  35  40  45
Gly Glu Thr Lys Pro Tyr Gly Gly Ser Val Thr Thr Ser Gly Glu Ile
  50  55  60
Gly Tyr Leu Pro Gln Asp Ser Arg Glu Gly Asn Ile Glu Gln Thr Ala
  65  70  75  80
Arg Asp Arg Val Leu Ser Ala Arg Gly Leu Asp Gln Leu Arg Ser Ser
  85  90  95
Met Glu Arg Gln Glu Ile Met Glu Thr Ala Thr Asp Pro Gly Lys
 100 105 110
Leu Asp Ala Ala Ile Arg Lys Tyr Ser Arg Leu Glu Glu Glu Phe Gln
 115 120 125
Ser Leu Gly Gly Tyr Glu Ala Asp Ala Glu Ala Ala Glu Ile Cys Asp
 130 135 140
Asn Leu Gly Leu Glu Ala Arg Ile Leu Asp Gln Glu Leu Lys Thr Leu
 145 150 155 160
Ser Gly Gly Gln Arg Arg Val Glu Leu Ala Gln Ile Leu Phe Ala
 165 170 175
Ala Thr Asn Gly Ser Gly Lys Ser Lys Thr Thr Leu Leu Leu Asp Glu
 180 185 190
Pro Thr Asn His Leu Asp Ala Asp Ser Ile Thr Trp Leu Arg Asp Phe
 195 200 205
Leu Ala Lys His Glu Gly Leu Ile Met Ile Ser His Asp Val Glu
 210 215 220
Leu Leu Gly Ala Val Cys Asn Lys Ile Trp Tyr Leu Asp Ala Val Arg
 225 230 235 240
Ser Glu Ala Asp Val Tyr Asn Met Gly Phe Ser Lys Tyr Val Asp Ala
 245 250 255
Arg Ala Leu Asp Glu Ala Arg Arg Arg Arg Glu Arg Ala Asn Ala Glu
 260 265 270
Lys Lys Ala Gly Ala Leu Lys Asp Glu Ala Ala Arg Leu Gly Ala Lys 275 280 285
Ala Thr Lys Ala Ala Ala Lys Gln Met Ile Ala Arg Ala Glu Arg 290 295 300
Met Ile Asp Asn Leu Asp Glu Ile Arg Val Ala Asp Arg Ala Ala Asn 305 310 315 320
Ile Val Phe Pro Glu Pro Ala Pro Cys Gly Lys Thr Pro Leu Asn Ala 325 330 335
Lys Gly Leu Thr Lys Met Tyr Gly Ser Leu Glu Val Phe Ala Gly Val 340 345 350
Asp Leu Ala Ile Asp Lys Gly Ser Arg Val Val Leu Gly Phe Asn 355 360 365
Gly Ala Gly Lys Thr Thr Leu Leu Leu Lys Leu Ala Gly Val Glu Arg 370 375 380
Thr Asp Gly Glu Gly Gly Ile Val Thr Gly Tyr Gly Leu Lys Ile Gly 385 390 395 400
Tyr Phe Ala Gln Glu His Asp Thr Ile Asp Pro Asp Lys Ser Val Trp 405 410 415
Gln Asn Thr Ile Glu Ala Cys Ala Asp Ala Asp Gln Gln Ser Leu Arg 420 425 430
Ser Leu Leu Gly Ser Phe Met Phe Ser Gly Glu Gln Leu Asp Gln Pro 435 440 445
Ala Gly Thr Leu Ser Gly Gly Glu Lys Thr Arg Leu Ala Leu Ala Thr 450 455 460
Leu Val Ser Ser Arg Ala Asn Val Leu Leu Leu Asp Glu Pro Thr Asn 465 470 475 480
Asn Leu Asp Pro Ile Ser Arg Glu Gln Val Leu Asp Ala Leu Arg Thr 485 490 495
Tyr Thr Gly Ala Val Val Leu Val Thr His Asp Pro Gly Ala Val Lys 500 505 510
Ala Leu Glu Pro Glu Arg Val Ile Val Leu Pro Asp Gly Thr Glu Asp 515 520 525
Leu Trp Asn Asp Gln Tyr Met Glu Ile Val Glu Leu Ala 530 535 540

<210> 3
<211> 19
<212> DNA
<213> Corynebacterium glutamicum
Primer atr43-int1

gcagcttaaa acacctgtcc

DNA
Corynebacterium glutamicum

Primer atr43-int2

gttgtgcgtc attcgttcc