

Method for the production of L-ornithine by fermentation

Prior art

L-Ornithine is known for its stimulatory action regarding liver function and is frequently utilized as an ingredient
5 of medicaments and in sports nutrition.

L-Ornithine is nowadays prepared by various processes. One method is the fermentative preparation with the aid of microorganisms. Another method is alkaline hydrolysis of arginine, for example with barium hydroxide (CN 1594282 A).
10 Another method is the biotransformation of arginine by immobilized microorganisms possessing an arginase activity (KR589121B1). A method of preparing L-ornithine from L-citrulline has also been described in the patent literature (JP 42007767 B4).

15 Microorganisms which are distinguished in that they excrete L-ornithine into the culture medium have been described in the literature. Examples of said microorganisms are bacteria of the genus Corynebacterium, Brevibacterium, Bacillus (JP 43010996 B4, JP 57041912 B), Escherichia
20 (US 3668072 A), Providencia (JP 03195494) or Arthrobacter (US 3574061).

L-Ornithine-producing microorganisms are often distinguished by being auxotrophic for the amino acids L-arginine or L-citrulline (described for Brevibacterium,
25 Bacillus, Corynebacterium in EP 392708 B1 and KR 161147 B1 and for Escherichia in US 366072 A). Furthermore, microorganisms have been described which are resistant to 2-thiazole-alanine, sulphaguanidine or 2-fluoropyruvate (Japanese Open-Laid publication No. 61-119194).
30 EP 0393708 B1 describes L-ornithine producers which are distinguished by a lower resistance to ornithine and mycophenolic acid. Said properties may also be in a combined form.

The release of basic amino acids such as L-lysine, L-arginine and L-ornithine by way of passive diffusion from the cell is very poor (Bellmann et al. (Microbiology 2001; 147: 1765-74)). This has been well described for lysine by way of example. Vrlijc et al. (Journal of Bacteriology 1995; 177(14): 4021-7) have studied a plurality of export-deficient *Corynebacterium glutamicum* mutants. For one mutant, an intracellular concentration of 174 mM L-lysine was measured, while a value of only 0.7 mM was measured extracellularly.

Vrlijc et al. (Molecular Microbiology 1996; 22(5): 815-26 and Journal of Molecular Microbiology and Biotechnology 1999; 1: 327-336) and EP 0868527 B1 identified and described a novel exporter as L-lysine exporter (LysE). A defined LysE null mutant was no longer capable of transporting L-lysine out of the cell. The polypeptide encoded by the *lysE* gene is 233 amino acids or amino acid residues in length and is represented in SEQ ID No. 2. After overexpression of the *lysE* gene in a lysine producer, an increase in L-lysine excretion was found.

Von Bellmann et al. (Microbiology 2001; 147: 1765-74) have characterized in more detail the LysE exporter with regard to the transport of various basic amino acids in *C. glutamicum*. The authors demonstrated that the transporter specifically exports the amino acids L-lysine and L-arginine out of the cell. The authors furthermore investigated whether LysE also exports L-ornithine out of the cell. For this purpose, first of all an L-arginine-auxotrophic *C. glutamicum* strain referred to as ATCC13032::argF was prepared.

The strain was cultured in 50 ml (batch culture) of a minimal medium referred to as CGXII which contained 40 g/l glucose. After an incubation period of 24 hours 60 mM L-ornithine, corresponding to 7.9 g/l, were measured. Intracellularly, an L-ornithine concentration of approx.

200 mM was measured in the cells of said strain over an incubation period of approx. 70 minutes. In order to clarify, whether LysE also transports L-ornithine out of the cell, the strain 13032::argF was transformed with the replicative plasmid pEC7lysE. This measure was intended to provide the strain with an increased LysE activity, thereby allowing the strain to transport L-ornithine into the medium at a higher rate of export. However, said measure did not increase the rate of L-ornithine export. The same rate of export ($0.6 \text{ nmol min}^{-1} (\text{mg of dry mass})^{-1}$) was determined both for the control strain (13032::argF) and in the transformant (13032::argF, harbouring pEC7lysE). From this the authors concluded that L-ornithine is not exported by the LysE exporter. They furthermore drew the conclusion that there must be another, unknown export function (export protein) for L-ornithine in *Corynebacterium glutamicum* (Bellmann et al., 2001, page 1771, fig. 5b) and page 1772, lines 21-28).

A variant LysE (see SEQ ID No. 4) was identified in *C. glutamicum* R, which differs from the amino acid sequence of the LysE exporter from strain ATCC 13032, depicted in SEQ ID No. 2, by an N terminus extended by three amino acid residues. The sequence of said amino acid residues is: methionine, valine, isoleucine (MVI). This LysE polypeptide from strain R has been described in EP 1266966 B1 as a variant which differs from the wild-type protein in the formation of a loop region or, more specifically, can no longer form said loop, and is therefore able to accomplish improved export of L-lysine and L-arginine.

Another LysE variant has been described by Gunji and Yasueda (Journal of Biotechnology 127, 2006, 1-13). The authors were interested in L-lysine formation by the obligately methylotrophic bacterium, *Methylophilus methylotrophus*. They transformed *M. methylotrophus* with a plasmid referred to as pSE which contained the

C. glutamicum ATCC13869 lysE gene in order to improve lysine formation by M. methylotrophus. However, the authors found that they were able to establish only a mutated form of the lysE gene (lysE24) in a stable manner in

5 M. methylotrophus. The open reading frame of the lysE gene has been shifted in the lysE24 allele due to the insertion of a thymine residue, resulting in the termination of the reading frame after 432 bp. The truncated reading frame codes for a LysE protein which is shorter by 92 aa residues

10 at the C terminus than the wild-type LysE protein of C. glutamicum ATCC13869. It is 141 amino acid residues in length. In addition, the last 6 C-terminal amino acids of the truncated protein (residues 135-141) differ from the amino acids of the wild-type LysE amino acid sequence. An

15 M. methylotrophus strain carrying the modified LysE allele on a plasmid (pSE24) was tested for lysine formation. To this end, the strain was assayed in 0.3 l of a minimal medium referred to as SEIIc in the form of a fed-batch culture for 50 hours. The authors found that the

20 transformant also formed small quantities (0.07 mM corresponding to 11.8 mg/l) of L-ornithine, in addition to 0.55 mM L-lysine and 0.19 mM L-arginine. As explained by the authors, this observed formation of L-ornithine is due to either an altered substrate specificity of the mutated

25 transporter or possibly the altered intracellular L-arginine pool of the strain. EP 1266966 B1 (inventors: Gunji and Yasueda) describes the positive action of the LysE24 transporter on the excretion of L-lysine and L-arginine.

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Object of the invention

It is an object of the invention to provide a novel process for the fermentative preparation of L-ornithine.

Summary

In a first aspect, there is provided a process for the preparation of L-ornithine, characterized in that the following steps are carried out: a) fermentation of an L-ornithine-excreting bacterium selected from the group consisting of *Corynebacterium*, *Bacillus*, *Streptomyces*, *Arthrobacter* and the *Enterobacteriaceae* which overexpresses a polynucleotide coding for a polypeptide which has the activity of an L-ornithine exporter and whose amino acid sequence is identical to the amino acid sequence of SEQ ID No. 2 or whose amino acid sequence can be obtained from SEQ ID NO:2 by a maximum of 25 deletions, insertions, substitutions, or N-respectively C-terminal additions of amino acids overall, in a medium, b) accumulation of said L-ornithine in said medium, wherein a fermentation broth is obtained, and c) wherein the plasmid pEC7lysE, deposited in DSM23239, is excluded for overexpression.

Description of the invention

The invention relates to a process for the preparation of L-ornithine, characterized in that the following steps are carried out:

- 5 a) fermentation of an L-ornithine-excreting bacterium selected from the group consisting of *Corynebacterium*, *Bacillus*, *Streptomyces*, *Arthrobacter* and the *Enterobacteriaceae* which overexpresses a polynucleotide coding for a polypeptide which has the
10 activity of an L-ornithine exporter and whose amino acid sequence is at least ($\geq$) 35%, $\geq$ 40%, $\geq$ 50%, $\geq$ 55%, $\geq$ 60%, $\geq$ 65%, $\geq$ 70%, $\geq$ 75%, $\geq$ 80%, $\geq$ 85%, $\geq$ 90%, $\geq$ 92%, $\geq$ 94%, $\geq$ 96%, $\geq$ 97%, $\geq$ 98%, $\geq$ 99% or 100%, preferably $\geq$ 70%, particularly preferably $\geq$ 90%,
15 very particularly preferably $\geq$ 96%, and most preferably 100%, identical to the amino acid sequence of SEQ ID No. 2, in a medium,
- b) accumulation of said L-ornithine in said medium, wherein a fermentation broth is obtained,
- 20 c) wherein the plasmid pEC71ysE, deposited in DSM23239, is not used for overexpression,
- d) and wherein the length of the encoded polypeptide, where appropriate, is $\geq$ 146 to $\leq$ 286 of amino acids or amino acid residues.
- 25 Preference is given to selecting length ranges from the group consisting of $\geq$ 171 to $\leq$ 286, $\geq$ 196 to $\leq$ 261, $\geq$ 203 to $\leq$ 258, $\geq$ 218 to $\leq$ 243, $\geq$ 228 to $\leq$ 236, and $\geq$ 228 to $\leq$ 233 amino acids or amino acid residues.

Particular preference is given to the length ranges

- 30 $\geq$ 203 to $\leq$ 258, $\geq$ 218 to $\leq$ 243, $\geq$ 228 to $\leq$ 236, and $\geq$ 228 to $\leq$ 233, and very particular preference is given to the

length ranges ≥ 228 to ≤ 236 and ≥ 228 to ≤ 233 .

Where L-ornithine is mentioned hereinbelow, the term also comprises its salts such as, for example, L-ornithine monohydrochloride or L-ornithine sulphate.

- 5 A process according to the invention makes use of bacteria selected from the group consisting of the genera *Corynebacterium*, *Bacillus*, *Streptomyces*, *Arthrobacter* and the *Enterobacteriaceae* family.

10 Within the genus *Corynebacterium*, preference is given to strains based on the following species:

Corynebacterium efficiens, for example the type strain DSM44549,

Corynebacterium glutamicum, for example the type strain ATCC13032 or the strain R, and

- 15 *Corynebacterium ammoniagenes*, for example the strain ATCC6871,

with very particular preference being given to the species *Corynebacterium glutamicum*.

- 20 Some representatives of the species *Corynebacterium glutamicum* are also known in the prior art under other names. These include for example:

strain ATCC13870, referred to as *Corynebacterium acetoacidophilum*,

- 25 strain DSM20137, referred to as *Corynebacterium lilium*,

strain ATCC17965, referred to as *Corynebacterium melassecola*,

strain ATCC14067, referred to as *Brevibacterium flavum*,

strain ATCC13869, referred to as *Brevibacterium lactofermentum*, and

strain ATCC14020, referred to as *Brevibacterium divaricatum*.

5 The term "*Micrococcus glutamicus*" for *Corynebacterium glutamicum* has likewise been in use. Some representatives of the species *Corynebacterium efficiens* have also been referred to in the prior art as *Corynebacterium thermoaminogenes*, for example the strain FERM BP-1539.

10 Within the genus *Bacillus*, preference is given to the species *Bacillus subtilis*.

Within the genus *Arthrobacter*, preference is given to the species *Arthrobacter citreus*.

Within the Enterobacteriaceae family, preference is given to
15 the genera *Escherichia*, *Erwinia*, *Providencia*, *Pantoea* and *Serratia*. Particular preference is given to the genera *Escherichia* and *Serratia*. Very particular preference is given to the species *Escherichia coli* in the genus *Escherichia*, to the species *Serratia marcescens* in the
20 genus *Serratia*, and to the species *Providencia rettgeri* in the genus *Providencia*.

The bacteria or strains (starting strains) employed for the measures of overexpressing the L-ornithine exporter preferably already have the ability to excrete L-ornithine
25 into the nutrient medium surrounding them and accumulate it there. The expression "to produce" is also used for this hereinbelow. More specifically, the strains employed for said overexpression measures have the ability to concentrate or accumulate in the nutrient medium ≥ 0.1 g/l,
30 ≥ 0.3 g/l, ≥ 1 g/l, ≥ 3 g/l, ≥ 10 g/l L-ornithine. The starting strains are preferably strains which have been prepared by mutagenesis and selection, by recombinant DNA technologies or by a combination of both methods.

It is obvious and requires no further explanations that a bacterium suitable for the measures of the invention may also be obtained by firstly overexpressing a polynucleotide coding for a polypeptide which has the activity of an L-
5 ornithine exporter and whose amino acid sequence is at least ($\geq$) 35% identical to that of SEQ ID No. 2, with the length of the encoded polypeptides, where appropriate, being within the length ranges described above, in a wild strain such as, for example, in the *Corynebacterium*
10 *glutamicum* type strain ATCC 13032 or in the strain ATCC 14067, and subsequently causing said bacterium, by further genetic measures described in the prior art, to produce L-ornithine. Transforming a wild type, such as e.g. the strain ATCC13032, ATCC14067, ATCC13869 or ATCC17965,
15 only with the polynucleotide mentioned does not result in a process according to the invention.

Examples of strains of the species *Corynebacterium glutamicum* which excrete or produce L-ornithine are:

Brevibacterium lactofermentum FERM-BP 2344, and
20 *Corynebacterium glutamicum* FERM-BP 2345 described in US 5188947.

An example of a strain of the species *Arthrobacter citreus* which excretes or produces L-ornithine is:

Arthrobacter citreus FERM-BP 2342 described in US 5188947.

25 An example of a strain of the species *Bacillus subtilis* which excretes or produces L-ornithine is:

Bacillus subtilis BOR-32 (FERM-P 3647) described in JP 57041912.

An example of a strain of the species *Providencia rettgeri*
30 which excretes or produces L-ornithine is:

Providencia rettgeri ARG6 (FERM P-11147) described in

JP 03195494.

An example of a strain of the species *Escherichia coli* which excretes or produces L-ornithine is:

Escherichia coli B-19-19 (ATCC 21104) described in
5 US 3668072.

L-Ornithine-producing bacteria typically are auxotrophic for the amino acids L-citrulline or L-arginine. As an alternative, L-orthinine-producing bacteria which are bradytrophic for L-citrulline or L-arginine may also be
10 contemplated. Definitions of the terms auxotrophic and bradytrophic can be found, for example, on page 9 of WO 01/09286. Bradytrophs are also referred to as leaky mutants in the art. Bradytrophic bacteria used are in particular those in which the activity of the gene products
15 ArgF (ornithine carbamoyl transferase), ArgG (argininosuccinate synthase) or ArgH (argininosuccinate lyase) is greater than ($>$) zero but equal to or less than ($\leq$) 10 per cent, preferably $>$ zero and $\leq$ 1%, compared to the activity in the wild type.

20 The prior art has disclosed polynucleotides which are referred to as *lysE* gene and which code for proteins or polypeptides having the activity of an L-lysine exporter. These polypeptides are also referred to by the abbreviation *LysE*.

25 An exporter is a protein which resides in the cell membrane of a cell and which transports a metabolite, for example L-lysine or L-ornithine, from the cytoplasm of said cell out into the surrounding medium. If the energy required for this is provided in the form of adenosine triphosphate
30 (ATP), this is referred to as primary active transport or export. It is referred to as secondary active transport or export if said energy is provided in the form of an ion gradient, for example of sodium ions (Jeremy M. Berg, John

L. Tymoczko and L. Stryer; Biochemie [Biochemistry], 5th edition, pages 378-384, Spektrum Akademischer Verlag [publisher], Heidelberg, Germany, 2003). Instructions for determining L-ornithine export activity can be found in
5 Bellmann et al. (Microbiology 2001; 147: 1765-74).

In the course of the work leading to the present invention the lysine exporters of the genera *Corynebacterium*, preferably *Corynebacterium glutamicum*, and *Micrococcus*, preferably *Micrococcus luteus*, were found to have the
10 activity of an L-ornithine exporter in addition to the L-lysine export activity.

The measures of the invention make use of genes coding for polypeptides which have export activity for L-ornithine and whose amino acid sequence is at least ($\geq$) 35%, $\geq$ 40%,
15 $\geq$ 50%, $\geq$ 55%, $\geq$ 60%, $\geq$ 65%, $\geq$ 70%, $\geq$ 75%, $\geq$ 80%, $\geq$ 85%, $\geq$ 90%, $\geq$ 92%, $\geq$ 94%, $\geq$ 96%, $\geq$ 97%, $\geq$ 98%, $\geq$ 99% or 100%, preferably $\geq$ 70%, particularly preferably $\geq$ 90%, very particularly preferably $\geq$ 96%, and most preferably $\geq$ 100%, identical to the amino acid sequence of SEQ ID No. 2, with
20 the length of the encoded polypeptide, where appropriate, being within the above-described length ranges.

Examples of suitable L-ornithine exporters are the lysine exporters or LysE polypeptides of *Corynebacterium glutamicum* ATCC13032 (SEQ ID No. 2), *Corynebacterium*
25 *glutamicum* R (SEQ ID No. 4), *Corynebacterium glutamicum* ATCC14067 (SEQ ID No. 5), *Corynebacterium glutamicum* ATCC13869 (SEQ ID No. 7), *Corynebacterium efficiens* YS-314 (SEQ ID No. 9), *Corynebacterium diphtheriae* NCTC 13129 (SEQ ID No. 10), *Corynebacterium striatum* ATCC6940 (SEQ ID
30 No. 11), *Corynebacterium aurimucosum* ATCC700975 (SEQ ID No. 12), *Corynebacterium matruchotii* ATCC33806 (SEQ ID No. 13), *Corynebacterium pseudogenitalium* ATCC33035 (SEQ ID No. 14), *Corynebacterium accolens* ATCC49725 (SEQ ID No. 15), *Corynebacterium glucuronalyticum* ATCC 51867 (SEQ
35 ID No. 16), *Micrococcus luteus* NCTC2665 (SEQ ID No. 17),

Corynebacterium tubuculostearicum SK141 (SEQ ID No. 18) and Corynebacterium matruchotii ATCC14266 (SEQ ID No. 19). SEQ ID No. 18 and SEQ ID No. 19 are also referred to as ArgO polypeptides in the art.

- 5 The nucleotide sequence of the lyse genes of Corynebacterium glutamicum ATCC14067 and Corynebacterium glutamicum ATCC13869 was determined in this study (SEQ ID No. 6 and SEQ ID No. 8). The amino acid sequences of the Lyse polypeptide of Corynebacterium glutamicum ATCC14067
10 and Corynebacterium glutamicum ATCC13869 are depicted in SEQ ID No. 5 and 7. They are identical to the amino acid sequence of C. glutamicum ATCC13032 Lyse, depicted in SEQ ID No. 2.

15 Table 1 lists the accession numbers of Lyse polypeptides of various representatives of the genus Corynebacterium and of Micrococcus luteus, which were taken from the databases of the National Center for Biotechnology Information (NCBI, Bethesda, MD, US). Furthermore, Table 1 makes reference to the amino acid sequences of the Lyse polypeptide that are
20 depicted in the sequence listing. Finally, Table 1 indicates the length (number of amino acids) of the encoded Lyse polypeptide.

Table 1

Bacterium	SEQ ID No.	Accession number	Length of polypeptide
C. glutamicum	2	YP_225551.1	233
C. efficiens	9	ZP_05749209.1	228
C. diptheriae	10	NP_939452.1	228
C. striatum	11	ZP_03933958.1	222
C. aurimucosum	12	YP_002834652.1	235

C. matruchotii	13	ZP_03711883.1	244
C. pseudogenitalium	14	ZP_03922319.1	230
C. accolens	15	ZP_03931790.1	241
C. glucuronolyticum	16	ZP_03918361.1	261
M. luteus	17	YP_002958101.1	204
C. tuberculostearicum	18	ZP_05365683.1	230
C. matruchotii	19	ZP_04835056.1	244

Figure 1 depicts a multiple sequence alignment of the amino acid sequences of the LysE polypeptides of the bacteria listed in Table 1. The alignments of the amino acid sequences depicted in figure 1 were produced by the program Clone Manager 9 Professional Edition (Scientific & Educational Software 600 Pinner Weald Way Ste 202 Cary NC 27513 USA). The reference molecule used for the alignment was the LysE polypeptide (LysE) of ATCC13032. For the scoring matrix, the setting "Blosum 62" (see: Jeremy M. Berg, John L. Tymoczko and L. Stryer; Biochemie, 5th edition, pages 194-197, Spektrum Akademischer Verlag, Heidelberg, Germany, 2003)) was chosen.

It is also possible, where appropriate, to employ programs described in the prior art, such as, for example, the ClustalX program (Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research, 25: 4876-4882).

The amino acid residues 4-236 of the LysE polypeptide of Corynebacterium glutamicum R (see SEQ ID No. 4) correspond to the C. glutamicum ATCC13032 LysE amino acid sequence

depicted in SEQ ID No. 2. The *C. glutamicum* R polypeptide has N-terminally an additional sequence of three amino acid residues (methionine-valine-isoleucine). These additional residues are produced when the start codon located 9 base
5 pairs further upstream of the *lyse* gene is used as an alternative to the start codon of the *lyse* gene in *C. glutamicum* ATCC13032 (see SEQ ID No. 1).

The amino acid sequence of the *Lyse* polypeptide of *C. efficiens* YS-314 is 71%, and that of *C. diptheriae*
10 NCTC 13129 is 44%, that of *Corynebacterium striatum* ATCC6940 is 44%, that of *Corynebacterium aurimucosum* ATCC700975 is 42%, that of *Corynebacterium matruchotii* ATCC33806 is 43%, that of *Corynebacterium pseudogenitalium* ATCC33035 is 43%, that of *Corynebacterium accolens*
15 ATCC49725 is 43%, that of *Corynebacterium glucuronalyticum* ATCC 51867 is 36%, that of *Micrococcus luteus* NCTC2665 is 40%, identical to the *C. glutamicum* ATCC13032 *Lyse* amino acid sequence depicted in SEQ ID No. 2. Furthermore, the amino acid sequence of the *ArgO* polypeptide of
20 *C. tuberculostearicum* SK141 is 43% identical to the amino acid sequence of SEQ ID No. 2. Furthermore, the amino acid sequence of the *ArgO* polypeptide of *C. matruchotii* ATCC14266 is 44% identical to the amino acid sequence of SEQ ID No. 2. The identity percentages were produced by
25 generating a global sequence alignment with the aid of the Clone Manager 9 program using the Blosum 62 setting (see fig. 2).

The *lyse* genes, i.e. the polynucleotides coding for polypeptides having the activity of an L-ornithine
30 exporter, may be isolated from the organisms with the aid of the polymerase chain reaction (PCR) using suitable primers. Instructions can be found inter alia in the laboratory manual "PCR" by Newton and Graham (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994), and in
35 WO 2006/100211 on pages 14 to 17.

Particular preference is given to employing for a process according to the invention genes coding for polypeptides which have L-ornithine export activity and whose amino acid sequence includes one or more of the features selected from the group consisting of

- a) amino acid sequence according to SEQ ID No. 2 or SEQ ID No. 4,
- b) amino acid sequence according to SEQ ID No. 2, including one or more, up to 25, 20, 15, 10, 5, 4, 3, 2, or 1, deletion(s) of amino acids,
- c) amino acid sequence according to SEQ ID No. 2, including one or more, up to 25, 20, 15, 10, 5, 4, 3, 2, or 1, insertion (s) of amino acids, and
- d) amino acid sequence according to SEQ ID No. 2, including one or more, up to 140, 130, 120, 110, 100, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15, 10, 5, 4, 3, 2, or 1, preferably up to 5, 4, 3, 2, or 1, replacement(s) (substitution(s)) of amino acids.
- e) amino acid sequence according to SEQ ID No. 2, including one or more, up to 25, 20, 15, 10, 5, 4, 3, 2, or 1, preferably up to 5, 4, 3, 2, or 1, addition(s) of amino acids on the N terminus and/or on the C terminus.

Where appropriate, preference is given to conservative amino acid substitutions. In the case of aromatic amino acids, conservative substitutions are those in which phenylalanine, tryptophan and tyrosine are substituted for each other. In the case of hydrophobic amino acids, conservative substitutions are those in which leucine, isoleucine and valine are substituted for one another. In the case of polar amino acids, conservative substitutions

are those in which glutamine and asparagine are substituted for one another. In the case of basic amino acids, conservative substitutions are those in which arginine, lysine and histidine are substituted for one another. In the case of acidic amino acids, conservative substitutions are those in which aspartic acid and glutamic acid are substituted for one another. In the case of amino acids containing hydroxyl groups, conservative substitutions are those in which serine and threonine are substituted for one another.

It is furthermore possible to use polynucleotides which hybridize under stringent conditions with the nucleotide sequence complementary to SEQ ID No. 1, preferably to the coding region of SEQ ID No. 1, and code for a polypeptide having L-ornithine export activity, with the amino acid sequence of the encoded protein being $\geq 70\%$ identical to the amino acid sequence of SEQ ID No. 2 and the length of the encoded polypeptide, where appropriate, being within the above-described length ranges.

Instructions regarding the hybridization of nucleic acids and polynucleotides, respectively, can be found by the skilled worker inter alia in the manual "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)). Hybridization takes place under stringent conditions, that is to say only hybrids are formed in which the probe, i.e. a polynucleotide comprising the nucleotide sequence complementary to SEQ ID No. 1, preferably the coding region of SEQ ID No. 1, and the target sequence, i.e. the polynucleotides treated with or identified by said probe, are at least 70% identical. The stringency of the hybridization, including the washing steps, is known to be influenced or determined by varying the buffer composition, temperature and salt concentration. The hybridization

reaction is generally carried out with relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

For example, a 5× SSC buffer at a temperature of approx.

5 50°C-68°C may be employed for the hybridization reaction. Here, probes can also hybridize with polynucleotides which are less than 70% identical to the nucleotide sequence of the probe employed. Such hybrids are less stable and are removed by washing under stringent conditions. This may be
10 achieved, for example, by lowering the salt concentration to 2× SSC or 1× SSC and, where appropriate, subsequently 0.5× SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995), with a temperature of approx. 50°C-68°C, approx.
15 52°C-68°C, approx. 54°C-68°C, approx. 56°C-68°C, approx. 58°C-68°C, approx. 60°C-68°C, approx. 62°C-68°C, approx. 64°C-68°C, approx. 66°C-68°C being set. Preference is given to temperature ranges of approx. 64°C-68°C or approx. 66°C-68°C. It is optionally possible to lower the salt
20 concentration to a concentration corresponding to 0.2× SSC or 0.1× SSC. The SSC buffer optionally contains sodium dodecyl sulphate (SDS) at a concentration of 0.1%. By gradually increasing the hybridization temperature in steps of approx. 1-2°C from 50°C to 68°C, it is possible to
25 isolate polynucleotide fragments which are at least 70%, at least 80%, at least 90%, at least 92%, at least 94%, at least 96%, at least 97%, at least 98%, or at least 99%, where appropriate 100%, identical to the sequence or complementary sequence of the probe employed and which code
30 for a polypeptide having L-ornithine export activity. Further instructions regarding hybridization are obtainable on the market in the form of "kits" (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalogue No. 1603558).

35 For the measures of the invention, a polynucleotide coding

for a protein which has L-ornithine export activity is overexpressed in a bacterium or starting or parent strain producing L-ornithine, with the amino acid sequence of the encoded protein being $\geq 35\%$ identical to the amino acid
5 sequence of SEQ ID No. 2 and the length of the encoded polypeptide, where appropriate, being within the above-described ranges.

Overexpression generally means an increase in the intracellular concentration or activity of a ribonucleic
10 acid, of a protein (polypeptide) or of an enzyme by comparison with the starting strain (parent strain) or wild-type strain, if the latter is the starting strain. A starting strain (parent strain) means the strain on which the measure leading to overexpression has been carried out.

15 The terms protein and polypeptide are considered to be interchangeable.

For overexpression, preference is given to the methods of recombinant overexpression. These include any methods in which a microorganism is prepared using a DNA molecule
20 provided in vitro. Examples of such DNA molecules include promoters, expression cassettes, genes, alleles, coding regions. They are transferred by methods of transformation, conjugation, transduction or similar methods into the desired microorganism.

25 The measures of overexpression increase the activity or concentration of the corresponding polypeptide generally by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, preferably by up to 1000%, 2000%, 4000%, 10 000% or 20 000%, based on the level of activity or concentration
30 of said polypeptide in the strain prior to the measure resulting in overexpression.

When using strains of the species *Corynebacterium glutamicum*, the L-ornithine export activity in strain

ATCC13032 or ATCC14067 or ATCC13869 or ATCC17965, where appropriate, is a suitable reference point for determining overexpression. When using strains based on or derived from ATCC13032, said strain ATCC13032 is a suitable reference
5 point. An example of this is the strain prepared in the course of the work leading to the present invention, ATCC13032_Delta_argFRGH/pVWEx1_lyeE, which is based on the strain ATCC13032. When using strains based on or derived from ATCC14067, said strain ATCC14067 is a suitable
10 reference point. When using strains based on or derived from ATCC13869, said strain ATCC13869 is a suitable reference point. Further suitable reference points are produced accordingly.

When using strains of the species *Escherichia coli*,
15 preferably *Escherichia coli* strain K12, the L-ornithine export activity in strain MG1655, where appropriate, is a suitable reference point for determining overexpression.

Overexpression is achieved by a multiplicity of methods available in the prior art.

20 These include increasing the copy number and modifying the nucleotide sequences directing or controlling expression of the gene. Transcription of a gene is controlled inter alia by the promoter and optionally by proteins which suppress (repressor proteins) or promote (activator proteins)
25 transcription. Translation of the RNA formed is controlled inter alia by the ribosome binding site and the start codon. Polynucleotides or DNA molecules which include a promoter and a ribosome binding site and optionally a start codon are also referred to as expression cassette.

30 Said methods also include the use of variants of polypeptides or enzymes, which have an increased catalytic activity.

The copy number may be increased by means of plasmids which

replicate in the bacterial cytoplasm. To this end, an abundance of plasmids are described in the prior art for very different groups of microorganisms, which plasmids can be used for setting the desired increase in the copy number of the gene. Plasmids suitable for the genus *Escherichia* are described, for example, in the manual *Molecular Biology, Labfax* (Ed.: T.A. Brown, Bios Scientific, Oxford, UK, 1991). Plasmids suitable for the genus *Corynebacterium* are described, for example, in Tauch et al. (*Journal of Biotechnology* 104 (1-3), 27-40, (2003)) or in Stansen et al. (*Applied and Environmental Microbiology* 71, 5920-5928 (2005)).

The use of plasmid pEC7lyseE, deposited in DSM 23239, for increasing the copy number in *Corynebacterium glutamicum* strains is excluded from the measures leading to the present invention. The nucleotide sequence of the pEC7lyseE plasmid was determined and is depicted in SEQ ID No. 29.

The copy number may furthermore be increased by at least one (1) copy by introducing further copies into the bacterium chromosome. Methods suitable for the genus *Corynebacterium*, preferably *Corynebacterium glutamicum*, are described, for example, in the patents WO 03/014330, WO 03/040373 and WO 04/069996. WO 03/014330 describes methods for tandem doubling of genes at the native gene locus. WO 03/040373 describes methods for incorporating a second or third copy of a gene at further gene loci, with the particular gene locus being non-essential for growth or production of the particular amino acid, L-ornithine in the case of the present invention. Examples of suitable gene loci for incorporating a second or further copy of the lyseE gene in a process according to the invention are the genes *odh*, *sucA*, *dapA*, *dapB*, *ddh*, *lysA*, *argR*, *argF*, *argG* and *argH*. WO 04/069996 (see Tables 12 and 13) describes *C. glutamicum* intergenic regions and genes coding for phages or phage components, which are suitable for

incorporating further copies of the *lysE* gene.

Examples of methods suitable for the genus *Escherichia* are incorporation of a gene copy into the *att* site of the phage (Yu and Court, *Gene* 223, 77-81 (1998)), chromosomal
5 amplification with the aid of the phage Mu, as described in EP 0 332 448, or the methods of gene replacement with the aid of conditionally replicating plasmids, as described by Hamilton et al. (*Journal of Bacteriology* 174, 4617-4622 (1989)) or Link et al. (*Journal of Bacteriology* 179, 6228-
10 6237 (1997)).

Gene expression may furthermore be increased by using a strong promoter which is functionally linked to the gene to be expressed. Preference is given to using a promoter which is stronger than the natural promoter, i.e. the one present
15 in the wild type or parent strain. To this end, the prior art has an abundance of methods available.

Suitable promoters and expression systems for the genus *Corynebacterium* can be found inter alia in the patents EP 0 629 699 A2, US 2007/0259408 A1 (gap promoter),
20 WO 2006/069711, EP 1 881 076 A1, WO 2008/088158, WO 2009/025470 (*butA* promoter, *pyk* promoter), US 6,861,246 (MC20 and MA16 variants of the *dapA* promoter), and EP 1 918 378 A1 (*sod* promoter), and in overviews such as the "Handbook of *Corynebacterium glutamicum*" (Eds.: Lothar
25 Eggeling and Michael Bott, CRC Press, Boca Raton, US (2005)), or the book "Corynebacteria, Genomics and Molecular Biology" (Ed.: Andreas Burkovski, Caister Academic Press, Norfolk, UK (2008)). Examples of promoters which allow controlled, i.e. inducible or repressible,
30 expression are described, for example, in Tsuchiya and Morinaga (*Bio/Technology* 6, 428-430 (1988)).

Promoters suitable for the genus *Escherichia* have been known for a long time. They include, inter alia, the classical promoters *lac* promoter, *trp* promoter, the hybrid

promoters *tac* and *trc*, the PL and PR promoters of phage λ . Similarly, it is possible to use the promoters of the T7 phage, the gear-box promoters, the *nar* promoter or the promoters of the genes *rrsG*, *rnpB*, *csrA*, *csrB*, *ompA*, *fusA*,
5 *pepQ*, *rplX* or *rpsG*. Controlled expression is permitted, for example, by the *cI857-PR* or the *cI857-PL* system of the λ phage (Götting et al., *BioTechniques* 24, 362-366 (1998)). Overviews can be found in Makrides (*Microbiological Reviews* 60(3), 512-538 (1996)) or in the manual "*Escherichia coli*
10 and *Salmonella*, Cellular and Molecular Biology" (F.C. Neidhardt (Editor in Chief), ASM Press, Washington, US (1996)).

Such promoters or expression cassettes are typically employed at a distance of from 1 to 1000, preferably 1 to
15 500, nucleotides upstream of the first nucleotide of the start codon of the coding region of the gene. At a distance of 1 means that the promoter or the expression cassette is positioned immediately in front of the first base of the start codon of the coding region.

20 To increase expression of the *lysE* gene in *C. glutamicum*, preference is given to inserting suitable promoters such as, for example, the *C. glutamicum* *sod* promoter (see SEQ ID No. 1 of EP 1918 378 A1) or the *C. glutamicum* *gap* promoter (see SEQ ID No. 3 of US 2007/0259408) between positions 930
25 and 990 of SEQ ID No. 1.

When using expression cassettes containing a promoter and a ribosome binding site (RBS), such as the expression unit of the *C. glutamicum* *sod* gene (see SEQ ID No. 2 of EP 1918 378 A1) or the expression unit of the *C. glutamicum*
30 *gap* gene, described in US 2007/0259408 and depicted in SEQ ID No. 28 (and referred to there as *PgapRBS*), for example, they are inserted, in the case of *C. glutamicum*, preferably between positions 930 and 1001, particularly preferably between positions 1000 and 1001, of SEQ ID No. 1. An
35 example of a suitable ribosome binding site in such an

expression cassette is the nucleotide sequence
5'-agaaaggagg-3' specified by Amador (Microbiology 145,
915-924 (1999)).

It is likewise possible to place a plurality of promoters
5 upstream of the desired gene or functionally link them to
the gene to be expressed and in this way achieve increased
expression. This is described, for example, in
WO 2006/069711.

10 The structure of *Corynebacterium glutamicum* and *Escherichia*
coli promoters is well known. It is therefore possible to
increase the strength of a promoter by modifying its
sequence by means of one or more substitution(s) and/or one
or more insertion(s) and/or one or more deletion(s) of
15 nucleotides. Examples of this can be found inter alia in
"Herder Lexikon der Biologie" [Herder's Encyclopaedia of
Biology] (Spektrum Akademischer Verlag, Heidelberg, Germany
(1994)).

Accordingly, a suitable measure for overexpressing the *lyeE*
20 gene is to modify or mutate the promoter of said *lyeE* gene.

The structure of the *Corynebacterium glutamicum* and
Escherichia coli ribosome binding sites is likewise well
known and is described, for example, in Amador
(Microbiology 145, 915-924 (1999)), and in manuals and text
25 books of genetics, for example "Gene und Klone" [Genes and
Clones] (Winnacker, Verlag Chemie, Weinheim, Germany
(1990)) or "Molecular Genetics of Bacteria" (Dale and Park,
Wiley and Sons Ltd., Chichester, UK (2004)). Well expressed
genes, i.e. the most important structural genes in an
30 organism, have a good ribosome binding site (Amador,
Microbiology 145, 915-924 (1999)), i.e. the latter is very
similar to or corresponds to the consensus sequence. It has
been demonstrated in the literature that highly expressed
genes have a strong ribosome binding site (Karlin and
35 Mrázek, Journal of Bacteriology 2000; 182(18): 5238-50).

Consequently, translation efficiency of a gene or of the mRNA can be achieved by adjusting the ribosome binding site.

It is also possible to increase translation efficiency by
5 adjusting the codon usage in the genes to be expressed
(e.g. Najafabadi et al., Nucleic Acids Research 2009, 37
(21): 7014-7023).

Overexpression can likewise be achieved by increasing the
expression of activator proteins or by reducing or
10 switching off the expression of repressor proteins.

The activator protein LysG for expressing lysE has been
described by Bellmann et al. (Microbiology 2001; 147: 1765-
74) and is referred to there as "positive regulator". The
amino acid sequence of *Corynebacterium glutamicum* ATCC13032
15 LysG is depicted in SEQ ID No. 30. In a global sequence
alignment, the amino acid sequence of the LysG polypeptide
of *Corynebacterium diptheriae* NCTC13129 is 62%, the amino
acid sequence of the LysG polypeptide of *Corynebacterium*
efficiens YS-314 is 81%, and the amino acid sequence of the
20 LysG polypeptide of *Corynebacterium glutamicum* R is 94%,
identical to that of SEQ ID No. 30.

For activator proteins, preference is given to a
polypeptide which is $\geq$ (at least) 55%, preferably $\geq$ 80%,
particularly preferably $\geq$ 90%, $\geq$ 92% or $\geq$ 94%, very
25 particularly preferably $\geq$ 99%, and most preferably 100%,
identical to the amino acid sequence depicted in SEQ ID
No. 30.

The overexpression measures mentioned, preferably selected
from the group consisting of increasing the copy number,
30 using a strong promoter, mutating the promoter, using a
suitable expression cassette and overexpressing an
activator protein, may be combined in a suitable manner.
Thus it is possible, for example, to combine using a

suitable promoter with increasing the copy number, or overexpressing an activator protein with using a suitable promoter or a suitable expression cassette.

It is likewise possible, in addition to the measures
5 relating to the polynucleotide coding for a protein having L-ornithine export activity, to attenuate individual biosynthesis genes.

To improve production of L-ornithine, it is thus convenient, where appropriate, to attenuate additionally
10 one or more of the genes selected from the group consisting of

- a) odhA gene coding for the E1 subunit of alpha-ketoglutarate dehydrogenase (EC 1.2.4.2),
- b) sucA gene coding for dihydrolipoamide succinyl
15 transferase (EC 2.3.1.61),
- c) dapA gene coding for a dihydrodipicolinate synthase (DapA, EC 4.2.1.52),
- d) dapB gene coding for a dihydrodipicolinate synthase (DapB, EC 1.3.1.26),
- 20 e) ddh gene coding for a meso-diaminopimelate dehydrogenase (Ddh, EC 1.4.1.16),
- f) lysA gene coding for a diaminopimelate decarboxylase (LysA, EC 4.1.1.20),
- g) argR gene coding for a/the repressor (ArgR) of L-
25 arginine biosynthesis,
- h) argF gene coding for an ornithine carbamoyl transferase (ArgF, EC 2.1.3.3),
- i) argG gene coding for an argininosuccinate synthase (ArgG, EC 6.3.4.5),

- j) argH gene coding for an argininosuccinate lyase (ASAL) (ArgH, EC 4.3.2.1),
- k) lysC gene coding for an aspartate kinase (LysC, EC 2.7.2.4), and
- 5 1) asd gene coding for an aspartate semialdehyde dehydrogenase (Asd, EC 1.2.1.11).

Preference is given to attenuating one or more of the genes selected from the group consisting of lysA, odhA, argR, argF, argG and argH. Particular preference is given to
10 attenuating one or more of the genes selected from the group consisting of lysA, odhA and argF. Very particular preference is given to attenuating the genes lysA and/or argF.

The term "attenuation" in this context describes reducing
15 or switching off the intracellular activity of one or more enzymes (proteins) in a bacterium, that are encoded by the corresponding DNA, by using, for example, a weak promoter or a gene or allele that codes for a corresponding enzyme having a low activity, or by inactivating the corresponding
20 gene or enzyme (protein), and optionally combining these measures.

An overview of known promoters of various strengths in *Corynebacterium glutamicum* can be found in Pátek et al. (Journal of Biotechnology 104, 311-323 (2003)). Other weak
25 promoters are described in the communication 512057 in the journal Research Disclosure from December 2006 (pages 1616 to 1618).

Mutations which may be considered for generating an attenuation are transitions, transversions, insertions and
30 deletions of at least one (1) base pair or nucleotide in the coding region of the gene in question. Depending on the effect of the amino acid substitution caused by the mutation on the activity of the protein or enzyme, the

mutations are referred to as missense mutations or nonsense mutations.

The missense mutation results in a replacement of a given amino acid in a protein with another one, said replacement
5 being in particular a non-conservative amino acid substitution. This impairs the functionality or activity of the protein and reduces it to a value of from ≥ 0 to 75%, ≥ 0 to 50%, ≥ 0 to 25%, ≥ 0 to 10% or ≥ 0 to 5%.

The nonsense mutation results in a stop codon in the coding
10 region of the gene and therefore in an early termination of translation and consequently to a switching-off. Insertions or deletions of at least one base pair in a gene lead to frame shift mutations resulting in wrong amino acids being incorporated or translation being terminated early. If the
15 mutation results in a stop codon in the coding region, this likewise leads to an early termination of translation. The measures of generating a nonsense mutation are preferably carried out in the 5'-terminal part of the coding region, which codes for the N terminus of the polypeptide. If the
20 overall length of a polypeptide (measured by way of the number of chemically linked L-amino acids) is referred to as 100%, then - within the scope of the present invention - the N terminus of the polypeptide includes that part of the amino acid sequence which, by calculation from the start
25 amino acid, L-formyl-methionine, onwards, contains 80% of the downstream L-amino acids.

In-vivo mutagenesis methods are described, for example, in the Manual of Methods for General Bacteriology (Gerhard et al. (Eds.), American Society for Microbiology, Washington,
30 DC, USA, 1981) or in Tosaka et al. (Agricultural and Biological Chemistry 42(4), 745-752 (1978)) or in Konicek et al. (Folia Microbiologica 33, 337-343 (1988)).

Suitable methods of in-vitro mutagenesis are, inter alia, the treatment with hydroxylamine according to Miller

(Miller, J.H.: A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and OxyRated Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1992), the use of mutagenic
5 oligonucleotides (T.A. Brown: Gentechnologie für Einsteiger [Genetic Engineering for Beginners], Spektrum Akademischer Verlag, Heidelberg, 1993 and R.M. Horton: PCR-Mediated Recombination and Mutagenesis, Molecular Biotechnology 3, 93-99 (1995)), and the use of a polymerase chain reaction
10 using a DNA polymerase with a high error rate. An example of such a DNA polymerase is the Mutazyme DNA polymerase (GeneMorph PCR Mutagenesis Kit, No. 600550) from Stratagene (LaJolla, CA, USA).

Further instructions and overviews on the generation of
15 mutations in vivo or in vitro can be found in the prior art and in known text books of genetics and molecular biology, such as the text book by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene and Klone", VCH
20 Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik" [General Genetics], Gustav Fischer Verlag, Stuttgart, 1986), for example.

With the aid of the known process of gene or allele replacement, the fundamentals of which are described in
25 Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)), it is possible to transfer a mutation prepared in vitro, or a polynucleotide containing the desired mutation, into the chromosome. Von Schäfer et al. (Gene 145, 69-73 (1994)) employed this method in order to incorporate a deletion
30 into the C. glutamicum hom-thrB operon. Von Nakagawa et al. (EP 1108790) and Ohnishi et al. (Applied Microbiology and Biotechnology 58(2), 217-223 (2002)) employed this method in order to incorporate various mutations, starting from the isolated alleles, into the C. glutamicum chromosome.

35 One method for targeted reduction of gene expression

consists of placing the gene to be attenuated under the control of a promoter which can be induced by addition of metered amounts of IPTG (isopropyl β -D-thiogalactopyranoside), such as, for example, the trc promoter or the tac promoter. Suitable for this purpose are vectors such as, for example, the Escherichia coli expression vector pXK99E (WO 0226787; deposited in accordance with the Budapest Treaty on 31st July 2001 in DH5alpha/pXK99E as DSM14440 with the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Brunswick, Germany)), pEKEx2 (NCBI Accession No. AY585307) or pVWEx2 (Wendisch, Ph. D. thesis, Berichte des Forschungszentrums Jülich, Jül-3397, ISSN 0994-2952, Jülich, Germany (1997)), which enable the cloned gene to be expressed in an IPTG-dependent manner in Corynebacterium glutamicum.

This method has been employed, for example, in the patent WO 02266787 for regulated expression of the dead gene by means of integration of the vector pXK99Edead into the genome of Corynebacterium glutamicum, and by Simic et al. (Applied and Environmental Microbiology 68: 3321-3327 (2002)) for regulated expression of the glyA gene by means of integration of the vector pK18mobglyA' into Corynebacterium glutamicum.

Another method for specifically reducing gene expression is the antisense technique which involves delivering into the target cells short oligodeoxynucleotides or vectors for synthesizing longer antisense RNA. There, the antisense RNA can bind to complementary sections of specific mRNAs and reduce their stability or block translatability. An example of this can be found by the skilled worker in Srivastava et al. (Applied Environmental Microbiology 2000 Oct.; 66 (10): 4366-4371).

The rate of elongation is influenced by the codon usage. Gene expression may be attenuated by using codons for t-RNAs which are rare in the parent strain. This is

described in detail in WO 2008049781 and in WO 2009133063.
For example, replacing an ATG start codon with the less
common codons GTG or TTG may impair translation, since the
AUG codon is twice to three times as effective as the GUG
5 and UUG codons, for example (Khudyakov et al., FEBS Letters
232(2): 369-71 (1988); Reddy et al., Proceedings of the
National Academy of Sciences of the USA 82(17): 5656-60
(1985)).

It is likewise possible, in addition to the measures
10 relating to the polynucleotide coding for a protein having
L-ornithine export activity, to enhance individual
biosynthesis genes.

To improve L-ornithine production, it is thus expedient,
where appropriate, additionally to enhance the enzyme
15 activity of one or more of the proteins selected from the
group consisting of

- a) glutamate dehydrogenase (EC 1.4.1.3) encoded by the
gdh gene,
- b) glutamate N-acetyltransferase (EC 2.3.1.35 and
20 EC 2.3.1.1) encoded by the argJ gene,
- c) acetyl glutamate kinase (EC 2.7.2.8) encoded by the
argB gene,
- d) N-acetyl-gamma-glutamyl-phosphate reductase
(EC 1.2.1.38) encoded by the argC gene,
- 25 e) acetylornithine aminotransferase (EC 2.6.1.11),
encoded by the argD gene,
- f) glucose-specific component EIIB (PtsG) (EC 2.7.1.69)
of the glucose uptake system, encoded by the ptsG
gene,
- 30 g) sucrose-specific component EIIB (PtsS) (EC 2.7.1.69)
of the sucrose uptake system, encoded by the ptsS

gene,

- h) glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49)
encoded by the zwf gene,
- i) glucose-6-phosphate isomerase (EC 5.3.1.9) encoded by
5 the pgi gene,
- j) phosphofructokinase (EC 2.7.1.11) encoded by the pfkA
gene,
- k) fructose-bisphosphate aldolase (EC 4.1.2.13) encoded
by the fda gene,
- 10 l) glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.59)
encoded by the gap gene,
- m) phosphoglycerate kinase (EC 2.7.2.3) encoded by the
pgk gene,
- n) pyruvate kinase (EC 2.7.1.40) encoded by the pyk gene,
- 15 o) E1 subunit of pyruvate dehydrogenase (EC 1.2.4.1),
encoded by the aceE gene,
- p) phosphoenolpyruvate carboxylase (EC 4.1.1.31) encoded
by the ppc gene,
- q) pyruvate carboxylase (EC 6.4.1.1), encoded by the pyc
20 gene,
- r) aconitase (EC 4.2.1.3) encoded by the acn gene, and
- s) isocitrate dehydrogenase (EC 1.1.1.42) encoded by the
icd gene.

The term enhancement comprises the overexpression measures
25 and the use of variants which have increased catalytic
activity compared to the protein of the wild type.

Particular preference is given to enhancing one or more of

the enzymes selected from the group consisting of glutamate dehydrogenase, glutamate N-acetyltransferase and acetylglutamate kinase.

5 The additional measures of attenuation listed may be combined with the additional measures of enhancement.

Instructions for the handling of DNA, digestion and ligation of DNA, transformation and selection of transformants can be found, inter alia, in the known manual by Sambrook et al. "Molecular Cloning: A Laboratory Manual,
10 Second Edition (Cold Spring Harbor Laboratory Press, 1989).

The extent of expression or overexpression can be determined by measuring the amount or concentration of mRNA transcribed from the gene, by determining the amount or concentration of the polypeptide and by determining the
15 level of enzyme activity.

The amount of mRNA may be determined, inter alia, by using the methods of "Northern blotting" and quantitative RT-PCR. In quantitative RT-PCR, the polymerase chain reaction is preceded by a reverse transcription. It is possible to use
20 for this purpose the LightCycler™ system from Roche Diagnostics (Boehringer Mannheim GmbH, Roche Molecular Biochemicals, Mannheim, Germany), as described in Jungwirth et al. (FEMS Microbiology Letters 281, 190-197 (2008)), for example. The concentration of the protein may be determined
25 by 1- and 2-dimensional protein gel fractionation and subsequent optical identification of the protein concentration in the gel using appropriate evaluation software. A common method of preparing the protein gels for coryneform bacteria and of identifying the proteins is the
30 procedure described by Hermann et al. (Electrophoresis, 22: 1712-23 (2001)). The protein concentration may likewise be determined by Western-blot hybridization using an antibody which is specific for the protein to be detected (Sambrook et al., Molecular cloning: a laboratory manual, 2nd Ed.

Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) and subsequent optical evaluation using appropriate concentration determination software (Lohaus and Meyer (1998) Biospektrum 5: 32-39; Lottspeich, 5 Angewandte Chemie 321: 2630-2647 (1999)).

The bacteria produced may be cultured continuously - as described, for example, in WO 05/021772 - or discontinuously in a batch process (batch cultivation) or in a fed-batch or repeated fed-batch process (described in 10 US 6,562,601 for example) for the purpose of producing L-ornithine. A summary of a general nature about known cultivation methods is available in the text book by Chmiel (Bioprozesstechnik [Bioprocess Technology] 1. Einführung in die Bioverfahrenstechnik [Introduction to Bioprocess 15 Engineering] (Gustav Fischer Verlag, Stuttgart, 1991)), or in the text book by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Brunswick/Wiesbaden, Germany 1994)).

The culture medium or fermentation medium to be used must 20 in a suitable manner satisfy the demands of the particular strains. The "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981) contains descriptions of culture media for various microorganisms. The terms growth medium, culture 25 medium and fermentation medium or medium are interchangeable.

It is possible to use, as carbon source, sugars and carbohydrates such as, for example, glucose, sucrose, lactose, fructose, maltose, molasses, sucrose-containing 30 solutions from sugar-beet or sugar-cane processing, starch, starch hydrolysate 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 35 example, glycerol, methanol and ethanol, and organic acids

such as, for example, acetic acid or lactic acid.

With sugars, preference is given to glucose, fructose, sucrose, mixtures of glucose and fructose, and mixtures of glucose, fructose and sucrose. Where appropriate,
5 particular preference is given to sucrose.

With alcohols, preference is given to glycerol.

It is possible to use, as nitrogen source, organic nitrogen-containing compounds such as peptones, yeast extract, meat extract, malt extract, corn steep liquor,
10 soybean flour and urea, or inorganic compounds such as ammonium sulphate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources may be used individually or by way of a mixture.

It is possible to use, as phosphorus source, phosphoric
15 acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts.

The culture medium must furthermore comprise salts, for example in the form of chlorides or sulphates of metals
20 such as, for example, sodium, potassium, magnesium, calcium and iron, such as magnesium sulphate or iron sulphate for example, which are necessary for growth. Finally, essential growth factors such as amino acids, for example homoserine and vitamins, for example thiamine, biotin or pantothenic
25 acid, may be employed in addition to the above-mentioned substances.

The starting materials mentioned may be added to the culture in the form of a single batch or be fed in in a suitable manner during cultivation.

30 The pH of the culture can be controlled by employing basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acidic compounds such as

phosphoric acid or sulphuric acid in a suitable manner. The pH is generally adjusted to a value of from 6.0 to 8.5, preferably 6.5 to 8. To control foaming, it is possible to employ antifoams such as, for example, fatty acid polyglycol esters. To maintain the stability of plasmids, it is possible to add to the medium suitable selective substances such as, for example, antibiotics. The fermentation is preferably carried out under aerobic conditions. In order to maintain these conditions, oxygen or oxygen-containing gas mixtures such as, for example, air are introduced into the culture. It is likewise possible to use liquids enriched with hydrogen peroxide. The fermentation is carried out, where appropriate, at elevated pressure, for example at an elevated pressure of from 0.03 to 0.2 MPa. The temperature of the culture is normally from 20°C to 45°C and preferably from 25°C to 40°C, particularly preferably from 30°C to 37°C. In batch processes, preference is given to continuing culturing until an amount of the desired L-ornithine sufficient for being recovered has formed. This aim is normally achieved within 10 hours to 160 hours. With continuous processes, longer culturing times are possible. The bacterial activity results in a concentration or an increase in the concentration (accumulation) of L-ornithine in the fermentation medium.

Example of suitable fermentation media can be found inter alia in the patents JP 43010996 B4 (for *B. subtilis*), US 3668072 A (for *E. coli*) and JP 57041912 B (for *B. flavum*).

Where appropriate, the volume of the fermentation medium in a process according to the invention is ≥ 0.5 l, ≥ 1 l, ≥ 5 l, ≥ 10 l, ≥ 50 l, ≥ 100 l, ≥ 500 l, ≥ 1000 l, preferably ≥ 1 l, particularly preferably ≥ 10 l, very particularly preferably ≥ 100 l and most preferably ≥ 1000 l.

To determine the concentration at one or more time point(s)

in the course of the fermentation, L-ornithine may be analysed by separating the L-amino acids by means of ion exchange chromatography, preferably cation exchange chromatography, with subsequent post-column derivatization using ninhydrin, as described in Spackman et al.

(Analytical Chemistry 30: 1190-1206 (1958)). It is also possible to employ ortho-phthaldialdehyde rather than ninhydrin for post-column derivatization. An overview article on ion exchange chromatography can be found in Pickering (LC.GC (Magazine of Chromatographic Science) 7(6), 484-487 (1989)).

It is likewise possible to carry out a pre-column derivatization, for example using ortho-phthaldialdehyde or phenyl isothiocyanate, and to fractionate the resulting amino acid derivatives by reversed-phase chromatography (RP), preferably in the form of high-performance liquid chromatography (HPLC). A method of this type is described, for example, in Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)). Detection is carried out photometrically (absorbance, fluorescence).

A review regarding amino acid analysis can be found inter alia in the text book "Bioanalytik" by Lottspeich and Zorbas (Spektrum Akademischer Verlag, Heidelberg, Germany 1998).

The performance of the processes or fermentation processes according to the invention, in respect of one or more of the parameters selected from the group consisting of L-ornithine concentration (L-ornithine formed per volume), L-ornithine yield (L-ornithine formed per carbon source consumed), L-ornithine formation (L-ornithine formed per volume and time), and specific L-ornithine formation (L-ornithine formed per dry cell matter or dry biomass and time, or L-ornithine formed per cellular protein and time), or else other process parameters and combinations thereof, is increased by at least 0.5%, at least 1%, at least 1.5%

or at least 2%, based on processes or fermentation processes using bacteria which contain a non-overexpressed protein having L-ornithine export activity or which have not been subjected to an overexpression measure.

- 5 The fermentation measures result in a fermentation broth which contains the desired L-ornithine.

A product containing L-ornithine is then provided or produced or recovered in liquid or solid form.

- A fermentation broth means a fermentation medium or growth
10 medium in which a microorganism has been cultured for a certain time and at a certain temperature. The fermentation medium or the media employed during fermentation comprises/comprise all the substances or components which ensure production of said L-ornithine and typically
15 propagation and viability.

When the fermentation is complete, the resulting fermentation broth accordingly comprises

- a) the bacterial biomass (cell mass) produced due to propagation of the bacterial cells,
- 20 b) the L-ornithine formed in the course of the fermentation,
- c) the organic by-products formed in the course of the fermentation, and
- d) the constituents of the fermentation medium employed
25 or of the starting materials, for example vitamins such as biotin or salts such as magnesium sulphate, which have not been consumed in the fermentation.

The organic by-products include substances which are produced by the bacteria employed in the fermentation in
30 addition to the L-ornithine and are optionally excreted. These also include sugars such as trehalose, for example.

The fermentation broth is removed from the culture vessel or fermentation tank, optionally collected, and used for providing an L-ornithine-containing product in liquid or solid form. The expression "recovering the L-ornithine-
5 containing product" is also used for this. In the simplest case, the L-ornithine-containing fermentation broth itself, which has been removed from the fermentation tank, constitutes the recovered product.

One or more of the measures selected from the group
10 consisting of

- a) partial ($> 0\%$ to $< 80\%$) to complete (100%) or virtually complete ($\geq 80\%$, $\geq 90\%$, $\geq 95\%$, $\geq 96\%$, $\geq 97\%$, $\geq 98\%$ or $\geq 99\%$ to $< 100\%$) removal of water,
- b) partial ($> 0\%$ to $< 80\%$) to complete (100%) or
15 virtually complete ($\geq 80\%$, $\geq 90\%$, $\geq 95\%$, $\geq 96\%$, $\geq 97\%$, $\geq 98\%$ or $\geq 99\%$ to $< 100\%$) removal of the biomass which is optionally inactivated before removal,
- c) partial ($> 0\%$ to $< 80\%$) to complete (100%) or
20 virtually complete ($\geq 80\%$, $\geq 90\%$, $\geq 95\%$, $\geq 96\%$, $\geq 97\%$, $\geq 98\%$, $\geq 99\%$, $\geq 99.3\%$ or $\geq 99.7\%$ to $< 100\%$) removal of the organic by-products formed in the course of the fermentation, and
- d) partial ($> 0\%$) to complete (100%) or virtually
25 complete ($\geq 80\%$, $\geq 90\%$, $\geq 95\%$, $\geq 96\%$, $\geq 97\%$, $\geq 98\%$, $\geq 99\%$, $\geq 99.3\%$ or $\geq 99.7\%$ to $< 100\%$) removal of the constituents of the fermentation medium employed or of the starting materials, which have not been consumed in the fermentation,

from the fermentation broth achieves concentration or
30 purification of the L-ornithine. Products having a desired L-ornithine content are isolated in this way.

The partial ($> 0\%$ to $< 80\%$) to complete (100%) or virtually

complete ($\geq 80\%$ to $< 100\%$) removal of water (measure a)) is also referred to as drying.

In one variant of the process, complete or virtually complete removal of water, of the biomass, of the organic
5 by-products and of the unconsumed constituents of the fermentation medium employed results in pure ($\geq 80\%$ by weight or $\geq 90\%$ by weight) or high-purity ($\geq 95\%$ by weight, $\geq 97\%$ by weight or $\geq 99\%$ by weight) L-ornithine product forms. An abundance of technical instructions for the
10 measures according to a), b), c) or d) are available in the prior art.

In the case of the amino acid L-ornithine or its salts, essentially three different products have been described in the prior art.

15 One group describes L-ornithine HCL, from which L-ornithine is purified from the fermentation solution, after removal of the cells by means of an ion exchanger, and then crystallized through crystallization as L-ornithine monochloride and recrystallization as L-ornithine
20 monochloride (US 2988489). The L-ornithine HCL obtained in this case has a purity of more than $> 90\%$, preferably more than 95% , particularly preferably more than 98% , and very particularly preferably more than 99% .

A further process is described in the patent application
25 EP 1995322. This involves applying the biomass-containing fermentation solution to the top of a weakly acidic ion exchanger with a particle diameter of $> 300 \mu\text{m}$ and purifying the L-ornithine by this step. The selection of an appropriate particle diameter prevents the biomass from
30 blocking the resin. The efficiency of cell removal was 99% .

The purified L-ornithine may then be employed for preparing various L-ornithine salts such as, for example, mono- or di-L-ornithine α -ketoglutarate, L-ornithine L-aspartate,

EP 0477 991, for example, describes a process for preparing L-ornithine L-aspartate. This involves adding to an aqueous solution of L-ornithine and L-aspartate a water-soluble solvent in order to arrive at a solution which is at least 90% saturated or over saturated. Said solution is heated under reflux until the formation of crystals has ended. A water-miscible solvent is then continued to be added under reflux until the salt crystals form. The crystals may be removed, for example, by centrifugation and are subsequently dried under vacuum. The product purity is typically above 98.5%.

JP 46003194 describes a process for preparing L-ornithine L-ketoglutarate. This involves, for example, converting ornithine HCL into the free base by means of adsorption to an acidic ion exchanger and elution with aqueous ammonia, adding α -ketoglutarate and evaporating the solution under vacuum until the product crystallizes.

The plasmid pEC7lyse has been deposited in the form of the strain *Escherichia coli* DH5alpha/pEC7lyse (DM2204) in accordance with the Budapest Treaty with the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Brunswick, Germany) under accession number DSM 23239 on 15 January 2010.

Examples

Example 1:

Cloning and sequencing of the lyse gene from *Corynebacterium glutamicum* ATCC 13032

The lyse gene of strain ATCC13032 was cloned into the *E. coli*/C. glutamicum shuttle and expression vector pVWEx1

(Peters-Wendisch et al., J. Mol. Microbiol. Biotechnol. (2001) 3(2): 295-300).

Cloning was carried out in two steps. First, a polymerase chain reaction (PCR) amplified the gene from

5 Corynebacterium glutamicum ATCC13032 by means of the following oligonucleotide primers derived from SEQ ID No. 1. Said oligonucleotides included additional restriction cleavage sites on their 5' end (underlined: EcoRV for lyse_1.p and AvrII or SspI for lyse_2.p).

10 lyse_1.p: 5'-[TCGATATCATGGAAATCTTCATTACAGG]-3'
(see SEQ ID No. 22)

lyse_2.p: 5'-[TGCCTAGGTCAATATTTGGGCGAAGGCCACCG]-3'
(see SEQ ID No. 23)

The PCR reaction was carried out in the presence of 200 μ M
15 deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP), 0.5 μ M each of the corresponding oligonucleotide, 100 ng of Corynebacterium glutamicum ATCC13032 chromosomal DNA, 1/5 volume of 5 times reaction buffer HF and 0.02 U/ μ l Phusion® Hot Start DNA polymerase (Biozym Scientific GmbH, D-31840
20 Hess. Oldendorf) in a thermocycler (Mastercycler, Eppendorf AG, Hamburg) under the following conditions: 98°C for 1 min; 30 cycles $\times$ (98°C, 20 s; 63°C, 20 s; 72°C, 40 s); 72°C for 6 min.

The 761 bp lyse PCR fragment (see SEQ ID No. 3) was cloned
25 into pVWEx1 as described below:

Preparation of the vector: 1 μ g of pVWEx1 plasmid DNA was cleaved in the enzyme-specific buffer system containing 10 units of the enzyme PstI by incubation at 37°C for 1 h. Immediately thereafter, the cleavage mix was treated with
30 the Quick Blunting Kit (New England Biolabs GmbH, Frankfurt am Main) according to the manufacturer's instructions and then purified using the QiaExII purification kit (Qiagen AG, Hilden, Germany) according to the manufacturer's

instructions. The vector pre-treated in this way was then cleaved with 10 units of XbaI in the enzyme-specific buffer system at 37°C for 1 h and then purified again using the QiaExII purification kit.

- 5 Preparation of the insert: the lyse PCR fragment was cleaved with 10 units each of the enzymes AvrII and EcoRV and then purified using the QiaExII purification kit according to the manufacturer's instructions.

Ligation: vector and insert were mixed at a 1:5 molar ratio
10 and ligated using T4 DNA ligase at 16°C for 1 h. Chemical competent E. coli DH5alpha cells (Subcloning efficiency, Invitrogen GmbH, Karlsruhe, Germany) were transformed with 3 µl of the ligation mix.

Transformants were identified on the basis of their
15 kanamycin resistance on LB-agar plates containing 50 µg/ml kanamycin sulphate. Plasmid DNA was isolated from 4 of said transformants, and the plasmids were assayed by restriction analysis for the presence of the 0.75 kb fragment as insert. The recombinant plasmid produced in this way was
20 referred to as pVWEx1_lyse.

The nucleotide sequence of the 0.75 kb fragment in plasmid pVWEx1-lyse was determined by the dideoxy chain termination method according to Sanger et al. (Proceedings of the
National Academy of Sciences of the United States of
25 America (1977) 74: 5463-5467). To this end, the complete insert of the pVWEx1_lyse plasmid was sequenced with the aid of the oligonucleotide primers pVW_1.p (5'-TGA GCG GAT AAC AAT TTC ACA C-3') and pVW_2.p (5'-CGA CGG CCA GTG AAT TCG AG-3') at Eurofins MWG Operon GmbH (Ebersberg,
30 Germany).

The nucleotide sequence obtained was analysed using the Clone Manager 9 Program and is depicted by way of SEQ ID No. 20.

Example 2:

Construction of the vector pK18mobsacB_DargFRGH for deletion of the argFRGH region in *Corynebacterium glutamicum*

5 To this end, firstly chromosomal DNA was isolated from
C. glutamicum ATCC13032 by the method of Tauch et al.
(1995, Plasmid 33: 168-179). The oligonucleotides listed
below were selected on the basis of the sequence of the
C. glutamicum argFRGH genes in order to prepare the argFRGH
10 deletion construct. Said deletion construct was generated
with the aid of the polymerase chain reaction (PCR), more
specifically by the gene SOEing method (Gene Splicing by
Overlap Extension, Horton, Molecular Biotechnology 3: 93-98
(1995)).

15 argFRGH_d1:

5'-GGT GGT GCT AGC CCG GCG ATT TCT TTG CAC AT-3'
(see SEQ ID No. 24)

argFRGH_d2:

5'-AAT GCT TAT CGA CGT ACC CCC CTG TGG TTG TGA AGT CAT A-3'
20 (see SEQ ID No. 25)

argFRGH_d3:

5'-GGG GTA CGT CGA TAA GCA TT-3'
(see SEQ ID No. 26)

argFRGH_d4:

25 5'-GGT GGT ATG CAT GGT GAT GGT TCC GAA TGT TG-3'
(see SEQ ID No. 27)

The oligonucleotide primers depicted were purchased from
Eurofins MWG Operon GmbH (Ebersberg, Germany). The PCR
reaction was carried out using the Phusion® Hot Start DNA
30 polymerase (Biozym Scientific GmbH, D-31840 Hess,
Oldendorf) in a thermocycler (Mastercycler, Eppendorf AG,
Hamburg).

The argFRGH_d2 primer is composed of two regions. One part of the nucleotide sequence is complementary to the region from 1 bp upstream to 19 bp downstream of the start codon of the argF gene. The other part of the nucleotide sequence is complementary to the region from nucleotide 1419 of the argH gene to 5 nucleotides downstream of the argH gene.

With the aid of the polymerase chain reaction, the primers argFRGH_1 and argFRGH_2 enable a 543 bp DNA fragment and the primers argFRGH_3 and argFRGH_4 enable a 513 bp DNA fragment to be amplified. The amplicons were produced by PCR, assayed by electrophoresis in a 0.8% strength agarose gel, isolated from said agarose gel using the High Pure PCR Product Purification Kit (Product No. 1732676, Roche Diagnostics GmbH, Mannheim, Germany), and employed as template for another PCR reaction using the primers argFRGH_1 and argFRGH_4. In this way, the 1036 bp DargFRGH deletion derivative was generated (see also SEQ ID No. 21). It includes 477 bp of the 3' end of the argD gene, 19 bp of the 5' end of the argF gene, 15 bp of the 3' end of the argH gene, and 420 bp of the 5' end of the cg1589 reading frame. The product amplified in this way was assayed by electrophoresis in a 0.8% strength agarose gel.

The 1.04 kb DargFRGH PCR product (SEQ ID No. 21) was cleaved completely by the enzymes NdeI and NsiI. The fragment was subsequently purified using the PCR purification kit (Qiagen, Hilden, Germany). The DargFRGH deletion derivative pre-treated in this way was employed together with the mobilizable cloning vector pK18mobsacB (Schäfer et al. (1994), Gene 14: 69-73) for ligation. Said cloning vector had previously been cleaved completely by the restriction endonucleases XbaI and PstI. This produced DNA ends compatible to the ends of the insert generated by NdeI and NsiI cleavage. The vector prepared in this way was mixed with the DargFRGH fragment at a 1:5 molar ratio and ligated using T4 DNA ligase (Amersham- Pharmacia, Freiburg,

Germany) at 16°C for 1 hour. Chemical competent *E. coli* DH5alpha cells (Subcloning efficiency, Invitrogen GmbH, Karlsruhe, Germany) were transformed with 3 µl of the ligation mix. Transformants were identified on the basis of their kanamycin resistance on LB-agar plates containing 50 µg/ml kanamycin sulphate. Plasmid DNA was isolated from 4 of said transformants (QIAprep Spin Miniprep Kit from Qiagen (Hilden)), and the plasmids were assayed by restriction analysis for the presence of the 1.04 kb fragment as insert. The recombinant plasmid produced in this way was referred to as pK18mobsacB_DargFRGH. The strain was referred to as *E. coli*_DH5alpha/pK18mobsacB_DargFRGH.

The nucleotide sequence of the 1.04 kb fragment (SEQ ID No. 21) in the pK18mobsacB_DargFRGH plasmid was determined by the dideoxy chain termination method according to Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America (1977) 74: 5463-5467). To this end, the complete insert of the pK18mobsacB_DargFRGH plasmid was sequenced and thus assayed for correctness with the aid of the oligonucleotide primers M13 uni (-21) (5'-TGT AAA ACG ACG GCC AGT-3') and M13 rev (-49) (5'-GAG CGG ATA ACA ATT TCA CAC AGG-3') at Eurofins MWG Operon (Ebersberg, Germany).

Example 3:

Preparation of the strain *Corynebacterium glutamicum* ATCC 13032_DargFRGH

The vector mentioned in Example 2, pK18mobsacB_DargFRGH, was transferred by means of conjugation according to a protocol by Schäfer et al. (Journal of Microbiology 172: 1663-1666 (1990)) into the *Corynebacterium glutamicum* strain ATCC13032. For this purpose, the vector had previously been transformed into the *E. coli* strain S17-1 (Simon et al., Biotechnology 1: 784-791). The vector in

S17-1 was assayed for identity similarly to detection in *E. coli* DH5alpha (see Example 2).

The vectors pK18mobsacB and pK18mobsacB_DargFRGH cannot self-replicate in *C. glutamicum* ATCC13032 and remain in the cell only if they have integrated into the chromosome following a recombination event. Clones with integrated pK18mobsacB_DargFRGH are selected by plating out the conjugation mix on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, New York, 1989) supplemented with 15 mg/l kanamycin and 50 mg/ml nalidixic acid. Established clones are struck out on LB-agar plates containing 25 mg/l kanamycin and incubated at 33°C for 16 hours. Mutants in which the plasmid has been excised due to a second recombination event are selected by culturing the clones in LB liquid medium without selection for 20 hours, then striking them out on LB agar containing 10% sucrose, followed by incubation for 24 hours.

The pK18mobsacB_DargFRGH plasmid, like the pK18mobsacB starting plasmid, contains in addition to the kanamycin resistance gene a copy of the sacB gene coding for *Bacillus subtilis* levansucrase. Sucrose-inducible expression leads to the formation of levansucrase which catalyses the synthesis of the product levan which is toxic to *C. glutamicum*. Consequently, only those clones in which the integrated pK18mobsacB_DargFRGH has been excised again establish growth on LB agar containing sucrose. Excision may comprise excision of the plasmid together with either the complete chromosomal copy of argFRGH or the incomplete copy having the internal argFRGH deletion.

Approximately 40 to 50 colonies were tested for the phenotype "growth in the presence of sucrose" and "no growth in the presence of kanamycin". In order to prove that the deleted argFRGH allele has remained in the chromosome, approximately 20 colonies having the phenotype

"growth in the presence of sucrose" and "no growth in the presence of kanamycin" were studied by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press) with the aid of the
5 polymerase chain reaction. This involved amplifying from the chromosomal DNA of the colonies a DNA fragment which carries the regions surrounding the deleted argFRGH region. The following primer oligonucleotides were selected for the PCR.

10 argFRGH_d1 (SEQ ID No. 24):

5'-GGT GGT GCT AGC CCG GCG ATT TCT TTG CAC AT-3'

argFRGH_d4 (SEQ ID No. 27):

5'-GGT GGT ATG CAT GGT GAT GGT TCC GAA TGT TG-3'

In control clones containing the complete argFRGH locus,
15 the primers enable an approx. 5.35 kb DNA fragment to be amplified. In clones having a deleted argFRGH locus, DNA fragments of approx. 1.04 kb in size are amplified.

The amplified DNA fragments were identified by means of electrophoresis in a 0.8% strength agarose gel. By this the
20 strain was shown to carry a deleted argFRGH allele on the chromosome. The strain was referred to as *Corynebacterium glutamicum* Delta_argFRGH.

Example 4:

Expression of the lyse gene in *Corynebacterium glutamicum*
25 ATCC 13032_Delta_argFRGH

The plasmid pVWEx1_Lyse and the empty plasmid pVWEx1 were introduced into the L-ornithine-forming strain ATCC 13032_Delta_argFRGH by means of electroporation (Haynes et al., FEMS Microbiology Letters (1989) 61: 329-334).
30 Transformants were identified on the basis of their kanamycin resistance on Caso agar plates containing 25 µg/ml kanamycin. 5 single clones were subsequently

tested for correctness of the transformed plasmid. For this purpose, plasmid DNA was isolated (Plasmid Isolation Kit, Qiagen), and this DNA was assayed by restriction analysis for the correct cleavage pattern. In this way, the

5 C. glutamicum strains ATCC 13032_Delta_argFRGH/pVWEx1_lyeE and ATCC 13032_Delta_argFRGH/pVWEx1 were produced.

Example 5:

Preparation of L-ornithine using *Corynebacterium glutamicum*

In order to study their ability to produce L-ornithine, in

10 each case three clones of strain ATCC 13032_Delta_argFRGH/pVWEx1_lyeE and three clones of strain ATCC 13032_Delta_argFRGH/pVWEx1 were pre-cultured in each case in 10 ml of test medium at 33°C for 16 h. For the production assay, in each case 10 ml of test medium were

15 inoculated with the pre-culture obtained in such a way that the OD₆₀₀ (optical density at 600 nm) at the start was 0.1. Each clone was tested in three shaker flasks so that each strain is represented at the respective harvesting time by nine shaker flasks in total. The test medium was identical

20 to the CgXII medium described in Keilhauer et al. (Journal of Bacteriology (1993) 175: 5593-5603) but additionally contained 7.5 g/l yeast extract (Difco), 25 µg/ml kanamycin, 1 mM IPTG (isopropyl beta-D-thiogalactopyranoside) and 40 g/l sucrose instead of

25 glucose. For reasons of simplicity, the composition of the test medium is summarized in Table 2 below.

Table 2

Component	Content per l
$(\text{NH}_4)_2\text{SO}_4$	20 g
Urea	5 g
KH_2PO_4	1 g
K_2HPO_4	1 g
$\text{MgSO}_4 \times 7\text{H}_2\text{O}$	0.25 g
3-Morpholinopropanesulphonic acid (MOPS)	42 g
CaCl_2	0.01 g
$\text{FeSO}_4 \times 7\text{H}_2\text{O}$	0.01 g
$\text{MnSO}_4 \times \text{H}_2\text{O}$	0.01 g
$\text{ZnSO}_4 \times 7\text{H}_2\text{O}$	0.001 g
CuSO_4	0.0002 g
$\text{NiCl}_2 \times 6\text{H}_2\text{O}$	0.00002 g
Biotin	0.0002 g
Protocatechuic acid	0.03 g
Sucrose	40 g
Yeast extract	7.5 g
Ph (with NaOH)	7

The cultivation was carried out in 100 ml shaker flasks at 33°C and 200 rpm. The deflection of the shaker was 5 cm.

- 5 Three cultures of a clone were harvested after 24 and 48 hours. To this end, samples were taken from the cultures and the optical density, the sucrose content and the L-ornithine content were determined. To determine the sucrose and L-ornithine contents the cells were removed by brief
- 10 centrifugation (table-top centrifuge type 5415D (Eppendorf) at 13 000 rpm, 10 min, room temperature).

- The optical density was determined at a wavelength of 660 nm, using a GENios microtitre plate photometer (Tecan, Reading, UK). The samples were diluted 1:100 with
- 15 demineralized water prior to the measurement.

Sucrose was determined using a test system (Cat. No. 10 716 251 035) from R-Biopharm AG (Darmstadt, Germany). This involves inversion of sucrose and the glucose formed being detected using a coupled enzyme assay (hexokinase/glucose-6-phosphate dehydrogenase) via NADH formation.

Quantitative determination of the extracellular amino acid concentration from the culture supernatant was carried out by means of reverse-phase HPLC (Lindroth et al., Analytical Chemistry (1979) 51: 1167-1174), using an HP1100 series HPLC instrument (Hewlett-Packard, Waldbronn, Germany) with connected fluorescence detector (G1321A); system control and data evaluation were carried out using a HP ChemStation (Hewlett-Packard). 1 μ L of the amino acid solution to be analysed was mixed in an automatic pre-column derivatization with 20 μ L of ready-to-use ortho-phthalaldehyde/2-mercaptoethanol reagent (Pierce Europe BV, Oud-Beijerland, Netherlands). The resulting fluorescent, thio-substituted isoindoles (Jones et al., Journal of Chromatography (1983) 266: 471-482) were fractionated on a pre-column (40 $\times$ 4 mm Hypersil ODS 5) and main-column combination (Hypersil ODS 5, both columns from CS-Chromatographie Service GmbH, Langerwehe, Germany) using a gradient program with an increasingly non-polar phase (methanol). The polar eluent was sodium acetate (0.1 M; pH 7.2); the flow rate was 0.8 mL per minute. The fluorescence of the derivatized amino acids was detected at an excitation wavelength of 230 nm and an emission wavelength of 450 nm. The L-ornithine and/or L-ornithine hydrochloride concentrations were calculated by way of comparison with an external standard and L-asparagine as additional internal standard.

The molecular weight of L-ornithine hydrochloride is 168.6 g $\times$ mol⁻¹ and that of L-ornithine is 132.1 g $\times$ mol⁻¹.

The yield was calculated by dividing the amount of L-

ornithine formed (measured as L-ornithine hydrochloride) by the amount of sucrose consumed.

The results are listed in Table 3.

Table 3: L-ornithine formation after 24 hours (Table 3A) and 48 hours (Table 3B) of incubation. Abbreviations:
*: ATCC 13032_Delta_argFRGH; Orn-HCl: L-ornithine hydrochloride.

Table 3A:

Time	24 hours		
Strain	Orn-HCl g/l	Yield g/g	OD
*/pVWEx1	9.83 ± 0.10	0.39 ± 0.01	10.78 ± 0.30
*/pVWEx1_lyseE	13.03 ± 0.16	0.44 ± 0.01	10.40 ± 0.41

Table 3B:

Time	48 hours		
Strain	Orn-HCl g/l	Yield g/g	OD
*/pVWEx1	15.50 ± 0.74	0.36 ± 0.01	11.69 ± 1.40
*/pVWEx1_lyseE	18.48 ± 0.51	0.42 ± 0.01	9.18 ± 0.48

Example 6: Sequencing and deposition of plasmid pEC7lyseE

Plasmid pEC7lyseE was made available in the form of an aqueous solution by Dr. Lothar Eggeling (Forschungszentrum Jülich GmbH, D-52425 Jülich), the corresponding author of the publication Bellmann et al. (Microbiology (2001) 147, 1765-1774).

An aliquot of the DNA solution obtained was employed for transforming competent Escherichia coli cells of the DH5alpha strain (subcloning efficiency, Genotype: F- Φ 80lacZAM15 Δ (lacZYA-argF) U169 recA1 endA1 hsdR17 (rK-,

mk+) *phoA supE44 λ- thi-1 gyrA96 relA1*) from Invitrogen GmbH (Paisley, UK) according to the manufacturer's instructions. The transformants were selected on Luria-Bertani agar supplemented with 50 µg/ml kanamycin.

- 5 One transformant referred to as *Escherichia coli* DH5alpha/pEC7lyse(DM2204) was deposited according to the Budapest Treaty with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Brunswick, Germany) under the deposition number DSM 23239 on 15 January 2010.
- 10 The pEC7lyse plasmid from the DSM 23239 strain was completely sequenced by custom DNA sequencing (Walking Service) at Eurofins MWG Operon GmbH (Martinsried, Germany). The sequence of pEC7lyse is listed as SEQ ID No. 29.

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1-1	page	40
1-2	line	12-16
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	DSMZ DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Inhoffenstr. 7B, D-38124 Brunswick, Germany
1-3-3	Date of deposit	15 January 2010 (15.01.2010)
1-3-4	Accession Number	DSMZ 23239
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Claims

- 1) Process for the preparation of L-ornithine, characterized in that the following steps are carried out:
 - a) fermentation of an L-ornithine-excreting bacterium selected from the group consisting of *Corynebacterium*, *Bacillus*, *Streptomyces*, *Arthrobacter* and the *Enterobacteriaceae* which overexpresses a polynucleotide coding for a polypeptide which has the activity of an L-ornithine exporter and whose amino acid sequence is identical to the amino acid sequence of SEQ ID No. 2 or whose amino acid sequence can be obtained from SEQ ID NO:2 by a maximum of 25 deletions, insertions, substitutions, or N-respectively C-terminal additions of amino acids overall, in a medium,
 - b) accumulation of said L-ornithine in said medium, wherein a fermentation broth is obtained, and
 - c) wherein the plasmid pEC71ysE, deposited in DSM23239, is excluded for overexpression.
- 2) Process according to Claim 1, characterized in that overexpression is achieved by one or more of the measures selected from the group consisting of
 - a) increasing the copy number,
 - b) using a strong promoter, and
 - c) mutating the promoter, and

- d) overexpressing an activator protein.
- 3) Process according to Claims 1 to 2, characterized in that the bacterium is *Corynebacterium*, preferably *Corynebacterium glutamicum*.
- 4) Process according to Claims 1 to 3, characterized in that additionally one or more of the genes selected from the group consisting of
- a) *odhA* gene coding for the E1 subunit of alpha-ketoglutarate dehydrogenase (EC 1.2.4.2),
 - b) *sucA* gene coding for dihydrolipoamide succinyl transferase (EC 2.3.1.61),
 - c) *dapA* gene coding for a dihydrodipicolinate synthase (DapA, EC 4.2.1.52),
 - d) *dapB* gene coding for a dihydrodipicolinate synthase (DapB, EC 1.3.1.26),
 - e) *ddh* gene coding for a meso-diaminopimelate dehydrogenase (Ddh, EC 1.4.1.16),
 - f) *lysA* gene coding for a diaminopimelate decarboxylase (LysA, EC 4.1.1.20),
 - g) *argR* gene coding for a/the repressor (ArgR) of L-arginine biosynthesis,
 - h) *argF* gene coding for an ornithine carbamoyl transferase (ArgF, EC 2.1.3.3),
 - i) *argG* gene coding for an argininosuccinate synthase, (ArgG, EC 6.3.4.5),
 - j) *argH* gene coding for an argininosuccinate lyase (ASAL) (ArgH, EC 4.3.2.1),

k) lysC gene coding for an aspartate kinase (LysC, EC 2.7.2.4), and

l) asd gene coding for an aspartate semialdehyde dehydrogenase (Asd, EC 1.2.1.11),

is/are attenuated.

5) Process according to Claims 1 to 4, characterized in that additionally one or more of the genes selected from the group consisting of

a) glutamate dehydrogenase (EC 1.4.1.3) encoded by the gdh gene,

b) glutamate N-acetyltransferase (EC 2.3.1.35 and EC 2.3.1.1) encoded by the argJ gene,

c) acetylglutamatekinase (EC 2.7.2.8) encoded by the argB gene,

d) N-acetyl-gamma-glutamyl-phosphate reductase (EC 1.2.1.38) encoded by the argC gene,

e) acetylornithine aminotransferase (EC 2.6.1.11), encoded by the argD gene,

f) glucose-specific component EIIB (PtsG) (EC 2.7.1.69) of the glucose uptake system, encoded by the ptsG gene,

g) sucrose-specific component EIIB (PtsS) (EC 2.7.1.69) of the sucrose uptake system, encoded by the ptsS gene,

h) glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49) encoded by the zwf gene,

i) glucose-6-phosphate isomerase (EC 5.3.1.9) encoded by the pgi gene,

- j) phosphofructokinase (EC 2.7.1.11) encoded by the pfkA gene,
 - k) fructose-bisphosphate aldolase (EC 4.1.2.13) encoded by the fda gene,
 - l) glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.59) encoded by the gap gene,
 - m) phosphoglycerate kinase (EC 2.7.2.3) encoded by the pgk gene,
 - n) pyruvate kinase (EC 2.7.1.40) encoded by the pyk gene,
 - o) E1 subunit of pyruvate dehydrogenase (EC 1.2.4.1), encoded by the aceE gene,
 - p) phosphoenolpyruvate carboxylase (EC 4.1.1.31) encoded by the ppc gene,
 - q) pyruvate carboxylase (EC 6.4.1.1), encoded by the pyc gene,
 - r) aconitase (EC 4.2.1.3) encoded by the acn gene, and
 - s) isocitrate dehydrogenase (EC 1.1.1.42) encoded by the icd gene,
- is/are enhanced.
- 6) Process according to Claims 1 to 5, characterized in that it is a process selected from the group consisting of batch process, fed-batch process, repetitive fed-batch process, and continuous process.
 - 7) Process according to Claims 1 to 6, characterized in that L-ornithine or a liquid or solid L-ornithine-containing product is recovered from the L-ornithine-containing fermentation broth.