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A family of microbial lysine transporter polypeptides

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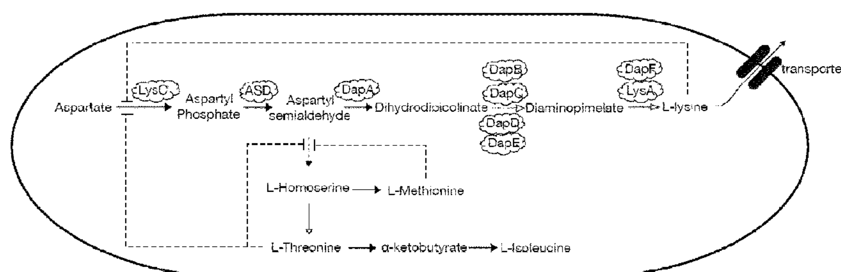
(54) **Title**: A FAMILY OF MICROBIAL LYSINE TRANSPORTER POLYPEPTIDES

Figure 1



(57) **Abstract**: The present invention provides a genetically modified microbial cell for production of lysine, comprising a transgene encoding a polypeptide capable of exporting lysine from the cell. The genetically modified microbial cell for production of lysine may be further characterized by genetic modifications that confer reduced lysine metabolism and/or enhanced lysine synthesis as compared to the parent cell from which said genetically modified cell was derived. The invention further provides a method for producing lysine using the genetically modified microbial cell. The invention further provides a novel family of lysine transporter polypeptides; and the use of said polypeptide to enhance production of extracellular lysine in a microbial cell.

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Title: A family of microbial lysine transporter polypeptides

Field of the invention

The invention provides a genetically modified microbial cell for production of lysine, comprising a transgene encoding a polypeptide capable of exporting lysine from the cell. The genetically modified microbial cell for production of lysine may be further characterized by genetic modifications that confer reduced lysine metabolism and/or enhanced lysine synthesis as compared to the parent cell from which said genetically modified cell was derived. The invention further provides a method for producing lysine using the genetically modified microbial cell. The invention further provides a novel family of lysine transporter polypeptides; and the use of said polypeptide to enhance production of extracellular lysine in a microbial cell.

Background of the invention

Current global demand for lysine market is about 2 million tons per year with an annual growth of 7% on average. Industrial stereospecific L-lysine bioprocesses are primarily based on *Escherichia coli* and *Corynebacterium glutamicum*, which are capable of producing lysine titers in excess of 100 g/L. The specific export rate of L-lysine is however inhibited these production microorganisms when the extracellular lysine reaches these titers. It has, for example been observed that when the extracellular lysine titer increases from 80 mM (11.7 g/L) to 400 mM (58.5 g/L), this causes a 50% reduction in lysine export rate, associated with the accumulation of intracellular L-lysine pool impeding the cell survivability. L-lysine toxicity arises conceivably due to i) feedback inhibition of aspartokinase by L-lysine inhibiting other necessary amino acids synthesis, ii) inhibition of L-arginine transporter reducing arginine uptake; and increased osmotic pressure and pH stress. Irrespective of the toxicity mechanism these issues could be mitigated by active secretion of L-lysine into culture media. This strategy has been deployed for L-lysine production by expressing a lysine exporter LysE from *C. glutamicu*m into *Methylophilis methylotrophus* (Gunji, Y. & Yasueda, H.; 2006), however, options for rational engineering are nearly exhausted and hence search for new genetic building blocks to further improve L-lysine tolerance are urgently needed. On the other hand, functional metagenomics selection is a powerful method for discovering

novel genes and enzymes from the environment because of its potential to access all the genetic elements present in a particular environmental niche.

Summary of the invention

5 According to a first embodiment, the invention provides a genetically modified microbial cell for production of lysine comprising a transgene encoding a lysine transporter polypeptide, wherein the amino acid sequence of the lysine transporter polypeptide has at least 85% sequence identity to SEQ ID No.: 2.

10 Alternatively, the genetically modified microbial cell of the invention comprises a transgene encoding a lysine transporter polypeptide, wherein the amino acid sequence of the lysine transporter polypeptide has at least 85 % sequence identity to SEQ ID No: 2, but with the proviso that amino acid residue at position 240 is valine and amino acid residue at position 278 is phenylalanine.

15

The genetically modified microbial cell of the invention may further be characterized by one or more genetic modification conferring: reduced lysine metabolism and/or enhanced lysine synthesis, as compared to the parent cell from which said genetically modified cell was derived.

20

The genetically modified microbial, characterized by reduced lysine metabolism, may comprise one or more endogenous genes encoding a polypeptide having lysine decarboxylase activity (EC 4.1.1.18) are deleted or inactivated.

25 The genetically modified microbial of the invention that is characterized by enhanced lysine synthesis, may further comprise one additional transgene or a combination of two or more additional transgenes encoding a polypeptide selected from the group consisting of: aspartate kinase (EC No.: 2.7.2.4); dihydrodipicolinate synthase (EC No.: 4.2.1.52 or 4.3.3.7); aspartate-
30 semialdehyde dehydrogenase (EC No.: 1.2.1.11); meso-diaminopimelate dehydrogenase (EC No.: 1.4.1.16); diaminopimelate decarboxylase (EC No.: 4.1.1.20); aspartate aminotransferase (EC No.: 2.6.1.1); dihydrodipicolinate reductase (EC No.: 1.3.1.26 or 1.17.1.8); succinyldiaminopimelate aminotransferase, AT class I (EC No.: 2.6.1.17); tetrahydrodipicolinate succinylase

(EC No.: 2.3.1.117); succinyl-diaminopimelate desuccinylase (EC No.: 3.5.1.18); and diaminopimelate epimerase (EC No.: 5.1.1.7).

The genetically modified microbial of the invention may be selected from among a bacterium, a yeast, and a filamentous fungus; preferably a species of bacterium selected from the group consisting of: *Corynebacterium, Escherichia, Brevibacteriaceae, Bacillus, Lactobacillus, Lactococcus, Acetobacter, Acinetobacter, Pseudomonas* and *Streptococcaceae*.

According to a second embodiment, the invention provides an isolated polynucleotide encoding a lysine transporter polypeptide, wherein the amino acid sequence of the polypeptide has at least 85% sequence identity to SEQ ID No.: 2.

Alternatively, the isolated polynucleotide encodes an amino acid sequence of the having at least 85 % sequence identity to SEQ ID No: 2, but with the proviso that amino acid residue at position 240 is valine and amino acid residue at position 278 is phenylalanine.

According to a third embodiment, the invention provides a method for producing lysine in *vivo* comprising: providing a microbial cell comprising a transgene encoding a lysine transporter polypeptide according to the invention; introducing said microbial cell into a growth medium to produce a culture; providing a substrate for lysine production; recovering the produced lysine from the culture and optionally purifying the lysine recovered.

According to a fourth embodiment, the invention provides for the use of a transgene encoding a lysine transporter polypeptide to enhance lysine export from a microorganism, wherein the amino acid sequence of said polypeptide has at least 85% sequence identity to SEQ ID No: 2.

The transgene encoding a lysine transporter may further be used to enhance lysine export from a microorganism, wherein said cell is characterized by one or more genetic modification conferring: reduced lysine metabolism and/or enhanced lysine synthesis as compared to the parent cell from which said genetically modified cell was derived; as detailed above.

The transgene encoding a lysine transporter may further be used to enhance lysine export from a microorganism of the invention, wherein the micro-organism is a species of a genus selected from the group consisting of: *Corynebacterium, Escherichia, Brevibacteriaceae, Bacillus, Lactobacillus, Lactococcus, Acetobacter, Acinetobacter, Pseudomonas* and *Streptococcaceae*.

Description of the invention

Description of the figures:

Figure 1. Cartoon illustrating the lysine biosynthesis pathway in a micro-organism. Metabolic steps in the lysine biosynthesis pathway are catalyzed by the following enzymes (and encoded by their respective genes) aspartate kinase (LysC); aspartate-semialdehyde dehydrogenase (ASD); dihydrodipicolinate synthase (DapA); dihydrodipicolinate reductase (DapB); succinyldiaminopimelate aminotransferase, AT class I (DapC); tetrahydrodipicolinate succinylase (DapD); succinyl-diaminopimelate desuccinylase (DapE); diaminopimelate epimerase (DapF) and diaminopimelate decarboxylase (LysA).

Figure 2 (A) L-lysine IC90 values for four selected lysine-tolerant *E.coli* C4860 clones (strains) harboring putative transporters (pZE-RCL-MglE; pZE-CGML1; pZE-CGML2; and pZE-CGML3) as compared to a clone harboring the vector (pZE21). Cells (1×10^4) from an overnight culture of each selected E. coli clones were inoculated into LB liquid media and grown at 30 °C and 300 RPM in 96-well microtiter plates containing 150 µl of medium per well. The lysine concentration in the wells followed a logarithmic L-lysine concentration gradient with two-fold serial dilutions. Growth in each well was determined as endpoint absorbance measurements (A600nm) taken after 24 h of incubation using a plate reader (Synergy H1, BioTek) and background-subtracted. Growth inhibition of the *E. coli* clones were plotted against L-lysine concentrations with a polynomial interpolation between neighboring data points using R software (http://www.r-project.org). The percentage of inhibition was calculated using the formula: 1 – [A_{600nm} L-lysine/$A_{600nm\ control}$]. The inhibitory concentration was defined as the lowest concentration of lysine that inhibited 90% (IC90) of the growth of the clone tested.

Figure 2 (B) Graphical presentation of growth rates of the four selected lysine-tolerant *E.coli* C4860 clones (strains) harboring putative transporters (pZE-RCL-MglE; pZE-CGML1; pZE-CGML2; and pZE-CGML3) as compared to a clone harboring the vector (pZE21) in the presence or absence of lysine. Single colonies of the

selected lysine-tolerant *E. coli* C4860 and control clones were grown overnight; and the cultures were diluted to adjust $(OD)_{600nm}$ of 0.1 and 5 µl of the cultures were inoculated in 150 µl of fresh media with kanamycin (50 µg/ml) and L-lysine (0 and 8 g/l) in a 96-well micro-titer plate. The plate was incubated at 37 °C for 24 h in an
5 automated spectrophotometer (ELx808, BioTek) which recorded the $(OD)_{630nm}$ at an interval of 60 min. Then the data points at the exponential phase of the growth curves were used to determine the growth rates by applying the formula $\ln(OD_2/OD_1)=\mu(t_2-t_1)$, where OD is the optical density of the cell culture at 630nm, t is the time in h and µ is the growth rate in h^{-1}. The data represents the average of
10 triplicate experiments, with error bars being representative of the standard error of the mean (SEM).

Figure 3 Bar graph showing the L-lysine tolerance of *E. coli* production strains: i) C4860, ii) MG1655, iii) W3110, iv) Crooks and v) W1116 harboring the plasmid pZE-RCL-MglE encoding a lysine transporter, or the vector (pZE21). Lysine
15 tolerance, measured as IC90, was determined as set out in Figure 2A.

Figure 4 Cartoon showing the structure of the metagenomics DNA insert in pZE-RCL-MglE.

Figure 5 Cartoon showing the predicted membrane topology of the MglE transporter protein showing 6 cytoplasmic regions, 5 periplasmic regions and 10
20 transmembrane α-helices.

Figure 6 Diagram showing the origin of 16 closest homologs to MglE, identified by BLASTp, with a ≥50% sequence identity compared to MglE and having the following GenBank numbers: *Bacteroides* sp. CAG:598 (GenBank: CCX61976.1); *Bacteroides* sp. CAG:443 (GenBank: CDB98382.1); *Bacteroides* sp. CAG:1076 (GenBank:
25 CCY93346.1); *Bacteroides coprocola* CAG:162 (GenBank:CDA70121.1); *Bacteroides coprocola* DSM 17136 (GenBank: EDV02549.1); *Bacteroides coprocola* (GenBank:WP_040311754.1); *Prevotella* sp., CAG:1031 (GenBank:CCX44039.1); *Bacteroides* sp. CAG:1060 (GenBank: CCX56076.1; SEQ ID No: 83); *Bacteroides* sp. CAG:545 (GenBank:CCZ43287.1; SEQ ID No: 81); *Bacteroides* sp. CAG:770
30 (GenBank:CDC66277.1; SEQ ID No: 79); *Bacteroides* sp. CAG 714 (GenBank:CDD32682.1); *Bacteroides barnesiae* (GenBank:WP_018711839.1); *Bacteroides plebeius* (GenBank:WP_007558717.1); *Bacteroides plebeius* CAG:211 (GenBank:CCZ87371.1); *Bacteriodes coprophilus* (GenBank:WP_008140691.1); and *Bacteriodes coprophilus* CAG:333 (GenBank: CDC57518.1);

Figure 7 Cartoon illustrating the lysine biosynthesis pathway in a genetically modified micro-organism adapted for lysine production, and harboring the plasmid pZE-RCL-MglE encoding a lysine transporter. Metabolic steps in the lysine biosynthesis pathway are catalyzed by the following enzymes (and encoded by their
5 respective genes) aspartate kinase (LysC); aspartate-semialdehyde dehydrogenase (ASD); dihydrodipicolinate synthase (DapA); dihydrodipicolinate reductase (DapB); succinyldiaminopimelate aminotransferase, AT class I (DapC); tetrahydrodipicolinate succinylase (DapD); succinyl-diaminopimelate desuccinylase (DapE); diaminopimelate epimerase (DapF) and diaminopimelate decarboxylase
10 (LysA); while the conversion of lysine to cadaverine is catalyzed by lysine decarboxylases (LdcC and CadA).

Figure 8 Histogram showing the L-lysine tolerance of cells of the *E.coli* W3110 Δ*ldcC*.Δ*cadA* strain (named as *E.coli* DMLC) (adapted for lysine production) transformed with the control vector pZE21; or the plasmids pZE-RCL-MglE, pZE-
15 LysE, and pZE-YbjE plasmids encoding the lysine exporters MglE, LysE and YbjE respectively. Lysine tolerance, measured as IC90, was determined as set out in Figure 2A.

Figure 9 Histogram showing the extracellular L-lysine content **(A)** and the specific lysine production (extracellular); **(B)** of cell cultures of the parent *E. coli* strain
20 W3110 transformed with the control vector pZE21; compared to the *E. coli* DMLC strain (adapted for lysine production) harboring the control vector pZE21 or the plasmid pZE-RCL-MglE encoding the lysine exporter MglE. The lysine content of the LB growth medium is shown.

Figure 10 Bar graph showing the fold change in L-lysine tolerance (IC90) of 2
25 strains of *E. coli* expressing a *MglE* gene encoding the lysine exporter MglE, or one of four genes showing homology to the *MglE* gene. (Left side) *E. coli* C4860 strain and (right side) *E. coli* DMLC strain (adapted for lysine production) transformed with the control vector (pZE21), or the plasmid pZE-RCL-MglE encoding the lysine exporter MglE, or a plasmid comprising one of the homologous genes: pZE-Gene 1,
30 pZE-Gene 2, pZE-Gene 3, and pZE-Gene 4. Lysine tolerance, measured as IC90, was determined as set out in Figure 2A.

Abbreviations and terms:

gi number: (genInfo identifier) is a unique integer which identifies a particular sequence, independent of the database source, which is assigned by NCBI to all sequences processed into Entrez, including nucleotide sequences from DDBJ/EMBL/GenBank, protein sequences from SWISS-PROT, PIR and many others.
5 **Amino acid sequence identity**: The term "sequence identity" as used herein, indicates a quantitative measure of the degree of homology between two amino acid sequences of substantially equal length. The two sequences to be compared must be aligned to give a best possible fit, by means of the insertion of gaps or alternatively, truncation at the ends of the protein sequences. The sequence
10 identity can be calculated as ((Nref-Ndif)100)/(Nref), wherein Ndif is the total number of non-identical residues in the two sequences when aligned and wherein Nref is the number of residues in one of the sequences. Sequence identity can alternatively be calculated by the BLAST program e.g. the BLASTP program (Pearson W.R and D.J. Lipman (1988)) (www.ncbi.nlm.nih.gov/cgi-bin/BLAST). In
15 one embodiment of the invention, alignment is performed with the sequence alignment method ClustalW with default parameters as described by Thompson J., et al 1994, available at http://www2.ebi.ac.uk/clustalw/.
Preferably, the numbers of substitutions, insertions, additions or deletions of one or more amino acid residues in the polypeptide as compared to its comparator
20 polypeptide is limited, i.e. no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 substitutions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 insertions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 additions, and no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 deletions. Preferably the substitutions are conservative amino acid substitutions: limited to exchanges within members of group 1: Glycine, Alanine, Valine, Leucine,
25 Isoleucine; group 2: Serine, Cysteine, Selenocysteine, Threonine, Methionine; group 3: Proline; group 4: Phenylalanine, Tyrosine, Tryptophan; Group 5: Aspartate, Glutamate, Asparagine, Glutamine.
Deleted gene: the deletion of a gene from the genome of a microbial cell leads to a loss of function of the gene and hence where the gene encodes a polypeptide the
30 deletion results in a loss of expression of the encoded polypeptide. Where the encoded polypeptide is an enzyme, the gene deletion leads to a loss of detectable enzymatic activity of the respective polypeptide in the microbial cell.
Lysine transporter protein: lysine transporter polypeptide; polypeptide capable of transporting lysine, more specifically polypeptide capable of exporting lysine
35 from a microbial cell.

Native gene: endogenous gene in a microbial cell genome, homologous to host micro-organism.

Parent cell: a cell of a microbial strain which is the subject of genetic modification to obtain a genetically modified microbial strain that is derived from the parent cell; whereby the properties of the derivative, attributable to the genetic modification, can be compared to that of the parent cell.

Detailed description of the invention

The present invention relates to the provision of a transporter protein capable of exporting lysine from a microbial cell, and thereby enhancing lysine export and/or production by the cell. Whereas the enzymes of the lysine biosynthesis pathway (figure 1) have been extensively studied and are conserved among diverse bacterial genomes, very few bacterial lysine transporters have been experimentally validated. A functional search for candidate lysine transporters, capable of exporting lysine, was initiated by screening for lysine transporters encoded by metagenomic DNA isolated from cow gut microbiota samples. DNA molecules derived from shearing the isolated metagenomic DNA were cloned into an expression plasmid compatible with expression in the host bacterium, *Escherichia coli*.

Bacterial cells, such as *E. coli*, are able to assimilate lysine provided in their growth medium, however, at high concentrations, lysine becomes toxic for growth due to three physiological factors:

i) lysine causes feedback inhibition of aspartokinase: impeding the synthesis of necessary amino acids for cell growth;

ii) lysine reduces L-arginine uptake; since L-lysine is a natural inhibitor of L-Arg transport,

iii) lysine increases osmotic pressure and pH stress due to the accumulation of intracellular L-lysine.

E. coli can be used as a host cell to screen for transporters capable of exporting lysine; since growth of a transformed host cell at toxic lysine concentration is dependent on the expression of a gene encoding a transporter capable of exporting lysine. A library of 10^6 *E. coli* cells, transformed with the metagenomic library, was screened, from which a clone (pZE-RCL-MglE) conferring a 43% increase in lysine tolerance was identified, when measured as the lowest concentration of lysine that inhibited growth by 90% (IC90).

I A novel family of lysine transporters that facilitate lysine export

The invention provides a new family of novel transporter proteins capable of exporting lysine from a microbial cell of the invention. One member of this family is the lysine transporter, designated MglE, which is expressed as a polypeptide having 297 amino acids and a deduced amino acid sequence having SEQ ID No: 2. Based on phylogenetic analysis, this new family of novel lysine transporters (including MglE) show structural features in common with the RhaT/EamA-like transporter family belonging to the drug/metabolite transporter (DMT) superfamily. The MglE protein contains two copies of the EamA domain common to members of this superfamily: of which one is located in the region of amino acid residues 9-143 and another in the region of amino acid residues 152-292. A two-dimensional topological model shows MglE transporter to possess six cytoplasmic domains, five periplasmic domains and ten transmembrane domains with both N- and C-terminals in the cytoplasmic region (Figure 5). The members of this new protein family of lysine transporters are characterized by an amino acid sequence having at least 85% sequence identity to SEQ ID No.:2; for example at least 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID No.:2.

Members of this family of novel lysine transporters are phylogenetically remote from known L-lysine specific transporters, and do not have any significant homology with the LysE lysine exporter from *C. glutamicum*. Accordingly, the MglE protein family comprises novel L-lysine transporters, whose function could not have been predicted by sequence homology to known L-lysine transporters.

The members of the family of novel lysine transporter proteins, when expressed in a microbial cell of the invention, e.g. *E. coli* cell, are capable of enhancing the cell's lysine tolerance. For example, when expressed in *E. coli* the cells lysine tolerance was increased by 43%, when measured as the lowest concentration of lysine that inhibited growth by 90% (IC90). The observed increase in lysine tolerance is due to the ability of the novel transporter protein to export lysine from the cell, at a rate that is sufficient to prevent the intracellular lysine concentration reaching levels that are toxic for microbial cell growth.

The members of the family of novel transporter proteins, are capable of enhancing lysine export when expressed in different microbial cells, for example, lysine export is enhanced in a number of different lysine production strains of *E. coli* (Example 2). When the novel transporter protein (MglE) is expressed in a microorganism adapted for lysine production, the extracellular lysine concentration is enhanced as compared to a control strain not expressing the transport protein MglE. The extracellular lysine concentration produced by Δ*ldcC*Δ*cadA E. coli* strain was increased by 8% in cells expressing the transport protein MglE as compared to control cells (Example 4). Surprisingly, the increased in lysine tolerance of a microorganism conferred by expression of the novel transporter protein (MglE) was greater than that conferred by expression of with the LysE or YbjE transporter proteins (Example 4).

Phylogenetic analyses revealed that this new family of novel lysine transporters showed partial amino acid sequence identity to a number of un-identified polypeptides reported in the GenBank, for example, the polypeptides identified as: GenBank:CDC57518.1 (SEQ ID No: 67); GenBank:WP_008140691.1 (SEQ ID No: 69); GenBank:CCZ87371.1 (SEQ ID No: 71); GenBank:WP_007558717.1 (SEQ ID No: 73); GenBank:WP_018711839.1 (SEQ ID No: 75); GenBank:CDD32682.1 (SEQ ID No: 77); GenBank:CCX44039.1 (SEQ ID No: 85); GenBank:WP_040311754.1 SEQ ID No: 87; GenBank: EDV02549.1 (SEQ ID No: 89); GenBank:CDA70121.1 (SEQ ID No: 91); GenBank: CCY93346.1 (SEQ ID No: 93); GenBank: CDB98382.1 (SEQ ID No: 95); and GenBank: CCX61976.1 (SEQ ID No: 97) (see figure 6). Surprisingly however, these un-identified polypeptides were not found to exhibit the same properties as the MglE lysine transporter, since they failed to provide a similar increase in lysine tolerance when expressed in a microorganism (see example 7). This leads to the surprising observation, that the new family of novel lysine transporters of the invention are indeed phylogenetically remote from known L-lysine specific transporters, and their properties could not have been predicted.

The members of the family of novel lysine transporter proteins, include variants or mutants thereof, having an amino acid sequence having at least 85% sequence identity to SEQ ID No: 2. More specifically, the family includes lysine transporter proteins produced by mutation, wherein the mutant proteins confer a higher levels of lysine tolerance (as compared to the parent protein from which the mutant was

derived) when expressed in a microbial cell; for example lysine transporter proteins MglE1* and MglE2*. As illustrated in Example 6, microbial cells expressing either MglE1* and MglE2*, showed a 61% increase in lysine tolerance as compared to cells expressing the parent lysine transport protein (MglE). The amino acid sequence of MglE1* [SEQ ID No.: 65] differs from the amino acid sequence of MglE [SEQ ID No.: 2] by 2 substitutions (A/V^{240} and L/F^{278}). Accordingly, in one embodiment, the amino acid sequence of the lysine transporter protein has at least 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID No: 2, but with the proviso that amino acid residues at the indicated positions are V^{240} and F^{278}.

II: A genetically modified cell expressing the novel lysine transporter

The present invention provides a genetically modified microbial cell capable of exporting enhanced levels of lysine. The microbial cell of the invention comprises a transgene encoding a lysine transporter, where the amino acid sequence of the transporter has at least 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID No: 2 that is capable of exporting lysine from a microbial cell. In one embodiment, the microbial cell of the invention comprises a transgene encoding a lysine transporter protein; where the amino acid sequence of the transporter has at least 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % (for example at least 85%) sequence identity to SEQ ID No: 2, but with the proviso that amino acid residues at the indicated positions are V^{240} and F^{278}.

In a preferred embodiment the microbial cell of the invention is adapted for lysine production by genetic modifications that reduce the depletion of the intracellular lysine pool and/or enhance the synthesis of lysine when compared to the cell from which the genetically modified cell was derived. The metabolic consumption of lysine in the microbial cell of the invention may for example be reduced by blocking the conversion of lysine to cadaverine by lysine decarboxylase (see example 4 and figure 7). The microbial cell of the invention, suitable for lysine production, may either be selected from a genotype having reduced lysine decarboxylase activity; or alternatively one or more genes encoding a polypeptide having lysine decarboxylase activity (EC 4.1.1.18) are deleted/inactivated from the genome of the microbial cell. A suitable microbial cell having reduced lysine decarboxylase activity (EC 4.1.1.18), includes a cell having a genome from which the *ldcC* and

cadA genes are deleted or are not present. For example, the amino acid sequence of the lysine decarboxylase whose activity is reduced in the microbial cell of the invention has at least 80, 85, 90, 95 or 100% sequence identity to SEQ ID No: 4 [GenBank: BAA77861.2] and SEQ ID No: 6 [GenBank: BAE78134.1].

5

The microbial cell of the invention, adapted for lysine production, may be characterized by genetic modifications that enhance the synthesis of lysine when compared to the cell from which the genetically modified cell was derived. For example the expression of one or more enzymatic steps in the lysine biosynthesis
10 pathway can be enhanced (Figure 1); either by upregulating expression of a native gene encoding the enzyme catalyzing the respective enzymatic step (e.g. substituting or complementing the native promoter with a DNA fragment conferring higher transcription rates of the cognate native gene), or by introducing a transgene encoding the respective enzyme. One or more genes, whose upregulated
15 expression in the microbial cell of the invention will enhance lysine production, may be selected from the group consisting of:

a) Polynucleotide (*lysC* gene) coding for an aspartate kinase (LysC, EC NO.: 2.7.2.4); for example wherein the encoded amino acid sequence has at least 80, 85, 90, 95 or 100% sequence identity to SEQ ID No: 8 [GenBank:
20 BAE78026.1] (for example in *E.coli* strains) and SEQ ID No: 10 [GenBank: CAA40502.1] (for example in *Corynebacterium glutamicum* strains);
b) Polynucleotide (*dapA* gene) coding for a dihydrodipicolinate synthase (DapA, EC No.: 4.2.1.52 or 4.3.3.7); for example wherein the encoded amino acid sequence has at least 80, 85, 90, 95 or 100% sequence identity to SEQ ID
25 No: 12 [GenBank: BAA16355.1] (for example in *E.coli* strains) and SEQ ID No: 14 [GenBank: X53993.1] (for example in *Corynebacterium glutamicum* strains)
c) Polynucleotide (*asd* gene) coding for an aspartate-semialdehyde dehydrogenase (ASD, EC No.: 1.2.1.11); for example wherein the encoded
30 amino acid sequence has at least 80, 85, 90, 95 or 100% sequence identity to SEQ ID No: 16 [GenBank: BAE77859.1] (for example in *E.coli* strains) and SEQ ID No: 18 [GenBank: CAA40504.1] (for example in *Corynebacterium glutamicum* strains);
d) Polynucleotide (*ddh* gene) coding for a meso-diaminopimelate
35 dehydrogenase (Ddh, EC No.: 1.4.1.16); for example wherein the encoded

amino acid sequence has at least 80, 85, 90, 95 or 100% sequence identity to SEQ ID No: 20 [GenBank: WP_038587080.1]

e) Polynucleotide (*lysA* gene) coding for a diaminopimelate decarboxylase (LysA, EC No.: 4.1.1.20); for example wherein the encoded amino acid sequence has at least 80, 85, 90, 95 or 100% sequence identity to SEQ ID No: 22 [GenBank: BAE76907.1] (for example in *E.coli* strains) and SEQ ID No: 24 [GenBank: WP_003861293.1] (for example in *Corynebacterium glutamicum* strains);

f) Polynucleotide (*aat* gene) coding for an aspartate aminotransferase (aspC or Aat, EC No.: 2.6.1.1); for example wherein the encoded amino acid sequence has at least 80, 85, 90, 95 or 100% sequence identity to SEQ ID No:26 [GenBank: BAA35674.1] (for example in *E.coli* strains) and SEQ ID No:28 [GenBank: WP_003862086.1] (for example in *Corynebacterium glutamicum* strains);

g) Polynucleotide (*dapB* gene) coding for a dihydrodipicolinate reductase (DapB, EC No.: 1.3.1.26 or 1.17.1.8), for example wherein the encoded amino acid sequence has at least 80, 85, 90, 95 or 100% sequence identity to SEQ ID No:30 [GenBank: BAB96600.1];

h) Polynucleotide (*dapC* gene) coding for a succinyldiaminopimelate aminotransferase, AT class I (DapC, EC No.: 2.6.1.17); for example wherein the encoded amino acid sequence has at least 80, 85, 90, 95 or 100% sequence identity to SEQ ID No:32 [GenBank: BAE77931.1] (for example in *E.coli* strains) and SEQ ID No:32 [GenBank: WP_040967251.1] (for example in *Corynebacterium glutamicum* strains);

i) Polynucleotide (*dapD* gene) coding for a tetrahydrodipicolinate succinylase (DapD, EC NO.: 2.3.1.117); for example wherein the encoded amino acid sequence has at least 80, 85, 90, 95 or 100% sequence identity to SEQ ID No: 36 [GenBank: BAB96742.1];

j) Polynucleotide (*dapE* gene) coding for a succinyl-diaminopimelate desuccinylase (DapE, EC NO.: 3.5.1.18); for example wherein the encoded amino acid sequence has at least 80, 85, 90, 95 or 100% sequence identity to SEQ ID No: 38 [GenBank: BAA16346.1] (for example in *E.coli* strains) and SEQ ID No: 40 [GenBank: WP_038583476.1] (for example in *Corynebacterium glutamicum* strains);

k) Polynucleotide (*dapF* gene) coding for a diaminopimelate epimerase (DapF, EC NO.: 5.1.1.7); for example wherein the encoded amino acid sequence has at least 80, 85, 90, 95 or 100% sequence identity to SEQ ID No: 42 [GenBank: BAE77491.1] (for example in *E.coli* strains) SEQ ID No: 44 [GenBank: WP_038584409.1] (for example in *Corynebacterium glutamicum* strains);

with particular preference being given to the genes *dapA, asd, ddh, lysA, dapB* and *lysC* and very particular preference being given to the genes *dapA* and *lysC*, since L-lysine production may be enhanced by using the two latter genes in particular.

Alternatively, enhanced synthesis of lysine in the micro-organism of the invention may be achieved by introducing a transgene encoding a feedback-resistant aspartate kinase (LysC) which, by comparison with the wild form (wild type), shows less sensitivity to inhibition by mixtures of lysine and threonine or mixtures of AEC (aminoethylcysteine) and threonine or lysine alone or AEC alone. The genes or alleles coding for aspartate kinases that are desensitized by comparison to the wild type allele are known as lysCFBR alleles. Numerous lysCFBR alleles coding for aspartate kinase variants that have amino acid substitutions by comparison with the wild-type protein are conventionally known (Kikuchi Y1, et al., 1999).

The genetically modified micro-organism according to the invention, for production and export of lysine, may be a bacterium. A non-exhaustive list of suitable bacteria is given as follows: a species belonging to a genus selected from the group consisting of *Corynebacterium, Escherichia, Bacillus, Lactobacillus, Lactococcus, Acetobacter, Acinetobacter, Pseudomonas, Streptococcaceae, and Brevibacteriaceae;* especially preferred group members being *Corynebacterium, Escherichia* and *Brevibacteriaceae*. Preferably the bacterium is a Generally Recognized As Safe (GRAS) strain.

Alternatively, the genetically modified micro-organism according to the invention, for production and export of lysine, may be a fungus, and more specifically a filamentous fungus belonging to the genus of *Aspergillus*, e.g. *A. niger, A. awamori, A. oryzae, A. nidulans*; a yeast belonging to the genus of *Saccharomyces*, e.g. *S. cerevisiae, S. kluyveri, S. bayanus, S. exiguus, S. sevazzi, S. uvarum*; a yeast

belonging to the genus *Kluyveromyces*, e.g. *K. lactis K. marxianus var. marxianus, K. thermotolerans*; a yeast belonging to the genus *Candida*, e.g. *C. utilis C. tropicalis, C. albicans, C. lipolytica, C. versatilis*; a yeast belonging to the genus *Pichia*, e.g. *P. stipidis, P. pastoris, P. sorbitophila*, or other yeast genera, e.g. *Cryptococcus, Debaromyces, Hansenula, Pichia, Yarrowia, Zygosaccharomyces* or *Schizosaccharomyces*. Concerning other micro-organisms a non-exhaustive list of suitable filamentous fungi is supplied: a species belonging to the genus *Penicillium, Rhizopus, Fusarium, Fusidium, Gibberella, Mucor, Mortierella*, and *Trichoderma*.

The preferred micro-organisms of the invention may be *Escherichia coli, Corynebacterium glutamicum, Brevibacterium flavum, Brevibacterium lactofermentum, Lactococcus lactis, Lactococcus plantarum, Bacillus subtilis, Aspergillus oryzae* and *Saccharomyces cerevisiae.*

III Methods for producing lysine using the genetically modified micro-organism of the invention

Lysine can be produced and exported using microbial cells of the invention (e.g. recombinant bacterial cells) by introducing the cells into a culture medium comprising a carbon source for biosynthesis of lysine or its precursors (see Figure 1); and finally recovering the lysine produced by the culture, as illustrated in the Examples.

The microbial cells of the invention will produce lysine when supplied with a suitable carbon source including glucose, maltose, galactose, fructose, sucrose, arabinose, xylose, raffinose, mannose, and lactose.

When the microorganism of the invention is an aerobic bacterial strain, the cells are grown under aerobic conditions; while the selection of a suitable temperature for growth of bacterial strain belonging to a given Genus lies within the competence of the skilled man.

IV Methods for producing a micro-organism of the invention

Integration and self-replicating vectors suitable for cloning and introducing a gene encoding a lysine transporter of the invention in a micro-organism of the invention are commercially available and known to those skilled in the art (see, e.g.,

Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989). Cells of a micro-organism are genetically engineered by the introduction into the cells of heterologous DNA. Heterologous expression of genes encoding a lysine transporter in a micro-organism of the
5 invention is demonstrated in Example 2, 5 and 6.

A nucleic acid molecule, that encodes a lysine transporter according to the invention, can be introduced into a cell or cells and integrated into the host cell genome using methods and techniques that are standard in the art. For example, nucleic acid molecules can be introduced by standard protocols such as
10 transformation including chemical transformation and electroporation, transduction, particle bombardment, etc. Expressing the nucleic acid molecule encoding the enzymes of the claimed invention also may be accomplished by integrating the nucleic acid molecule into the genome.

Methods for obtaining a micro-organism of the invention adapted for lysine
15 production can be obtained by genetic modifications that reduce the depletion of the intracellular lysine pool and/or enhance the synthesis of lysine, as described in Section II. For example the metabolic consumption of lysine in the microbial cell of the invention may be reduced by blocking the conversion of lysine to cadaverine by lysine decarboxylase (EC 4.1.1.18). The genome of the microbial cell is deleted for
20 one or more genes encoding a polypeptide having lysine decarboxylase activity [SEQ ID No: 4 and/or 6] such as the *ldcC* and *cadA* genes as described in example 4.

Methods for enhancing the synthesis of lysine in the micro-organism of the invention may be carried out by introducing one or more genes into the micro-
25 organism, whose upregulated expression will enhance lysine production. Suitable genes may be selected from the group listed/named in section II.

V Use of the lysine transporter in a genetically modified micro-organism of the invention for production of a lysine

The invention further encompasses the use of a transgene encoding a lysine
30 transporter to enhance the extracellular production of lysine in a microbial cell. The transgene encoding a lysine transporter, encodes an amino acid sequence having at least 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence

identity to SEQ ID No: 2 that is capable of exporting lysine from a microbial cell. In one embodiment, microbial cell of the invention comprises a transgene encoding a lysine transporter protein, wherein the encoded amino acid sequence has at least 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence
5 identity to SEQ ID No: 2 or 65, but with the proviso that amino acid residues at the indicated positions are V^{240} and F^{278}.

VI A method of detecting lysine production

Methods for detecting and quantifying extracellular and intracellular lysine produced
10 by a micro-organism of the invention include LC-MS Fusion (Thermo Fisher Scientific, USA) with positive electrospray ionization (ESI+). One suitable method for quantitating lysine is detailed in Example 5.2.

Examples

15 **Example 1 Metagenomic Library Construction and Functional Screening for L-lysine**

The microbial population of cow fecal samples comprises a wide range of bacteria, including both bacteria that are auxotrophic and bacteria that are prototrophic for lysine. Common for many bacterial cells is a limited capacity to catabolize lysine
20 and lysine dipeptides, while the accumulation of inhibitory levels of lysine is avoided by the export of lysine by means of transporters. Accordingly, metagenomic libraries constructed from DNA extracted from fecal samples provides a potentially rich source of novel lysine transporters.

The growth of *E.coli* strain C4860 (DSM 13127), is inhibited by elevated levels of
25 lysine in the growth medium, with a determined IC90 of 8.28 g/L extracellular L-lysine after 24 h culture. Accordingly *E.coli* strain C4860 was used as a host cell to screen for plasmids with a metagenomic DNA fragment encoding a lysine transporter, whereby only those host cells transformed with a plasmid expressing a lysine transporter (encoded by metagenomic DNA) will survive and grow when
30 cultivated on a medium comprising 12 or 14 g/L lysine.

1.1 Metagenomic library construction:

A metagenomic expression library of total DNA extracted from a cow fecal sample was constructed by the steps of: i) isolation of total DNA from 5 g of fecal material using the PowerMax Soil DNA Isolation Kit (Mobio Laboratories Inc.), ii) fragmentation of extracted DNA into pieces of an average size of 2 kb by sonication using a Covaris E210 (Massachusetts, USA) followed by end-repairing using the End-It end repair kit (Epicentre) and iii) blunt-end ligation into pZE21 MCS1 expression vector (Lutz and Bujard, 1997) at the HincII site using the Fast Link ligation kit (Epicentre), and iv) transformation of the ligated sample into *E. coli* top10 cells by electroporation.

After electroporation, cells were recovered in 1ml of SOC medium for 1 h at 37 °C and the library was titered by plating out 1 µl, 0.1 µl and 0.01 µl of recovered cells onto LB-km plates. The insert size distribution was estimated by gel electrophoresis of PCR products obtained by amplifying insert using primers site annealing to the vector backbone, flanking the HincII site. The average insert size for the library was found to be 1.7 kb. The total size of the metagenomics library was determined by multiplying the average insert size with the number of colony forming units (CFU) per ml, which is $5x10^8$ bp.

1.2 Functional screening for L-lysine transporters in *E. coli* C4860

Plasmids comprising the metagenomics library (see 1.1) were isolated; and 400 ng of the plasmid DNA were transformed into electro-competent cells of *E.coli* C4860 strain. On the basis of the determined library sizes and titer of the library, 10^6 cells (i.e., 100 µl of the library cells) were plated out on LB-km agar supplemented with L-lysine at the selective concentration (12 and 14 g/L). Plates were incubated at 37 °C and growth of colonies (potential lysine-tolerant clones) was assayed after 48-65 h of incubation. Colonies appeared only on the plates with the metagenomic library but not on the control plates with *E.coli* C4860 harboring the vector, pZE21. Eighty L-lysine tolerant clones were selected for further analysis. The metagenomic inserts present in the L-lysine-tolerant clones were Sanger sequenced using pZE21_F and pZE21_R primer pairs (Table 3) annealing to the vector backbone. Annotation of the sequenced inserts indicated that lysine tolerance in the transformed strains was conferred by either degradation/modification of L-lysine or active L-lysine export. Six unique metagenomic inserts encoding putative lysine transporters. Four inserts were selected for further testing based on the absence of

putative degradation enzymes flanking the putative transporters on the metagenomic insert.

1.3 Functional comparison of putative lysine transporters

5 The transport properties of the four putative lysine transporters were compared by measuring the concentration of lysine needed to inhibit the growth by 90% [IC90] of *E.coli* C4860 clones expressing each of the putative lysine transporters.

The L-lysine IC90s of the selected *E. coli* C4860 clones ranged from 10.44 ± 1.277
10 g/L to 14.25 ± 0.415 g/L; corresponding to a 43% increase in IC90 for the clone harboring pZE-RCL-MglE (nucleotide sequence of DNA insert in pZE-RCL-MglE: SEQ ID No.: 1) compared to the control cells of the *E. coli* C4860 comprising the vector pZE21 (Figure 2A).

15 The growth of each selected *E. coli* C4860 clones in LB-media was compared and found to be similar, whereas in the presence of 8 g/L of L-lysine, the growth rate of the clone harboring pZE-RCL-MglE (0.31 h^{-1}) was ~30 % higher than the control strain comprising the vector pZE21 (0.24 h^{-1}) (Figure 2B).

20 **Example 2 Lysine transporter MglE confers enhanced lysine tolerance on a range of *E. coli* strains**

The pZE-RCL-MglE plasmid encoding the MglE transporter [SEQ ID No.: 2] was introduced into the following *E. coli* production strains; i) MG1655, ii) Crooks, iii) W1116 and iv) W3110. The lysine tolerance (measured as IC90) of each *E.coli*
25 strains was increased when harboring and expressing the pZE-RCL-MglE plasmid (Figure 3). The L-lysine tolerance of strain *E. coli* W3110, which is widely used for the commercial production of L-lysine, was increased by 29.5%. Hence the lysine tolerance conferred by this transporter is widely applicable to industrial *E. coli* based L-lysine fermentation.

30

Example 3 Structural properties of the Lysine transporter MglE

Sequence analysis of the metagenomics insert in pZE-RCL-MglE [SEQ ID No.: 1] showed an operon with an open reading frame (coined as mglE, **m**etagenomics **g**ene for **l**ysine **e**xport) flanked by a promoter and a terminator sequences (figure
35 4). The deduced amino acid sequence of MglE transporter is 297 amino acids [SEQ

ID No: 2], and showed the closest amino acid sequence identity (82%) to a hypothetical protein from *Bacteroides coprophilus* (Genbank accession no. WP_022277040) [SEQ ID No: 45].

5 The MglE protein is predicted to be a member of a new family of RhaT/EamA-like transporters related to the drug/metabolite transporter (DMT) superfamily. EamA family members are very diverse and most of their functions are unknown, although PecM from *Erwinia chrysanthemi* and YdeD in *E. coli*, are characterized as exporters (Franke et al., 2003). MglE also contains two copies of the EamA domain 10 found in members of this superfamily: one in the region of 9-143 amino acids and another in 152-292 amino acids. Two-dimensional topological model of MglE transporter possesses six cytoplasmic domains, five periplasmic domains and ten transmembrane domains with both N- and C-terminals in the cytoplasmic region (Figure 5).

15

Phylogenetic analysis of selected bacterial transporter protein sequences shows that the MglE protein shows homology to some hypothetical proteins of *Bacteroides* species (Figure 6) sharing an amino acid sequence identity of ≥50%; the majority of this group sharing an amino acid sequence identity of ≥74%. In contrast, the 20 known L-lysine specific transporters from various strains formed a group that is phylogenetically very remote from MglE. MglE does not have any significant homology with the LysE lysine exporter from *C. glutamicum*. Accordingly, the MglE protein is a novel L-lysine transporter, whose function could not have been predicted by sequence homology to known L-lysine transporters.

25

Example 4 Lysine transporter, MglE confers enhanced lysine tolerance in an *E. coli* strain adapted for lysine production

E. coli comprises genes encoding lysine decarboxylases that degrade intracellular lysine. The *E. coli* strain W3110 was genetically modified to knock-out the 30 constitutive gene (*ldcC*) and the acid-inducible gene (*cadA*) encoding two lysine decarboxylases to produce the strain *E.coli* DMLC, having a reduced lysine degradation capacity. Cells of this knock-out strain were used as a host to compare the effect of the lysine transporter MglE on lysine tolerance with two known lysine transporters, the L-Lysine efflux permease (LysE) from *Corynebacterium*

glutamicum [SEQ ID No.: 47] and the lysine exporter (YbjE; LysO protein) from *E. coli* [SEQ ID No.: 49].

Disruption of the *ldcC* and *cadA* genes was carried out using the ampicillin resistant pSJI8 helper plasmid containing genes for λ Red recombinase (γ, β, exo) enhancing recombination rate under control of arabinose promoter; and the FLP recombinase to eliminate the resistance cassette under the control of rhamnose promoter.

Table 1: Primers and genes for generating a ΔldcC.ΔcadA bacterial strain

Primer/gene	Oligonucleotides (5'-3')/genes	SEQ ID No.*
LdcC_F1	TCAGCGCCTGATGAGCTACG	50
LdcC_F2	AGTTCTGAAAAAGGGTCACTTC	51
LdcC_R	TCGCAATATGGTGAACCTGTT	52
ldcC gene	Encoding GenBank Acc: BAA77861.2	3
CadA_F1	TGAAGTACTCCCAGATTTGGATC	53
CadA_F2	CGGCTGTGAGGGTGTTTTCA	54
CadA_R	TTAATTAAAAGTATTTTCCGAGGCTCC	55
cadA gene	Encoding GenBank Acc: BAE78134.1	5

* Nucleotide sequence

4.1 *ldcC* disruption: Chemically competent cells of *E. coli* W3110 strain were transformed with the pSJI8 plasmid and the transformed cells were selected in LB-amp solid media and incubated at 30 °C. The *E. coli* W3110/pSJI8 transformants were grown in LB-amp at 30°C to an OD_{600nm} of 0.3 and 1 mM arabinose was added to induce the λ Red system.

A PCR product was generated by using primer pair LdcC_F2/LdcC_R (Table 1; positioned 64 bp from the *ldcC* gene start/stop codon) to amplify a kanamycin cassette from the genomic DNA of *ldcC* in-frame knocked-out Kieo strain b0186.

Electro-competent cells were prepared from the induced *E. coli* W3110/pSJI8 transformants; which were then transformed with about 200 ng of the purified PCR product. The transformed cells were plated in LB-km, amp solid media. The replacement of the *ldcC* gene (2.1 kb) by the kanamycin (km) cassette was confirmed by colony PCR using a primer pair LdcC_F1/LdcC_R that gave a 1.6 kb DNA band corresponding to the km cassette. The colony, confirmed to have the *ldcC* gene replaced by the km cassette, was grown in 1 ml of LB-km-amp at 30 °C

for 3-4 h. The cells were collected by centrifugation and re-suspended in 100 µl of sterilized water and 10 µl of the re-suspended cells were inoculated into 1 ml of LB-amp and 50 mM of rhamnose, followed by incubation at 30 ºC for 4-6 h. The cells were then spread onto LB-amp plates and incubated at 30 °C for overnight. To confirm the removal of the km cassette, colony PCR was performed using a primer pair LdcC_F1/LdcC_R which gave band at ~0.25 kb instead of 1.6 kb, confirming the successful resistant markerless *ldcC* deletion. The strain was named as *E.coli* W3110::ΔldcC/pSJI8.

4.2 *cadA* disruption: Similarly, primer pair CadA_F2/CadA_R (Table 1; located 56 bp from the *cadA* gene start/stop codon) was used to amplify the kanamycin cassette from the genomic DNA of a *cadA* inframe knocked-out Kieo strain b4131. The purified PCR product was transformed into *E. coli* W3110::ΔldcC/pSJI8 and a similar protocol was followed for the confirmation of the *cadA* deletion. To confirm the replacement of *cadA* by the km cassette, as well as the removal of the km cassette in the double deletion mutant, a primer pair CadA_F1/CadA_R was used for colony PCR.

The helper plasmid pSJI8 was removed from the double deletion mutant, by growing the *E. coli* W3110::ΔldcC.ΔcadA/pSJI8 strain on LB plates incubated at 37 °C overnight and then streaking colonies on LB and LB-amp plates. Cells of *E. coli* W3110::ΔldcC.ΔcadA that had lost pSJI8 grew on LB but not on LB-amp media. The double (*ldcC* and *cadA*) deletion strain was designated *E.coli* DMLC.

4.3 Construction of pZE-LysE and pZE-YbjE plasmids: Oligonucleotides LysE-F and LysE-R (Table 2) were synthesized to amplify nucleotide sequence of *lysE* gene (Genbank accession no. AGT05251) from genomic DNA of *C. glutamicum*. Similarly oligonucleotides YbjE-F and YbjE-R were used to amplify the *ybjE* gene (Genbank accession no. CAQ31402) from *E. coli* BL21 (DE3). The *lysE* and *ybjE* PCR products were cloned into the pZE21 vector excised with KpnI and BamHI restriction enzymes to construct pZE-LysE and pZE-YbjE expression plasmids, respectively.

Table 2 Primers and genes for constructing *lysE* and *ybjE* plasmids

Primer/gene	Oligonucleotides (5’-3’)/ genes	SEQ ID No*
LysE-F	ATGGGTACCATGGAAATCTTCATTACAGGTC	56
LysE-R	CCGGGATCCCTAACCCATCAACATCAGTTTG	57

lysE gene	Encoding GenBank Acc: AGT05251	46
YbjE-F	AAAGGTACCATGTTTTCTGGGCTGTTAATCA	58
YbjE-R	TATGGATCCTTACGCAGAGAAAAAGGCGAT	59
ybjE gene	Encoding GenBank Acc: CAQ31402	48

* Nucleotide sequence

4.4 *E.coli* DMLC expressing the lysine transporter MglE: Cells of the Δ*ldcC*.Δ*cadA* strain *E.coli* DMLC were then transformed with either the pZE-RCL-MglE, pZE-LysE, pZE-YbjE plasmids or the control vector pZE21.

The lysine tolerance (measured as IC90) of the Δ*ldcC*.Δ*cadA* strain *E.coli* DMLC was enhanced by expressing of each of the lysine transporters; however, cells harboring and expressing the pZE-RCL-MglE plasmid showed the highest lysine tolerance (Figure 8).

In conclusion, the lysine exporter MglE confers a higher lysine tolerance in the *E. coli* DMLC strain adapted for lysine production than the known lysine transporters *lysE* and *ybjE*.

Example 5 Lysine productivity of recombinant *E. coli* cells adapted for lysine production is enhanced by the lysine transporter MglE

Cells of the recombinant Δ*ldcC*.Δ*cadA* strain *E.coli* DMLC, adapted for lysine production, were also used to demonstrate the effect of lysine transporter MglE on lysine production and export.

5.1 L-lysine production: Recombinant *E.coli* DMLC were transformed with either the pZE-RCL-MglE plasmid (*E.coli* DMLC/pZE-RCL-MglE) or the control vector pZE21 (*E. coli* DMLC/pZE21) and their lysine production was analyzed and compared to the control strain *E.coli* W3110/pZE21. Cells of each strain were grown for 24 h in LB media; and the lysine levels were quantitated as described below (5.1). Both extracellular L-lysine levels and specific L-lysine titers were enhanced in the *E.coli* DMLC strains confirming that knock-out of the lysine degradation genes promotes lysine accumulation. Additionally, the level of extracellular L-lysine produced by the DMLC strain harboring the pZE-RCL-MglE plasmid was increased 8.3% as compared to the DMLC/pZE21 vector control strain (Figure 9A); while the specific L-lysine titer (Figure 9B) were increased 2.1 fold. In conclusion, MglE functions as a lysine exporter in *E. coli*, and can enhance L-lysine excretion from bacteria adapted for lysine production (e.g. *E.coli* DMLC/pZE-RCL-MglE).

5.2 L-lysine quantitation: A calibration curve of authentic L-lysine standard was drawn using concentrations ranging from 0.4 to 77 mg/L. The accurate mass of L-lysine from 20-fold diluted samples (supernatants containing L-lysine to be
5 quantified) was analyzed using an LC-MS Fusion (Thermo Fisher Scientific, USA) with positive electrospray ionization (ESI+). The final concentration was adjusted for the dilution factor. Bracketing calibration was used for quantifying the external lysine concentration. The quantification of L-lysine was determined with an LC-MS/MS, EVOQ (Bruker, Fremont USA), using multiple reaction monitoring (MRM)
10 transition in positive ionisation mode (ESI+), with quantified transition 147→84 (CE 10), and qualifier transition 147→130 (CE 7).

Example 6 Mutant lysine transporter MglE confers increased lysine tolerance on *E. coli* strains

15 Random mutagenesis was used to generate a library of mutated genes encoding mutant MglE lysine transporters in order to isolate a more efficient MglE lysine transporter.

6.1 Random mutagenesis: Following primer pairs were used for amplification the *mglE* insert and the pZE21 vector backbone, respectively.

20

Table 3 Primers for amplification of mglE gene and pZE21 backbone

Primer names	Oligonucleotides (5'-3')	SEQ ID No
pZE21_F	GAATTCATTAAAGAGGAGAAAGGT	60
pZE21_R	TTTCGTTTTATTTGATGCCTCTAG	61
pZE21_EP-Gib.F	CTAGAGGCATCAAATAAAACGAAA	62
pZE21_EP-Gib.R	ACCTTTCTCCTCTTTAATGAATTC	63

The primer pair pZE21_F/pZE21_R (Table 3) was used for amplification of *mglE* gene from pZE-MglE plasmid DNA while the primer pair
25 pZE21_EP_Gib.F/pZE21_EP_Gib.R (Table 3) was used to amplify the vector backbone using the pZE21 vector as a template.

Error prone PCR of *mglE* gene was carried out using the GeneMorph II Random Mutagenesis Kit (Agilent) according to the protocol (using 20 ng template DNA in 50 μl reaction volume) to create random mutants of the mglE gene. Then the error
30 prone PCR product of *mglE* (*mglE**) was gel purified and ligated into pZE21 vector backbone using the Gibson assembly composition:

i. PCR product of *mglE** = 76 ng,
ii. PCR product of vector backbone = 48 ng
iii. Gibson assembly master mix= 5 µl.
iv. MQ-Water= adjusted to final volume of 10 µl

5 The ligation mixture was briefly centrifuged and the reaction was carried out in a thermocycler held constant at 50 °C for 1 h. The ligation mixture was then desalted for an hour using a Millipore (type VSWP) drop dialysis film with 0.025 µm on Milli-Q water (Desalting DNA Drop dialysis method). The purified ligation product (pZE-MglE* library) was transformed into electro-competent cells of *E. coli* C4860 strain
10 to give a library of 75-80,000 CFU/ml.

6.2 Screening lysine transport mutants: The library was screened by plating 100 µl (10^7 cells) of the amplified random mutant library (*E. coli* C4860/pZE-MglE*) and the control parent strain *E. coli* C4860/pZE-MglE on LB-km agar plates supplemented with different concentrations of L-lysine. Then the plates were
15 incubated at 37 °C for 48 to 68 h. Colonies having the highest L-lysine tolerance were sequenced using the primer pair pZE21_F/pZE21_R to characterize the relevant mutation (s). In parallel, the L-lysine MICs of the selected clones were determined.

Eight mutants MglE lysine transporters, each conferring higher levels of lysine
20 tolerance that the parent MglE lysine transporter were identified on the basis of the MIC of cells expressing the mutant transporters and a corresponding mutation in the amino acid sequence of the expressed transporter. The mutant conferring the highest L-lysine tolerance in the *E. coli* C4860 was MglE1* [SEQ ID No.: 65]; which was characterized by 2 amino acid mutations as indicated in Table 4.

25

Table 4 Mutations in mutant MglE lysine transporter

Amino acid position										**n**	**MglE mutant**	**IC90 at 24h**	**STDEV**
64	**146**	**197**	**200**	**240**	**249**	**265**	**278**	**281**	**296**				
S	G	V	G	**A**	A	T	**L**	C	R	4	Wildtype	9.79	0.24
S	G	V	G	**V**	A	T	**F**	C	R	23	MglE1*	15.83	0.24
S	G	V	**E**	**V**	A	T	**F**	C	**K**	1	MglE2*	15.79	
S	G	V	G	A	A	T	L	**Y**	**K**	8	MglE3*	13.27	0.46

S	G	V	G	A	**V**	T	L	C	**K**	1	MglE4*	13.31	
S	G	V	G	A	**V**	T	L	C	R	8	MglE5*	12.63	0.70
S	**D**	V	**V**	A	A	T	L	C	R	2	MglE6*	10.98	0.54
S	G	**L**	G	A	A	T	L	C	R	1	MglE7*	11.25	
G	G	V	G	A	A	**S**	L	C	R	1	MglE8*	10.41	

In conclusion, the lysine tolerance of *E. coli* C4860 can be further enhanced by 61% (IC90: 9.79/15.83 or 15.83) by the expression of the mutant lysine transporters MglE1* and MglE2*as compared to the parent lysine transporter, MglE; while *E. coli* C4860 cells expressing variant MglE lysine transporters having a number of different amino acid substitutions either retain lysine tolerance or also exhibit enhanced lysine tolerance as compared to the parent lysine transporter.

Example 7 Comparison of Lysine tolerance conferred by expression of the MglE gene and homologous genes encoding hypothetical proteins

Recombinant plasmids were constructed for the expression of four homologous genes encoding polypeptides showing different levels of amino acid sequence identity to the lysine transporter, MglE. The four genes were: i) Gene1 (891 bp, Genbank accession no. CDC57518.1) [SEQ ID No: 66] from *Bacteroides coprophilus* CAG:333, ii) Gene2 (891 bp, Genbank accession no. WP_018711839) [SEQ ID No: 76] from *Bacteroides barnesiae*, iii) Gene3 (870 bp, Genbank accession no. CDC66277) [SEQ ID No: 78] from *Bacteroides* sp. CAG:770, and iv) Gene4 (912 bp, 43% identity, Genbank accession no. CCZ76555) [SEQ ID No: 98, encoding polypeptide with SEQ ID No.:99] from *Alistipes finegoldii* CAG:68, were synthetized along with 5´and 3′ linker sequences (Table 5) including uracil easing direct USER cloning. These four synthetized gene fragments were cloned into the linear PCR fragment of pZE21 vector amplified by using oligonucleotides pZE21_User-F4 and pZE21_User-R4 (Table 5) to construct pZE-Gene1, pZE-Gene2, pZE-Gene3 and pZE-Gene4 recombinant plasmids, respectively. The hypothetical proteins encoded by genes 1, 2, 3 and 4 showed an amino acid sequence identity of 82, 78, 54 and 43% respectively to the lysine transporter, MglE.

Table 5 Primers for amplification of mglE gene

Primers	Oligonucleotide sequences (5'-3')	Cloning strategy	SEQ ID No.:
pZE21_User_F4	ATAAGCT**U** GATATCGAATTCCTGCAGCCC	USER cloning	100
pZE21_User_R4	AATGAAT**U** CGGTCAGTGCGTCCTGCTGAT	USER cloning	101
5′ linker	AATTCAT**U** AAAGAGGAGAAAGGTACC	USER cloning	102
3′ linker	GTCGACGGTATCG **A**TAAGCTT (in the complementary sequence, T is replaced by U for pairing with **A**)	USER cloning	103

The lysine tolerance conferred by expression of the gene encoding the lysine transporter MglE, as compared to that of the four genes encoding the hypothetical proteins, was determined by introducing the four constructed plasmids, pZE-MglE and the pZE21 vector as control, into *E.coli* C4860 and *E.coli* DMLC strains. The transformed strains were then cultured under conditions for determining their IC90 for L-lysine as described in Example 6.2. Surprisingly, none of the four genes encoded proteins that conferred lysine tolerance at the same level as expression of the MglE gene in either *E.coli* C4860 and *E.coli* DMLC strains. The relative L-lysine tolerance conferred by introducing the recombinant plasmids containing the four genes in *E.coli* C4860 and *E.coli* DMLC is shown in figure 10.

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Claims

1. A genetically modified microbial cell for production of lysine, comprising a transgene encoding a lysine transporter polypeptide, wherein the amino acid sequence of the lysine transporter polypeptide has at least 85% sequence identity to SEQ ID No.: 2.

2. The genetically modified microbial cell of claim 1, wherein the amino acid sequence of the lysine transporter polypeptide has at least 85 % sequence identity to SEQ ID No: 2, but with the proviso that amino acid residue at position 240 is valine and amino acid residue at position 278 is phenylalanine.

3. The genetically modified microbial cell of claim 1 or 2, wherein the cell is further characterized by one or more genetic modification conferring:
   a. reduced lysine metabolism and/or
   b. enhanced lysine synthesis

   as compared to a parent cell from which said genetically modified cell was derived.

4. The genetically modified microbial cell of claim 3, wherein one or more genes encoding a polypeptide having lysine decarboxylase activity (EC 4.1.1.18) are deleted or inactivated to confer reduced lysine metabolism.

5. The genetically modified microbial cell of claim 3 or 4, wherein the cell comprises one additional transgene or a combination of two or more additional transgenes conferring enhanced lysine synthesis and wherein said transgenes encode polypeptides selected from the group consisting of:
   a. aspartate kinase (EC No.: 2.7.2.4);
   b. dihydrodipicolinate synthase (EC No.: 4.2.1.52 or 4.3.3.7);
   c. aspartate-semialdehyde dehydrogenase (EC No.: 1.2.1.11);
   d. meso-diaminopimelate dehydrogenase (EC No.: 1.4.1.16);
   e. diaminopimelate decarboxylase (EC No.: 4.1.1.20);
   f. aspartate aminotransferase (EC No.: 2.6.1.1);
   g. dihydrodipicolinate reductase (EC No.: 1.3.1.26 or 1.17.1.8);

h. succinyldiaminopimelate aminotransferase, AT class I (EC No.: 2.6.1.17);
i. tetrahydrodipicolinate succinylase (EC No.: 2.3.1.117);
j. succinyl-diaminopimelate desuccinylase (EC No.: 3.5.1.18); and
5 k. diaminopimelate epimerase (EC No.: 5.1.1.7).

6. The genetically modified microbial cell according to any one of claims 1 to 5, wherein said micro-organism is selected from among a bacterium, a yeast, and a filamentous fungus.

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7. The genetically modified microbial cell according to claim 6, wherein said micro-organism is a species of a genus selected from the group consisting of: *Corynebacterium, Escherichia, Brevibacteriaceae, Bacillus, Lactobacillus, Lactococcus, Acetobacter, Acinetobacter, Pseudomonas* and
15 *Streptococcaceae*.

8. An isolated polynucleotide encoding a lysine transporter polypeptide, wherein the amino acid sequence of the polypeptide has at least 85% sequence identity to SEQ ID No.: 2.

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9. The isolated polynucleotide of claim 8, wherein the amino acid sequence of the lysine transporter polypeptide has at least 85 % sequence identity to SEQ ID No: 2, but with the proviso that amino acid residue at position 240 is valine and amino acid residue at position 278 is phenylalanine.

25

10. A method for producing lysine in *vivo* comprising:
    a. providing a genetically modified microbial cell comprising a transgene encoding a lysine transporter polypeptide according to any one of claims 1 to 7;
30 b. introducing said microbial cell into a growth medium to produce a culture;
    c. providing a substrate for lysine production;
    d. recovering the produced lysine from the culture and optionally
    e. purifying the lysine recovered.

35

11. Use of a transgene encoding a lysine transporter polypeptide to enhance lysine export in a microorganism, wherein the amino acid sequence of said polypeptide has at least 85% sequence identity to SEQ ID No: 2.

12. Use of a transgene encoding a lysine transporter to enhance lysine export in a microorganism according to claim 11, wherein said microorganism is characterized by one or more additional genetic modification conferring:

    a. reduced lysine metabolism and/or

    b. enhanced lysine synthesis

    as compared to the parent cell from which said genetically modified cell was derived.

13. Use of a transgene encoding a lysine transporter to enhance lysine export in a microorganism according to claim 12, wherein one or more genes encoding a polypeptide having lysine decarboxylase activity (EC 4.1.1.18) are deleted or inactivated to confer reduced lysine metabolism.

14. Use of a transgene encoding a lysine transporter to enhance lysine export from a microorganism according to claim 12 or 13, wherein the microorganism comprises one additional transgene or a combination of two or more additional transgenes conferring enhanced lysine synthesis and wherein said transgenes encode polypeptides selected from the group consisting of:
    a. aspartate kinase (EC No.: 2.7.2.4);
    b. dihydrodipicolinate synthase (EC No.: 4.2.1.52 or 4.3.3.7);
    c. aspartate-semialdehyde dehydrogenase (EC No.: 1.2.1.11);
    d. meso-diaminopimelate dehydrogenase (EC No.: 1.4.1.16);
    e. diaminopimelate decarboxylase (EC No.: 4.1.1.20);
    f. aspartate aminotransferase (EC No.: 2.6.1.1);
    g. dihydrodipicolinate reductase (EC No.: 1.3.1.26 or 1.17.1.8);
    h. succinyldiaminopimelate aminotransferase, AT class I (EC No.: 2.6.1.17);
    i. tetrahydrodipicolinate succinylase (EC No.: 2.3.1.117);
    j. succinyl-diaminopimelate desuccinylase (EC No.: 3.5.1.18); and

k. diaminopimelate epimerase (EC No.: 5.1.1.7).

15. Use of a transgene encoding a lysine transporter to enhance lysine export from a microorganism according to any one of claims 11 to 14, wherein said micro-organism is a species of a genus selected from the group consisting of: *Corynebacterium, Escherichia, Brevibacteriaceae, Bacillus, Lactobacillus, Lactococcus, Acetobacter, Acinetobacter, Pseudomonas* and *Streptococcaceae*.

Figure 1

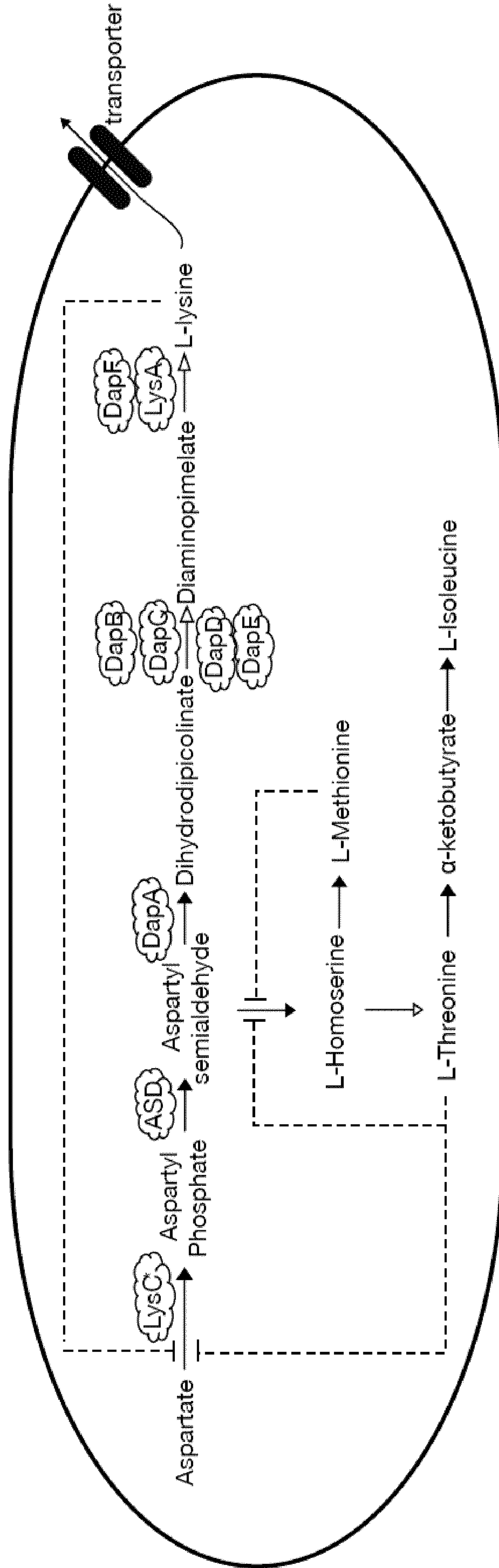

Figure 2A

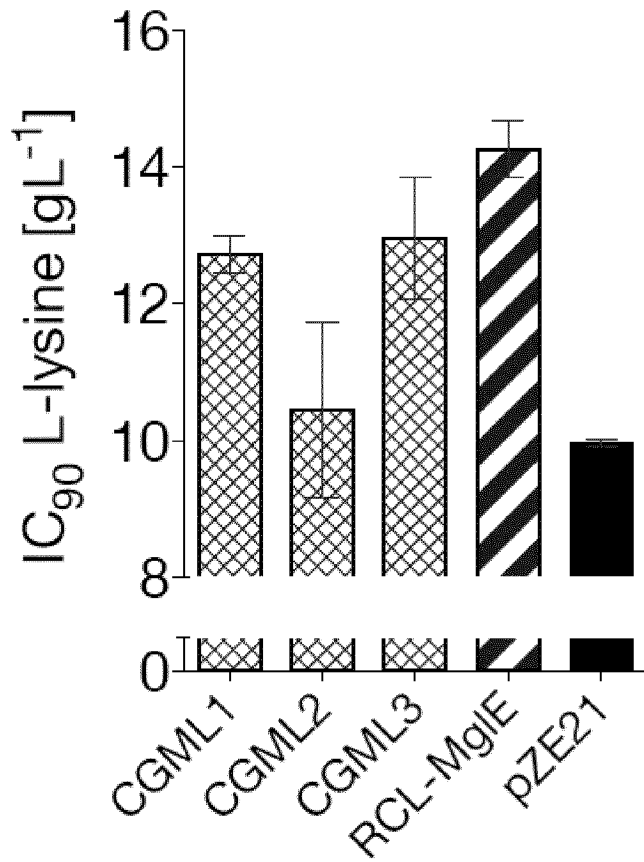

Figure 2B

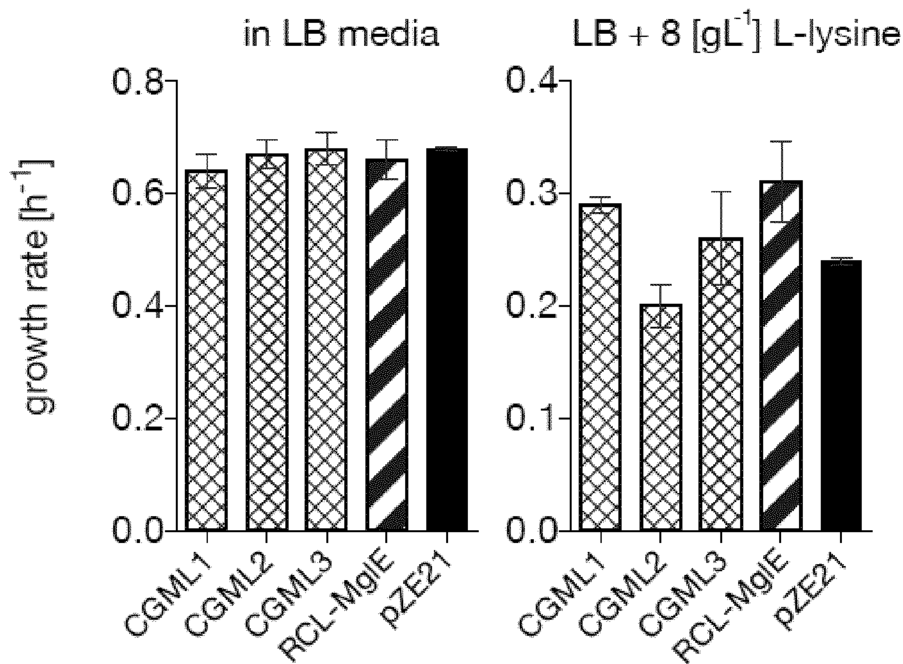

Figure 3

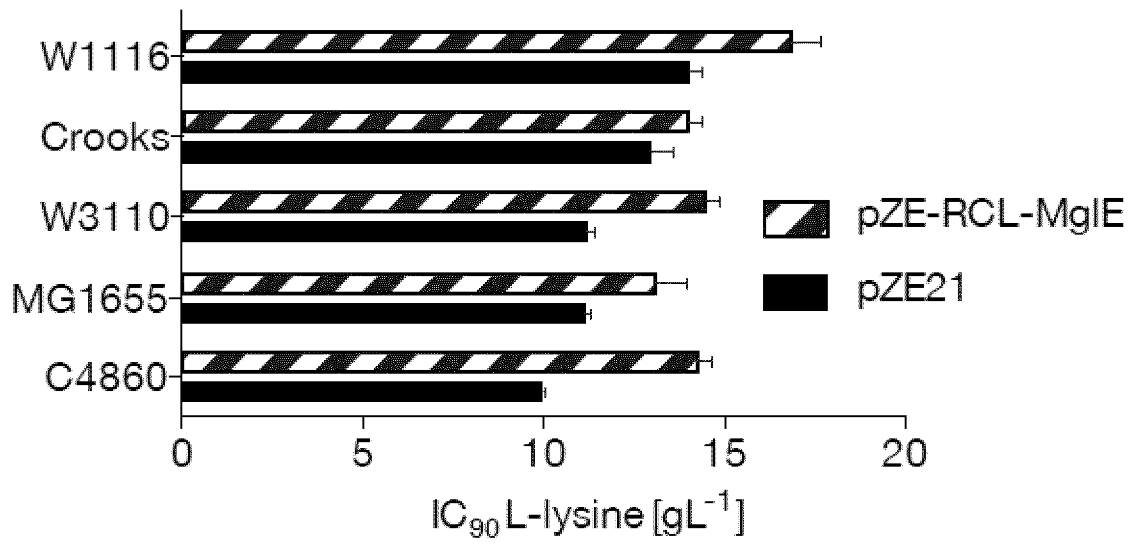

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Figure 4

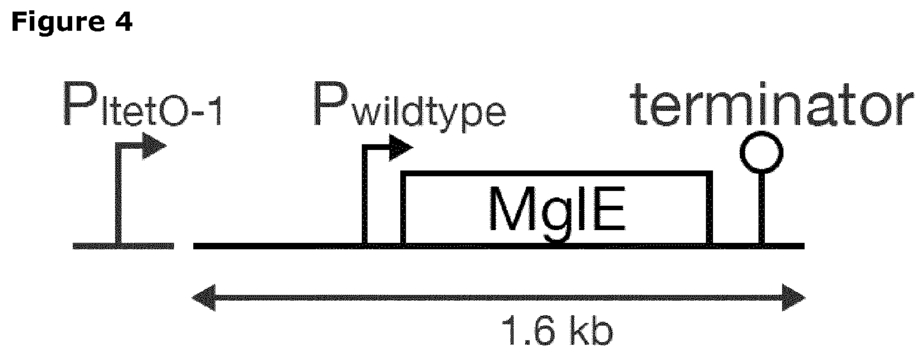

Figure 5

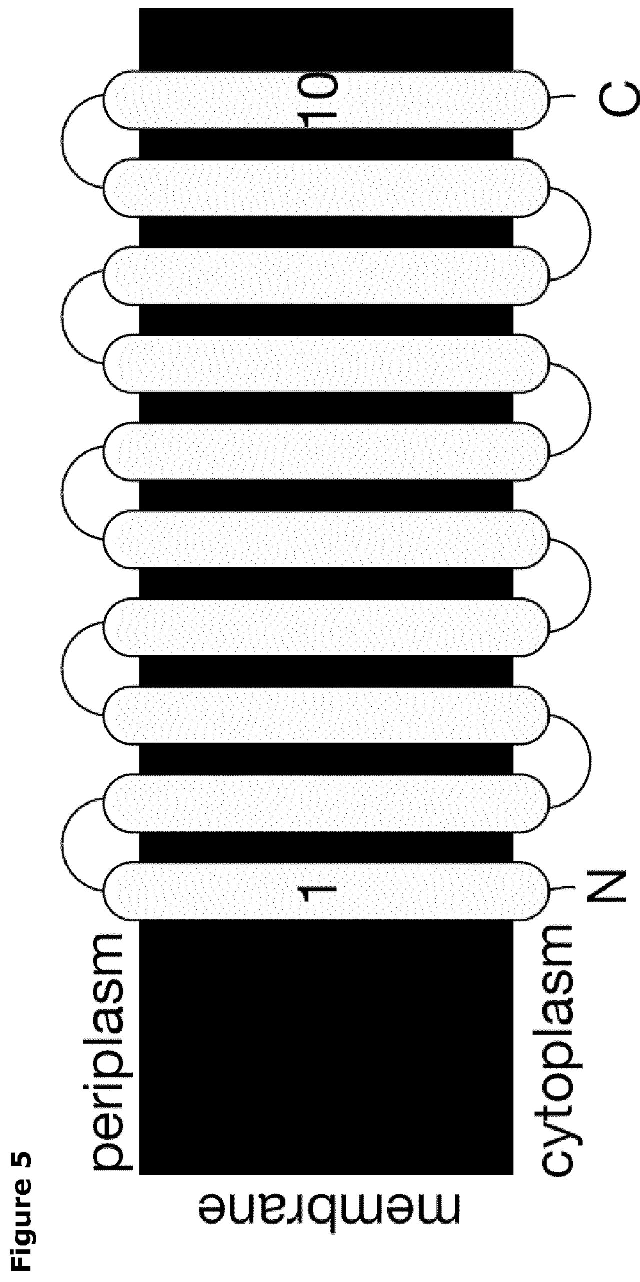

Figure 6

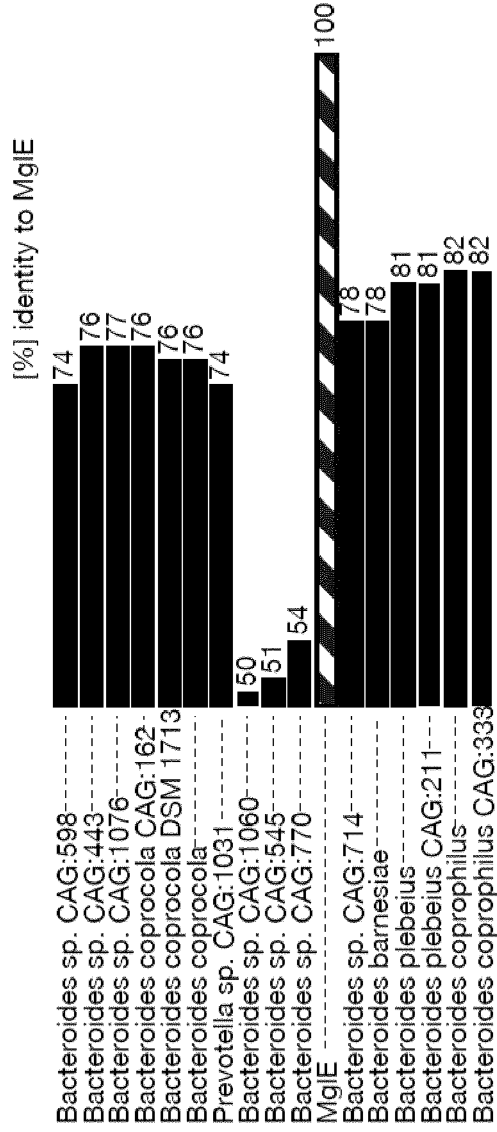

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Figure 7

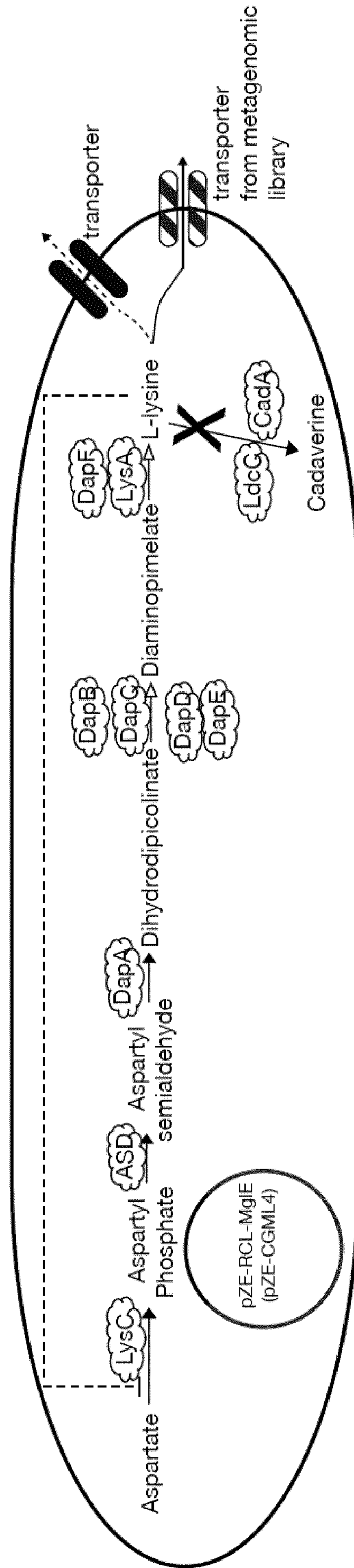

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Figure 8

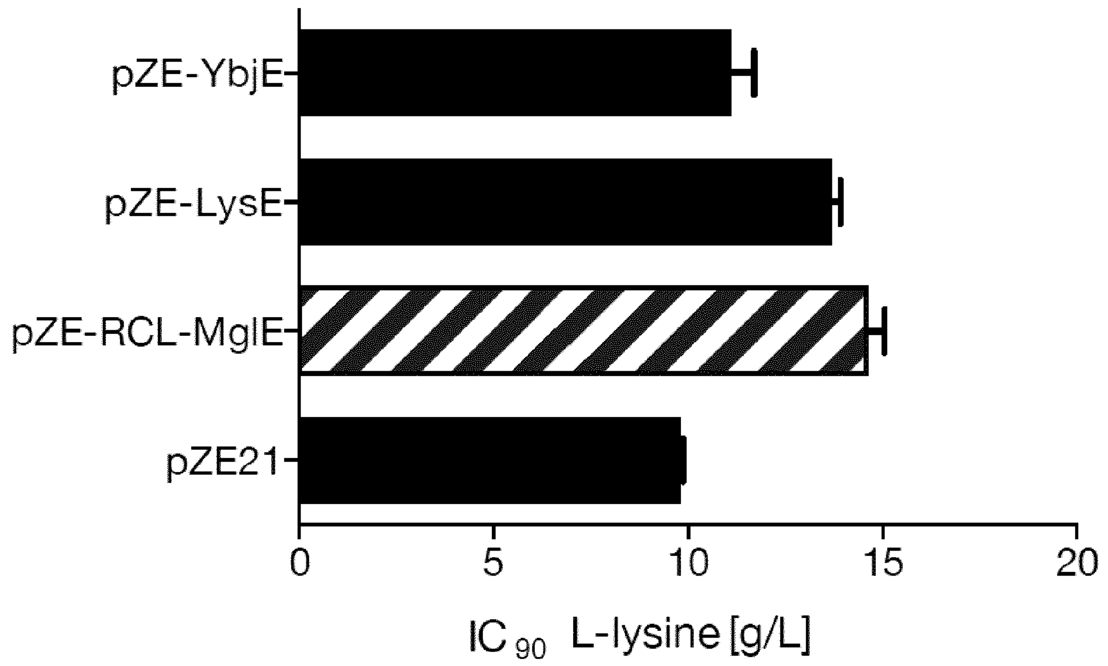

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Figure 9 A

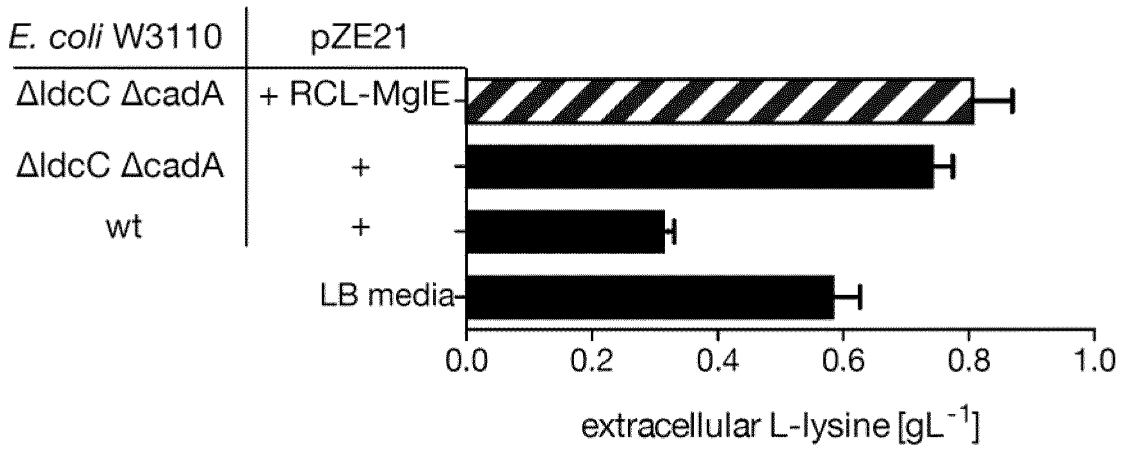

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Figure 9 B

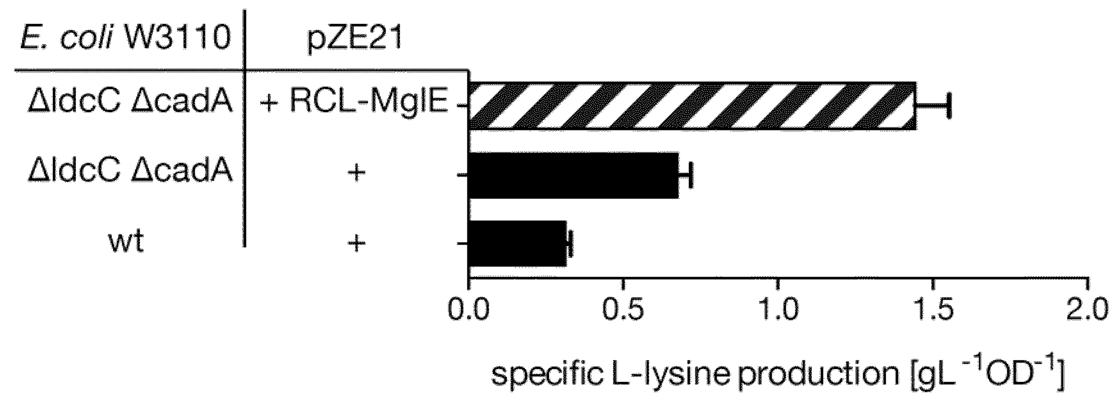

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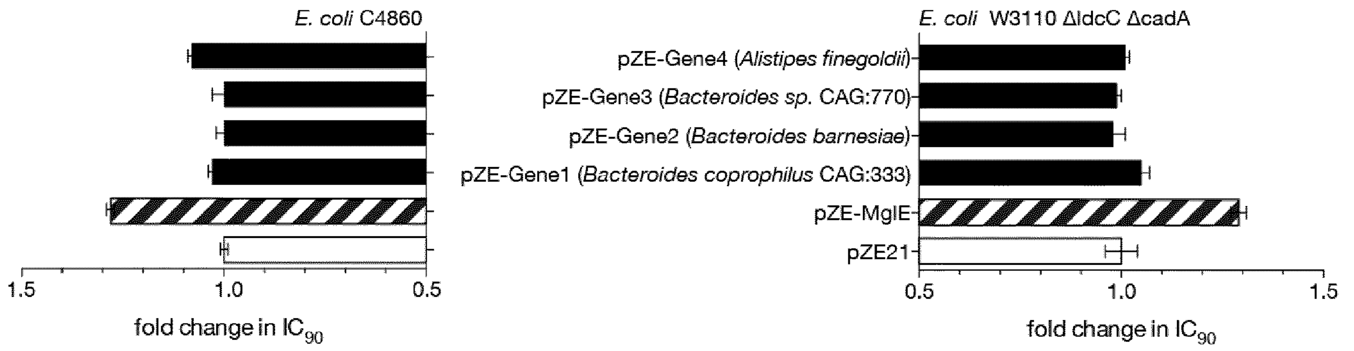
Figure 10



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/078110

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/195 C12N1/20 C12P13/08
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N C12P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, Sequence Search, EMBASE, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE UniProt [Online] 18 September 2013 (2013-09-18), "SubName: Full=Putative membrane protein {ECO:0000313¦EMBL:EEF75136.1};", XP002756696, retrieved from EBI accession no. UNIPROT:S0F9D6 Database accession no. S0F9D6 sequence ----- -/--	1,2,6-9

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

[X] See patent family annex.

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6 February 2017	15/02/2017

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE UniProt [Online] 24 July 2013 (2013-07-24), "SubName: Full=Uncharacterized protein {ECO:0000313¦EMBL:CDC57518.1};", XP002756695, retrieved from EBI accession no. UNIPROT:R6S8M7 Database accession no. R6S8M7 sequence -----	1,2,6-9
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

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