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(54) Title: MICROORGANISMS COMPRISING ENZYMES EXPRESSED WITH LOW GAMMA-ELIMINATION ACTIVITY

(57) Abstract: The present invention concerns a microorganism in which enzymes are expressed that have one or several of the following activities cystathionine--synthases and/or phosphohomoserine sulfhydrylase and/or acylhomoserine sulfhydrylases and that have at the same time low -elimination activity. The present invention also relates to recombinant enzymes that have one or several of the following activities cystathionine- γ -synthase and/or phosphohomoserine and/or acylhomoserine sulfhydrylase, have at the same time low γ -eliminase activity and are used for the fermentative production of amino acids, in particular methionine.



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Microorganisms comprising enzymes expressed with low gamma-elimination activity**Field of the invention**

The present invention concerns a microorganism in which enzymes are expressed
5 that have one or several of the following activities cystathionine- γ -synthases and/or
phosphohomoserine sulfhydrylase and/or acylhomoserine sulfhydrylases and that have at
the same time low γ -elimination activity. The present invention also relates to recombinant
enzymes that have one or several of the following activities cystathionine- γ -synthase
and/or phosphohomoserine and/or acylhomoserine sulfhydrylase, have at the same time
10 low γ -eliminase activity and are used for the fermentative production of amino acids, in
particular methionine.

Prior art

Sulfur-containing compounds such as cysteine, homocysteine, methionine or S-
15 adenosylmethionine are critical to cellular metabolism and are produced industrially to be
used as food or feed additives and pharmaceuticals. In particular methionine, an essential
amino acid, which cannot be synthesized by animals, plays an important role in many body
functions. Aside from its role in protein biosynthesis, methionine is involved in
transmethylation and in the bioavailability of selenium and zinc. Methionine is also
20 directly used as a treatment for medical disorders like allergy and rheumatic fever.
Nevertheless most of the methionine that is produced is added to animal feed.

Chemically, D,L-methionine is commonly produced from acrolein, methyl
mercaptan and hydrogen cyanide. However, the racemic mixture does not perform as well
as pure L-methionine, e.g. in chicken feed additives (Saunderson, C.L., (1985) British
25 Journal of Nutrition 54, 621-633). Pure L-methionine can be produced from racemic
methionine e.g. through the acylase treatment of N-acetyl-D,L-methionine which increases
production costs dramatically. The increasing demand for pure L-methionine coupled to
environmental concerns renders microbial production of methionine attractive.

Only microorganisms and plants are capable of methionine biosynthesis. The
30 pathway for L-methionine synthesis is well known in many microorganisms and plants
(Fig. 1). Methionine in *Escherichia coli* is derived from the amino acid aspartate, but its
synthesis requires the convergence of two additional pathways, cysteine biosynthesis and
C1 metabolism (N-methyltetrahydrofolate). Aspartate is converted into homoserine by a
sequence of three reactions. Homoserine can subsequently enter the threonine/isoleucine or
35 methionine biosynthetic pathway. In *E. coli* entry into the methionine pathway requires the
acylation of homoserine to succinyl-homoserine. This activation step allows subsequent
condensation with cysteine, leading to the thioether-containing cystathionine, which is

hydrolyzed to give homocysteine. The final methyl transfer leading to methionine is carried out by either a B₁₂-dependent or a B₁₂-independent methyltransferase.

Methionine biosynthesis in *E. coli* is regulated by repression and activation of methionine biosynthetic genes via the MetJ and MetR proteins. In addition to this
5 transcriptional regulation the entry into the methionine specific pathway is tightly controlled by *metA* encoding homoserine transsuccinylase (EC 2.3.1.46). Aside from the transcriptional control of *metA* by MetJ and MetR the enzyme is also feedback regulated by the major end-products of the pathway methionine and S-adenosylmethionine (Lee, L.-W et al. (1966) Multimetabolite control of a biosynthetic pathway by sequential
10 metabolites, JBC 241 (22), 5479-5780). Feedback inhibition by these two products is synergistic meaning that low concentrations of each metabolite alone are only slightly inhibitory, while in combination a strong inhibition is exerted.

Whereas in *E. coli* homoserine is activated to succinyl-homoserine that subsequently is transformed to γ -cystathionine by cystathionine- γ -synthase, gram-positive
15 bacteria and spirochetes activate homoserine to acetyl-homoserine and are able to incorporate sulfur from H₂S by transforming acetyl-homoserine directly into homocysteine using acetyl-homoserine sulfhydrylase, called MetY in gram-positives and MetZ in spirochetes.

In contrast to methionine biosynthesis in eubacteria, in plants the branch point
20 between methionine and threonine/isoleucine biosynthesis lies at the level of phosphohomoserine. Homoserine is phosphorylated to yield phosphohomoserine, which can either react to threonine catalyzed by the enzyme threonine synthase or to γ -cystathionine and/or homocysteine using the plant enzyme METB having cystathionine- γ -synthase and phosphohomoserine sulfhydrylase activity. Recent data indicate that a similar
25 pathway operates in archaea (White, 2003, The biosynthesis of cysteine and homocysteine in *Methanococcus jannaschii*, Biochim Biophys Acta. 1624(1-3):46-53), but the corresponding enzymes have not yet been characterized. Thus in plants and most likely in some archaea the committed step for methionine production consists in the synthesis of γ -cystathionine from phosphohomoserine. In plants methionine biosynthesis is controlled
30 through the activity of the two key-enzymes cystathionine- γ -synthase (CGS) and threonine synthase (TS). CGS is regulated posttranscriptionally via an N-terminal regulatory region not found in the bacterial enzyme (Hacham et al. 2002 Plant Physiol. 128, 454-462). TS is feed-back activated by S-adenosyl methionine, a methionine derivative that signals high methionine concentrations. Both of these regulatory mechanisms direct the carbon flux
35 away from methionine towards threonine, but are non-functional in prokaryotes.

Expression of different cystathionine- γ -synthases has been taught in patent application WO 93/17112 (Genencor). Precise knowledge about cystathionine- γ -synthases was lacking at that time and sequences from plants were not known. Patent application

JP2000-139471 (Ajinomoto) describes the overexpression of *E. coli* cystathionine- γ -synthase, but the use of cystathionine- γ -synthase from different organisms has not been evaluated for the production of methionine in any organism.

MetB enzymes and their counterparts MetY/MetZ can accept a variety of different substrates, listed in Table 1. These enzymes can catalyze four different reactions, which all require activated homoserine. Activated homoserine can be either phospho-, acetyl or succinyl-homoserine. (i) Together with cysteine cystathionine- γ -synthase produces cystathionine, (ii) together with hydrogen sulfide sulfhydrylase synthesizes homocysteine, (iii) together with methylmercaptan methionine synthase produces methionine and (iiii) in the absence of a second substrate γ -eliminase catalyzes the dissociation of the substrate to α -ketobutyrate, ammonia and the activating group (acetate, succinate, phosphate). Several enzymes can catalyze more than one reaction, but with varying catalytic efficiencies. For example *E. coli* MetB has the highest catalytic efficiency with cysteine and succinyl-homoserine as substrates and a relatively high γ -eliminase activity in the absence of cysteine (Aitken et al. 2003, Biochemistry 42, 11297-11306). Plant enzymes are equally good cystathionine producers, but have low γ -eliminase activity. In fact the k_{cat} of *A. thaliana* METB for the γ -eliminase activity is only 1/1500 of the *E. coli* enzyme. (Ravanel et al. 1998 Biochem. J. 331, 639-648). Some enzymes with low γ -elimination activity should favor high methionine production when introduced into *E. coli*.

substrates	Cystathionine- γ -synthase	sulfhydrylase	Methionine synthase	γ -eliminase
Phosphohomoserine	Plants METB archaea <i>Chloroflexus</i>	plants		plants METB
O-acetyl-homoserine	<i>Bacillus metZ</i>	<i>Corynebacterium metY</i> Spirochetes <i>metZ</i> yeast MET25		
O-succinyl-homoserine	<i>Escherichia metB</i> <i>Xanthomonas metB</i>	α -proteobacteria <i>metZ</i>	<i>Escherichia metB</i>	<i>Escherichia metB</i>

Table 1. Preferred substrates for enzymes with cystathionine- γ -synthase and/or phosphohomoserine sulfhydrylase and/or acylhomoserine sulfhydrylase activity from different organisms.

The type of activated homoserine and favored reaction are indicated for enzymes from different species or groups. For details see (Hacham et al. 2003, Mol. Biol. Evol. 20:1513-1520)

Summary of the invention

The invention is based on the discovery that methionine production is enhanced when enzymes with cystathionine- γ -synthase and/or phosphohomoserine sulfhydrylase and/or acylhomoserine sulfhydrylase activity are expressed that have a low γ -elimination activity. Use of these enzymes reduces the production of α -ketobutyrate that can be transformed into isoleucine, which accumulates in the fermentation broth. The invention thus relates to DNA fragments comprising genes encoding enzymes with cystathionine- γ -synthase and/or phosphohomoserine sulfhydrylase and/or acylhomoserine sulfhydrylase activity that have a low γ -eliminase activity. The present invention also relates to the expression, especially overexpression of these enzymes in the production of methionine. The invention relates further to microorganisms, preferentially enterobacteriaceae, coryneform bacteria or yeast, in which the aforementioned enzymes are expressed. In addition, the invention describes a process for the fermentative production of methionine its precursors or derivates using the microorganisms with the described properties.

Detailed description of the invention

Methionine is used in animal nutrition and pharmaceutical applications. Often a specific stereoisomer, in this case the biologically active L-form, is the preferred species. Since chemical synthesis can only provide racemic mixtures that are hard to resolve, it is of general interest to produce L-methionine by fermentation. One object of this invention is thus the optimization of a strain by genetic engineering and the improvement of a fermentative process that yields L-methionine, its precursors or derivative in high quantities. In plants and microorganisms several enzymes are required for the fermentative production of methionine. Cystathionine- γ -synthase in *E. coli* (SEQ ID NO 1) is one of the key enzymes involved in methionine biosynthesis.

>*E. coli* |EG10582|MetB: 386 aa - Cystathionine gamma-synthase

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MTRKQATIAV RSGLNDDEQY GCVVPPIHLS STYNFTGFNE PRAHDYSRRG
NPTRDVVQRA LAELEGGAGA VLTNTGMSAI HLVTTVFLKP GDLLVAPHDC
YGGSYRLFDS LAKRGCYRVL FVDQGDEQAL RAALAEKPKL VLVESPSNPL
LRVVDIAKIC HLAREVGAVS VVDNTFLSPA LQNPLALGAD LVLHSCTKYL
NGHSDVVAGV VIAKDPDVVT ELAWWANNIG VTGGAFDSYL LLRGLRTLVP
RMELAQRNAQ AIVKYLQTQP LVKKLYHPSL PENQGHEIAA RQQKGFGAML
SFELDGDEQT LRRFLGGLSL FTLAESLGGV ESLISHAATM THAGMAPEAR
AAAGISETLL RISTGIEDGE DLIADLENGF RAANKG
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In addition to its major activity cystathionine γ -synthase it has an undesired side-activity, succinyl-homoserine γ -eliminase, which transforms succinyl-homoserine into α -ketobutyrate, succinate and ammonia. This activity is especially pertinent if only low amounts of cysteine are present. In *E. coli* it leads to a loss in methionine production, a

problem that can be avoided by using cystathionine- γ -synthase that has a reduced γ -eliminase activity when compared to the *E. coli* enzyme.

The object of the invention is thus a microorganism for the fermentative production of amino acids in which one or several enzymes with cystathionine- γ -synthase and/or
5 phosphohomoserine sulfhydrylase and/or acylhomoserine sulfhydrylase are expressed that have a low γ -eliminase activity.

Low γ -eliminase activity means according to the invention, preferentially a γ -eliminase activity lower than the activity observed with the native *E. coli* enzyme. In addition, it is desired that the enzymes with reduced γ -eliminase activity have specific
10 cystathionine- γ -synthase and/or acylhomoserine sulfhydrylase and/or phosphohomoserine sulfhydrylase activities. Said activity can eventually be lower than the activities of the native *E. coli* enzymes.

The said expressed enzymes with cystathionine- γ -synthase and/or phosphohomoserine sulfhydrylase and/or acylhomoserine sulfhydrylase that have a low γ -
15 eliminase activity are preferably different from the native enzymes present in the same organism. They may be either mutated genes of the same species or native or mutated genes of other species.

According to a preferred embodiment of the present invention, the microorganism is transformed to introduce a gene coding for an enzyme with such low γ -eliminase activity.

20 Such gene may be introduced by different means available to the man skilled in the art:

- modification of the native gene by homologous recombination to introduce mutations in the enzyme encoded by the said gene to reduce γ -eliminase activity and keep the enzyme activity;
- 25 - integrating into the genome of the microorganism a foreign gene coding for the selected enzyme known to have low γ -eliminase activity; said foreign gene being under control of regulatory elements functional in the host microorganism;
- introducing a plasmid comprising a foreign gene coding for the selected
30 enzyme known to have low γ -eliminase activity under control of regulatory elements functional in the host microorganism.

When the gene is integrated into the genome of the microorganism, it may advantageously be introduced in a locus selected to replace the native gene.

Methods used to transform microorganisms are well known in the art, including
35 homologous recombination.

It is known that plant cystathionine- γ -synthases have lower γ -eliminase activities than the *E. coli* enzyme (Ravanel *et al.* 1998, Biochem. J 331, 639-648). Nevertheless it has never been shown that the use of plant enzymes in the fermentative production of

methionine presents an advantage compared to the use of the native *E. coli* enzyme. This invention relates thus to the use of plant cystathionine- γ -synthase in order to increase the fermentative production of methionine.

Plant enzymes use phosphohomoserine as a substrate whereas the *E. coli* enzyme
5 accepts succinyl-homoserine and to some extent acetyl-homoserine. Recently it was shown that methionine biosynthesis in archaea probably proceeds via phosphohomoserine. So far the enzymes have not been characterized. Thus the invention also relates to the use of archaeal enzymes with cystathionine- γ -synthase and/or phosphohomoserine sulfhydrylase activity in the fermentative production of methionine.

10 The closest homolog of plant cystathionine- γ -synthases in bacteria is found in the photosynthetic bacterium *Chloroflexus*. Alignments of this enzyme with plant enzymes demonstrate that the amino acids required for the binding of the phospho-group, which have been determined from structural comparisons between the *E. coli* and the plant enzymes (Steegborn et al. 2001 J. Mol Biol 311, 789-801) are conserved in the
15 *Chloroflexus* enzyme (Fig. 2).

Thus a microorganism expressing a homolog of the *Chloroflexus* gene, e.g. the gene >gi|53798754|ref|ZP_00020132.2| COG0626 from *Chloroflexus aurantiacus*, is also object of this invention.

Further objects of the invention are enzymes with cystathionine- γ -synthase/
20 phosphohomoserine sulfhydrylase activity that have conserved the following amino acids at positions 107E, 111Y, 165K, 403S (see alignment Fig.2) permitting the use of phosphohomoserine as a substrate and thus exhibiting a reduced γ -elimination activity. Amino acid positions are given by reference to the sequence of *Nicotiana tabacum*. The same amino acid position can be identified without undue experimentation by simple
25 sequence alignment (see Fig. 2).

As part of the biosynthetic pathway of threonine, phosphohomoserine is produced in *E. coli* and could thus be transformed directly into γ -cystathionine and/or homocysteine.

In contrast to plants and enterobacteriaceae, several gram-positive bacteria use a mechanism to activate homoserine that relies on the transfer of an acetyl group onto
30 homoserine yielding acetyl-homoserine. Subsequently acetyl-homoserine is transformed into homocysteine by sulfhydrylation using an acetyl-homoserine sulfhydrylase MetY or into γ -cystathionine using cystathionine- γ -synthase. This reaction has been used to produce methionine by fermentation in coryneform bacteria as described in patent applications WO2004024933 and EP1313871. Nevertheless the corresponding genes have never been
35 tested in *E. coli* and their γ -eliminase activity has not been determined.

Thus the present invention also relates to the use of MetY enzymes in *E. coli* with low γ -eliminase activity but comparable or increased sulfhydrylase activity with respect to the *E. coli* enzyme.

According to another embodiment of the present invention, decrease in the γ -eliminase activity of the native or introduced enzyme can also be obtained by optimizing the enzyme, e.g. by directed evolution or site directed mutagenesis as described in Sambrook et al. (1989 Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Lab., Cold Spring Harbor, New York.). The enzyme can also be optimized through random mutagenesis using mutagens as NTG or EMS or by *in vivo* evolution as described in patent application PCT/FR04/00354. Optimized enzymes can also be synthetic genes that are based on natural genes and have an optimized codon usage and GC-content for the host organism. Therefore the use of optimized enzymes with reduced γ -eliminase activity or increased cystathionine- γ -synthase and/or phosphohomoserine sulphydrylase and/or acylhomoserine sulphydrylase activity is object of the invention.

A particular example of an optimized enzyme is an optimized cystathionine- γ -synthase and/or phosphohomoserine sulphydrylase and/or acylhomoserine sulphydrylase enzyme, in particular the *E. coli* MetB enzyme, in which the alanine at position 337 has been replaced by a proline that is highly conserved in enzymes from different bacterial genera and/or comprising an alanine at position 335. Except stated otherwise, positions are given by reference to the native *E. coli* enzyme.

The present invention furthermore relates to nucleotide sequences, DNA or RNA sequences, which encode cystathionine- γ -synthase and/or phosphohomoserine sulphydrylase and/or acylhomoserine sulphydrylase according to the invention as defined above.

The cystathionine- γ -synthases and phosphohomoserine sulphydrylases and/or acylhomoserine sulphydrylases are advantageously selected among the enzymes corresponding to PFAM references PF01053 and to COG reference COG0626 and COG2873.

The PFAM (protein families database of alignments and hidden Markov models; <http://www.sanger.ac.uk/Software/Pfam/>) form a large collection of alignments of protein sequences. Each PFAM makes it possible to visualize multiple alignments, see protein domains, evaluate distribution among organisms, gain access to other databases, and visualize known protein structures.

The COGs (clusters of orthologous groups of proteins; <http://www.ncbi.nlm.nih.gov/COG/>) are obtained by comparing the protein sequences from 43 fully sequenced genomes representing 30 major phylogenetic lines. Each COG is defined from at least three lines, which thus makes it possible to identify ancient conserved domains.

The preferred enzymes are the cystathionine- γ -synthase of *Arabidopsis thaliana* (accession number gi:1389725), the putative cystathionine- γ -synthase and/or sulphydrylase of *Methanosarcia barkeri* (accession number gi:48839517), the acetyl-homoserine

sulphydrylase of *Saccharomyces cerevisiae* (accession number YLR303W), the putative cystathionine- γ -synthase and/or sulphydrylase from *Chloroflexus aurantiacus* (gi:53798753) and the acetyl-homoserine sulphydrylase from *Corynebacterium glutamicum* (gi:41324877) as synthetic gene adapted to *E. coli*. These sequences are aligned with the

5 following representative sequences:

- >gi|8439541|gb|AAF74981.1|AF082891_1 cystathionine gamma-synthase isoform 1 [Solanum tuberosum]
- >gi|4959932|gb|AAD34548.1|AF141602_1 cystathionine-gamma-synthase precursor [Glycine max]
- 10 >gi|2198853|gb|AAB61348.1| cystathionine gamma-synthase [Zea mays]
- >gi|4322948|gb|AAD16143.1| cystathionine gamma-synthase precursor [Nicotiana tabacum]
- >gi|11602834|gb|AAG38873.1|AF076495_1 cystathionine gamma-synthase [Oryza sativa]
- >gi|305042|gb|AAB03071.1| cystathionine gamma-synthase [Escherichia coli]

15 The homologous sequences of these sequences that present a cystathionine- γ -synthase and/or phosphohomoserine sulphydrylase and/or acylhomoserine sulphydrylase activity, and present at least 80% homology, and preferably 90% homology, and more preferably 95% homology with the amino acid sequences described above are equally object of the this invention.

20 The means of identifying homologous sequences and their percentage homology are well-known to those skilled in the art, and include in particular the BLAST program, and in particular the BLASTP program, which can be used from the web site <http://www.ncbi.nlm.nih.gov/BLAST/> with the default parameters indicated on that site.

To determine which enzymes may be beneficial to methionine production when

25 introduced into the corresponding strain, γ -eliminase activities may be evaluated enzymatically. For example, cystathionine- γ -synthase, γ -eliminase activity and sulphydrylase activities can be determined in enzymatic tests with activated homoserine and (i) no other substrate, or (ii) cysteine, or (iii) H₂S as substrate. The reaction is started by adding the protein extract containing the corresponding enzymatic activity, and the

30 formation of homocysteine and/or γ -cystathionine is monitored by GC-MS after protein precipitation and derivatization with a silylating reagent.

The gene(s) encoding cystathionine- γ -synthase and/or phosphohomoserine and/or acylhomoserine sulphydrylase may be encoded chromosomally or extrachromosomally. Chromosomally there may be one or several copies on the genome that can be introduced

35 by methods of recombination known to the expert in the field. Extrachromosomally genes may be carried by different types of plasmids that differ with respect to their origin of replication and thus their copy number in the cell. They may be present as 1-5 copies, ca 20 or up to 500 copies corresponding to low copy number plasmids with tight replication

(pSC101, RK2), low copy number plasmids (pACYC, pRSF1010) or high copy number plasmids (pSK bluescript II)

The *metB* genes may be expressed using promoters with different strength that need or need not to be induced by inducer molecules. Examples are the promoters P_{trc}, P_{tac},
5 Plac, the lambda promoter cI or other promoters known to the expert in the field.

Expression of the target genes may be boosted or reduced by elements stabilizing or destabilizing the corresponding messenger RNA (Carrier and Keasling (1998) Biotechnol. Prog. 15, 58-64) or the protein (e.g. GST tags, Amersham Biosciences)

The present invention also relates to microorganisms that contain one or several
10 alleles encoding a cystathionine- γ -synthase and/or acylhomoserine and/or phosphohomoserine sulfhydrylase according to the invention.

Such strains are characterized by the fact that they possess a methionine metabolism which permits an increased flux towards methionine by either exclusively using phosphohomoserine accepting enzymes and thus reducing the amount of α -
15 ketobutyrate produced or by using phosphohomoserine accepting enzymes in addition to acylhomoserine accepting enzymes thus increasing the flow towards methionine.

In particular the invention relates to the preparation of L-methionine, its precursors or compounds derived thereof, by means of cultivating novel microorganisms and isolating the produced sulfur containing compounds.

20 An increase in the production of L-methionine, its precursors or compounds derived thereof, can be achieved by reducing the expression levels or deleting one of the following genes.

<i>Gene</i>	<i>Genbank entry</i>	<i>activity</i>
<i>ackA</i>	1788633	acetate kinase
25 <i>pta</i>	1788635	phosphotransacetylase
<i>acs</i>	1790505	acetate synthase
<i>aceE</i>	1786304	pyruvate dehydrogenase E1
<i>aceF</i>	1786305	pyruvate dehydrogenase E2
<i>lpd</i>	1786307	pyruvate dehydrogenase E3
30 <i>sucC</i>	1786948	succinyl-CoA synthetase, beta subunit
<i>sucD</i>	1786949	succinyl-CoA synthetase, alpha subunit
<i>pck</i>	1789807	phosphoenolpyruvate carboxykinase
<i>pykA</i>	1788160	pyruvate kinase II
<i>pykF</i>	1787965	pyruvate kinase I
35 <i>poxB</i>	1787096	pyruvate oxidase
<i>ilvB</i>	1790104	acetohydroxy acid synthase I, large subunit
<i>ilvN</i>	1790103	acetohydroxy acid synthase I, small subunit
<i>ilvG</i>	1790202	acetohydroxy acid synthase II, large subunit

	1790203	
<i>ilvM</i>	1790204	acetohydroxy acid synthase II, small subunit
<i>ilvI</i>	1786265	acetohydroxy acid synthase III, large subunit
<i>ilvH</i>	1786266	acetohydroxy acid synthase III, small subunit
5 <i>aroF</i>	1788953	DAHP synthetase
<i>aroG</i>	1786969	DAHP synthetase
<i>aroH</i>	1787996	DAHP synthetase

An additional increase in the production of L-methionine, its precursors or compounds derived thereof can be achieved by overexpressing one or several of the following genes: the pyruvate carboxylase from *Rhizobium etli* (pyc, U51439), or one of its homologs, the homoserine synthesizing enzymes encoded by the genes *thrA* (homoserine dehydrogenase/ aspartokinase, 1786183), preferably with reduced feed-back sensitivity, *metL* (homoserine dehydrogenase/aspartokinase, g1790376) or *lysC* (aspartokinase, 15 1790455) and *asd* (aspartate semialdehyde dehydrogenase, 1789841) or a combination thereof.

A further increase in the production of L-methionine, its precursors or compounds derived thereof, is possible by overexpressing genes involved in sulfate assimilation and production of cysteine. An increased amount of sulfur containing compounds will equally reduce the γ -eliminase activity. This can be achieved by overexpressing the following genes (see below) or by deregulating the pathway through the introduction of a constitutive *cysB* allele as described by Coyler and Kredich (1994 Mol Microbiol **13** 797-805) and by introducing a *cysE* allele encoding a serine acetyl transferase with decreased sensitivity for its inhibitor L-cysteine (US patent application US 6,218,168, Denk & Bock 1987 J Gen 20 Microbiol **133** 515-25). The following genes need to be overexpressed.

	<i>CysA</i>	1788761	sulfate permease
	<i>CysU</i>	1788764	cysteine transport system
	<i>CysW</i>	1788762	membrans bound sulphate transport system
30	<i>CysZ</i>	1788753	ORF upstream of <i>cysK</i>
	<i>cysN</i>	1789108	ATP sulfurylase
	<i>cysD</i>	1789109	sulfate adenylyltransferase
	<i>cysC</i>	1789107	adenylylsulfate kinase
	<i>cysH</i>	1789121	adenylylsulfate reductase
35	<i>cysI</i>	1789122	sulfite reductase, alpha subunit
	<i>cysJ</i>	1789123	sulfite reductase, beta subunit
	<i>cysE</i>	1790035	serine acetyltransferase
	<i>cysK</i>	1788754	cysteine synthase

	<i>cysM</i>	2367138	O-acetyl-sulphydrolase
	<i>cysW</i>	1788762	sulfate transport
	<i>cysT</i>		sulfate transport
	<i>cysZ</i>	1788753	sulfate transport
5	<i>sbp</i>	1790351	Periplasmic sulfate-binding protein

In addition genes involved in the production of C1 (methyl) groups may be enhanced by overexpressing the following genes:

10	<i>serA</i>	1789279	phosphoglycerate dehydrogenase, preferably feed-back resistant
	<i>serB</i>	1790849	phosphoserine phosphatase
	<i>serC</i>	1787136	phosphoserine aminotransferase
	<i>glyA</i>	1788902	serine hydroxymethyltransferase
	<i>metF</i>	1790377	5,10-Methylenetetrahydrofolate reductase

15 In addition genes directly involved in the production of methionine may be overexpressed:

	<i>metB</i>	1790375	Cystathionine- γ -synthase
	<i>metC</i>	1789383	Cystathionine- β -lyase
	<i>metH</i>	1790450	B12-dependent homocysteine-N5-methyltetrahydrofolate transmethylase
20	<i>metE</i>	2367304	Tetrahydropteroyltriglutamate methyltransferase
	<i>metF</i>	1790377	5,10-Methylenetetrahydrofolate reductase
	<i>metR</i>	1790262	Positive regulatory gene for <i>metE</i> and <i>metH</i> and autogenous regulation

25 Furthermore expression of genes in pathways degrading methionine or deviating from the methionine production pathway may be reduced or the genes may be deleted.

	<i>speD</i>	1786311	S-Adenosylmethionine decarboxylase
	<i>speC</i>	1789337	Ornithine decarboxylase
	<i>astA</i>	1788043	Arginine succinyltransferase
30	<i>dapA</i>	1788823	Dihydrodipicolinate synthase

Anaplerotic reactions may be boosted by expressing

	<i>ppc</i>	1790393	phosphoenolpyruvate carboxylase
	<i>pps</i>	1787994	phosphoenolpyruvate synthase

35 A further increase in the production of L-methionine, its precursors or compounds derived thereof, is achieved by means of deleting the gene for the repressor protein MetJ, responsible for the down-regulation of the methionine regulon as was suggested in JP 2000157267-A/3 (see also GenBank 1790373).

Production of methionine may be further increased by using an altered *metB* allele that uses preferentially or exclusively H₂S and thus produces homocysteine from O-succinyl-homoserine as has been described in the patent application PCT N° PCT/FR04/00354, which content is incorporated herein by reference.

5 In a preferred application the organism is either *E. coli* or *C. glutamicum* or *Saccharomyces cerevisiae*.

The invention also concerns the process for the production of L-methionine, its precursors or compounds derived thereof, which is/are usually prepared by fermentation of the designed bacterial strain.

10 According to the invention, the terms 'culture' and 'fermentation' are used indifferently to denote the growth of a microorganism on an appropriate culture medium containing a simple carbon source.

According to the invention a simple carbon source is a source of carbon that can be used by those skilled in the art to obtain normal growth of a microorganism, in particular
15 of a bacterium. In particular it can be an assimilable sugar such as glucose, galactose, sucrose, lactose or molasses, or by-products of these sugars. An especially preferred simple carbon source is glucose. Another preferred simple carbon source is sucrose.

Those skilled in the art are able to define the culture conditions for the microorganisms according to the invention. In particular the bacteria are fermented at
20 temperature between 20°C and 55°C, preferentially between 25°C and 40°C, and more specifically about 30°C for *C. glutamicum* and about 37°C for *E. coli*.

The fermentation is generally conducted in fermenters with an inorganic culture medium of known defined composition adapted to the bacteria used, containing at least one simple carbon source, and if necessary a co-substrate necessary for the production of the
25 metabolite.

In particular, the inorganic culture medium for *E. coli* can be of identical or similar composition to an M9 medium (Anderson, 1946, *Proc. Natl. Acad. Sci. USA* 32:120-128), an M63 medium (Miller, 1992; A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria, Cold Spring Harbor
30 Laboratory Press, Cold Spring Harbor, New York) or a medium such as defined by Schaefer *et al.* (1999, *Anal. Biochem.* 270: 88-96).

Analogously, the inorganic culture medium for *C. glutamicum* can be of identical or similar composition to BMCG medium (Liebl *et al.*, 1989, *Appl. Microbiol. Biotechnol.* 32: 205-210) or to a medium such as that described by Riedel *et al.* (2001, *J. Mol.*
35 *Microbiol. Biotechnol.* 3: 573-583). The media can be supplemented to compensate for auxotrophies introduced by mutations.

After fermentation L-methionine, its precursors or compounds derived thereof, is/are recovered and purified if necessary. The methods for the recovery and purification of

the produced compound such as methionine in the culture media are well known to those skilled in the art.

The sulfur source used for the fermentative production of L-methionine, its precursors or compounds derived thereof, may be any of the following: sulfate, thiosulfate, hydrogen sulfide, methylmercaptam.

Figure legends:

Fig. 1. Metabolic pathways converting oxaloacetate into threonine, isoleucine and methionine. MetB homologs can catalyze at least three reactions: synthesis of γ -cystathionine, sulfhydrylation or γ -elimination of activated homoserine. MetB homologs accepting preferentially phosphohomoserine are shown in red, succinylhomoserine accepting enzymes are represented in blue and acetylhomoserine transforming enzymes (MetB/metZ) are indicated in green.

Fig. 2. Alignment of enzymes with cystathionine- γ -synthase and/or phosphohomoserine sulfhydrylase and/or acetylhomoserine sulfhydrylase activity of plants and bacterial species. The residues shown in grey boxes render the enzyme specific for the binding of phosphohomoserine. Highly conserved amino acids are shown in red, conserved residues in blue.

Example 1: Construction of a strain using exclusively plant phosphohomoserine accepting METB with low γ -eliminase activity for methionine production

To verify that plant CGS have a lower γ -eliminase activity than the *E. coli* enzyme, ratios between γ -eliminase activity and CGS activity of the *Arabidopsis* METB enzyme and the *E. coli* enzyme were determined in crude extracts. For this purpose *E. coli* strains were constructed in which the chromosomal copy of the *metB* gene was deleted and either *E. coli* or *Arabidopsis* CGS was expressed from a plasmid. Concomitantly with the *metB* deletion the *metJ* gene was also eliminated (see below).

E. coli $\Delta metJB$ strains carrying either wild-type or heterologous acylhomoserine or phosphohomoserine accepting cystathionine- γ -synthase and/or sulfhydrylases were cultured in minimal medium with 5 g/l glucose and harvested at late log phase. Cells were resuspended in cold potassium phosphate buffer and sonicated on ice (Branson sonifier, 70W). After centrifugation, proteins contained in the supernatants were quantified (Bradford, 1976).

Ten μ l of the extracts were incubated for 10 minutes at 30°C with either 5 mM O-succinyl-homoserine or O-acetyl-homoserine and either 1.5 mM sodium sulfide or cysteine. Proteins were precipitated with acetone and homocysteine or γ -cystathionine produced during the enzymatic reaction was quantified by GC-MS, after derivatization with tert-butyldimethylsilyltrifluoroacetamide (TBDMS-TFA). L-Serine [1-13C] was

included as an internal standard. Alternatively the disappearance of cysteine was measured using the same protocol.

To inactivate the *metB* and *metJ* gene the homologous recombination strategy described by Datsenko & Wanner (2000) was used. This strategy allowed the insertion of a chloramphenicol resistance cassette, while deleting most of the genes concerned. For this

purpose the following oligonucleotides were used:
DmetJR (SEQ ID NO 2): tgacgtaggc ctgataagcg tagcgcatca ggcgattcca ctccgcgccg ctctttttg
cttttagtatt cccacgtctc TGTAGGCTGG AGCTGCTTCG

with

- a region (lower case) homologous to the sequence (4125596-4125675) of the gene *metJ* (reference sequence on the website <http://genolist.pasteur.fr/Colibri/>),

a region (upper case) for the amplification of the chloramphenicol resistance cassette (reference sequence in Datsenko, K.A. & Wanner, B.L., 2000, PNAS, 97: 6640-6645),

DmetJBF (SEQ ID NO 3): tatgcagctg acgaccttc gccctgcct gcgcaatcac actcatttt acccctgtt
tgcagcccg aagccattt CAGGCACCAG AGTAAACATT

with

- a region (lower case) homologous to the sequence (4127460-4127381) of the gene *metB* and a region (4126116-4126197) homologous to the promoter of *metL*

- a region (upper case) for the amplification of the chloramphenicol resistance cassette.

The oligonucleotides DmetJBR and DmetJBF were used to amplify the chloramphenicol resistance cassette from the plasmid pKD3. The PCR product obtained was then introduced by electroporation into the strain MG1655 (pKD46) in which the Red recombinase enzyme was expressed permitting the homologous recombination. Chloramphenicol resistant transformants were selected and the insertion of the resistance cassette was verified by a PCR analysis with the oligonucleotides MetJR and MetJF defined below. The strain retained was designated MG1655 ($\Delta metJB::Cm$).

MetJR (SEQ ID NO 4): ggtacagaaa ccagcaggct gaggatcagc (homologous to the sequence from 4125431 to 4125460).

MetLR (SEQ ID NO 5): aaataacact tcacatcagc cagactactgc caccaaattt (homologous to the sequence from 4127500 to 4157460).

The chloramphenicol resistance cassette was then eliminated. The plasmid pCP20 carrying recombinase FLP acting at the FRT sites of the chloramphenicol resistance cassette was introduced into the recombinant strains by electroporation. After a series of cultures at 42°C, the loss of the chloramphenicol resistance cassette was verified by a PCR analysis with the same oligonucleotides as those used previously.

For the production of methionine using phosphohomoserine-accepting METB from *Arabidopsis thaliana* the following *Escherichia coli* strain was constructed. The methionine repressor *metJ* together with *E. coli metB* were deleted as described above.

Subsequently the gene *metA* encoding succinyl-homoserine transferase and/or the gene *thrC* encoding threonine synthase were deleted. The deletion strategy has been exemplified for the *metJB* deletion. The deletion of all other genes was based on the same strategy using the oligonucleotides indicated. The oligonucleotides for the deletions of the *metA* and *thrC* genes are indicated below. The numbers in parentheses indicate the regions that are homologous to the *E. coli* chromosome. Oligonucleotides beginning with D were used for the actual deletion, other oligonucleotides for the verification of the constructs or for specific purposes as indicated.

For the deletion of the *metA* gene the following oligonucleotides were used:

- 10 DmetAF (4211866-4211945 ; SEQ ID NO 6): ttcgtgtgcc ggacgagcta cccgccgtca atttcttgcg tgaagaaaac gtccttga tgacaacttc tcgtgcgtct TGTAGGCTGG AGCTGCTTCG
DmetAR (4212785-4212706; SEQ ID NO 7): atccagcgtt ggattcatgt gccgtagatc gtagggcgtg atctggtaga cgtaatatgt gagccagttg gtaaacagta CATATGAATA TCCTCCTTAG
MetAF (4211759-4211788; SEQ ID NO 8): tcacctcaa catgcaggct cgacattggc
15 MetAR (4212857-4212828; SEQ ID NO 9): ataaaaaagg caccgaagg tgcctgaggt

For the deletion of the *thrC* gene the following oligonucleotides were used:

- DthrCF (3740-3821; SEQ ID NO 10): ctctacaatc tgaaagatca caacgagcag gtcagctttg cgcaagccgt aaccagggg ttgggcaaaa atcaggggcT GTAGGCTGGAG CTGCTTCG
DthrCR (5012-4932; SEQ ID NO 11): gattcatcat caattacgca acgagcaaaa atcgggggg
20 agattatgtg aaagcaaggg taaatcagca cgttctgcCA TATGAATATC CTCCTTAG
thrCF (3490-3511; SEQ ID NO 12): cgctgaaccc tacgtgaac gg
thrCR (5284-5260; SEQ ID NO 13): gcgaccagaa ccagggaag tgcg

To further boost the production of homoserine the aspartokinase/homoserine a *thrA** allele with reduced feed-back resistance to threonine was expressed from the plasmid pCL1920 (Lerner & Inouye, 1990, NAR 18, 15 p 4631) using the promoter P_{trc}. For the construction of plasmid pME101-*thrA**1 *thrA* was PCR amplified from genomic DNA using the following oligonucleotides:

*Bsp*H1*thrA* (SEQ ID NO 14): ttaTCATGAgagtgtgaagttcggcggtacatcagttggc

*Sma*1*thrA* (SEQ ID NO 15): ttaCCCGGGccgccccgagcacatcaaaccggacgc

- 30 The PCR amplified fragment was cut with the restriction enzymes *Bsp*HI and *Sma*I and cloned into the *Nco*I / *Sma*I sites of the vector pTRC99A (Stratagene). For the expression from a low copy vector the plasmid pME101 was constructed as follows. The plasmid pCL1920 was PCR amplified using the oligonucleotides PME101F and PME101R and the *Bst*Z17I-*Xmn*I fragment from the vector pTRC99A harboring the *lacI* gene and the P_{trc} promoter was inserted into the amplified vector. The resulting vector and the vector harboring the *thrA* gene were restricted by *Apa*I and *Sma*I and the *thrA* containing fragment was cloned into the vector pME101. To relieve ThrA from feed-back inhibition the mutation F318S was introduced by site-directed mutagenesis (Stratagene) using the

oligonucleotides ThrAF F318S and ThrAR F318S, resulting in the vector pME101-thrA*1, called pSB1.

PME101F (SEQ ID NO 16): Ccgacagtaagacgggtaagcctg

PME101R (SEQ ID NO 17): Agcttagtaaagccctcgctag

5 ThrAF F318S (SmaI) (SEQ ID NO 18):

Ccaatctgaataacatggcaatg**tc**cagcgttctg**cccg**gg

ThrAR F318S (SmaI) (SEQ ID NO 19):

Cccgggccagaaacgct**gg**acattgccatgtattcagattgg

10 To transform part of the homoserine produced into phosphohomoserine the *thrB* gene was cloned into the vector pSB1. *thrB* was PCR amplified using the oligonucleotides thrA'BF and thrA'BR

thrA'BF (SEQ ID NO 20): tacgat**gtac** atggccttaa tctggaaaac tggc

thrA'BR (SEQ ID NO 21): tcc**cccg**ggT TAGTTTTCCA GTACTCGTGC GCCC

15 The PCR fragment was digested with *Bsr*G1 and *Sma*I and cloned into the vector pSB1 cut by the same restriction enzymes resulting in plasmid pSB2.

Subsequently the enzyme METB from *Arabidopsis thaliana* was PCR amplified from a cDNA clone that is commercially available (TAIR, <http://arabidopsis.org/contact/>) using the oligonucleotides gapA-cgsAF and cgsAR. The oligonucleotides gapA-cgsAF consisted of nucleotides 1 to 38 of METB (fat) and nucleotides 1860761 to 1960799 (underlined) of *E. coli* pertaining to the GapA promoter region.

gapA-cgsAF (SEQ ID NO 22): ccttttattc actaacaat agctggtgga atatatgttg agctccgatg
ggagcctcac tgttcatgcc gg

cgsAR (SEQ ID NO 23): AATCGC**GGAT** **CCGAATCCGG** TCAGATGGCT

25 TCGAGAGCTT GAAGAATGTC AGC

At the same time the GapA promoter region of the *E. coli* gene GapA was amplified using the oligonucleotides gapA-cgsAR and GapAF. The oligonucleotide gapA-cgsAR harbors base 1 to 39 of the MetB gene and bases 1860799 to 1860761 from the *E. coli* chromosome corresponding to the GapA promoter region. The oligonucleotide GapAF corresponds to bases 1860639 to 1860661 of the *E. coli* genome.

gapA-cgsAR (SEQ ID NO 24): ccggcatgaa cagtgaggct cccatcggag ctcaacatat attccaccag
ctatttgtta gtgaataaaag g

GapAF (SEQ ID NO 25): acgt**cccg**gg caagcccaaa ggaagagtga ggc

Both fragments were subsequently fused using the oligonucleotides cgsAR and GapAF (Horton et al. 1989 Gene 77:61-68), cut with the restriction enzymes *Bam*HI and *Sma*I and cloned into the corresponding restriction sites of vectors pSB1 and pSB2 giving vector pSB3 (pME101thrA* -met*Bat*) and pSB4 (pME101thrA* -thrB-met*Bat*), respectively.

The vectors pSB3 and pSB4 were subsequently introduced into the strain $\Delta metBJ$ $\Delta metA \Delta thrC$.

Example 2: Construction of a strain using exclusively plant METB with low γ -eliminase activity for methionine production via O-succinylhomoserine.

For the use of plant METB for the production of methionine via succinyl homoserine the strain $\Delta metBJ$ $metA^*$ was constructed. The construction is initiated with the strains described in example 1 in which a feed-back resistant homoserine transsuccinylase $metA^*11$ (described in patent application PCT IB2004/001901) is introduced into the genome. For this purpose the $metA^*11$ allele was amplified from the *E. coli* chromosome using the following oligonucleotides.

MetArcF (4211786-4211883 ; SEQ ID NO 26): ggcaaattt ctggttatct tcagctatct ggatgtctaa acgtataagc gtatgtatg aggtaatcag gttatgccga ttcgtgtgcc ggacgagc

MetArcR (4212862-4212764; SEQ ID NO 27): cggaaataaa aaaggcacc gaagggtgcct gaggaaggt gctgaatcgc ttaacgatcg actatcacag aagattaatc cagcgttgga ttcattgtgc

The plasmid pKD46 was introduced into strains MG1655 $\Delta metA \Delta thrC$ and MG1655 $\Delta metA$ and transformed with the DNA fragment harboring the $metA^*11$ allele. Clones were selected for methionine prototrophy on modified M9 plates. Subsequently the mutation $\Delta metBJ$ was added as described above. The plasmid pSB3 and pSB4 were introduced into the two strains.

Example 3: Construction of a strain using plant phosphohomoserine accepting METB with low γ -eliminase activity and simultaneous use of *E. coli* MetB.

In order to further boost methionine synthesis a strain was constructed that simultaneously produces methionine via O-succinyl homoserine and phosphohomoserine. The construction was initiated with the strains $\Delta metA$ and $\Delta metA \Delta thrC$ described in example 1 in which the gene $metJ$ was deleted using the following oligonucleotides:

DmetJF with 100 bases (SEQ ID NO 28): caggcaccag agtaaacatt gtgtaaatgg acgtcaatac atctggacat ctaaacttct ttgcgtatag attgagcaaa CATATGAATA TCCTCCTTAG

DmetJR with 100 bases (SEQ ID NO 29): tgacgtaggc ctgataagcg tagcgcatca ggcgattcca ctccgcgccg ctcttttttg cttagtatt cccacgtctc TGTAGGCTGG AGCTGCTTCG

MetJR (SEQ ID NO 30): ggtacagaaa ccagcaggct gaggatcagc (homologous to the sequence from 4125431 to 4125460).

MetBR (SEQ ID NO 31): ttcgtcgtca ttaaccgcg tacgcactgc (homologous to the sequence from 4126305 to 4126276).

Subsequently a feed-back resistant homoserine transsuccinylase $metA^*11$ (described in patent application PCT IB2004/001901) was introduced into the genome. For

this purpose the *metA**11 allele was amplified from the *E. coli* chromosome using the following oligonucleotides.

MetArcF (4211786-4211883 ; SEQ ID NO 32): ggcaaatttt ctggttatct tcagctatct ggatgtctaa acgtataagc gtatgtagtg aggtaatcag gttatgccga ttcgtgtgcc ggacgagc

- 5 MetArcR (4212862-4212764; SEQ ID NO 33): cggaaataaa aaaggcaccc gaaggtgcct gaggttaaggt gctgaatcgc ttaacgatcg actatcacag aagattaatc cagcgttgga ttcattgtgc

The plasmid pKD46 was introduced into strains MG1655 $\Delta metJ \Delta metA \Delta thrC$ and MG1655 $\Delta metJ \Delta metA$ that were transformed with the DNA fragment harboring the *metA**11 allele. Clones were selected for methionine prototrophy on modified M9 plates
10 (supplemented with threonine if necessary).

Similarly to the construction of pSB3 and pSB4 using *metB*At in example 1, the *metB* gene of *E. coli* with its proper promoter was cloned into vectors pSB1 and pSB2 by amplifying it with the oligonucleotides MetBF and MetBR and cloning the PCR amplificate into the restriction sites *Pst*I and *Hind*III.

- 15 MetBF (4125957-4125982) (SEQ ID NO 34): ttgacagaa ctgcagcgcc gctccattca gccatgagat ac

MetBR (4127500-4127469) (SEQ ID NO 35): cgtaacgccc aagcttaaat aacacttcac atcagccaga ctactgcc

The resulting vectors are named pSB5 (pME101*thrA**-*metB*Ec) and pSB6
20 (pME101*thrA**-*thrB*-*metB*Ec).

Subsequently the plasmids pSB3, pSB4, pSB5 and pSB6 were introduced into the MG1655 $\Delta metJ metA$ *11 $\Delta thrC$ and MG1655 $\Delta metJ metA$ *11 strains.

25 **Example 4: Production of methionine via phosphohomoserine and/or O-succinylhomoserine using sulfate or hydrogen sulfide as sulfur source**

Amino acid production of the strains constructed in example 1 to 3 is analyzed in small Erlenmeyer flask cultures using modified M9 medium (Anderson, 1946, *Proc. Natl. Acad. Sci. USA* 32:120-128) that is supplemented with 5 g/l MOPS 5 g/l glucose and possibly 2 mM threonine. If hydrogensulfide is used as sulfur source all sulfate containing
30 salts are replaced by equal molar amounts of chloride containing salts. Sulfide is supplied as ammonium sulfide (10 mM). Spectinomycin is added if necessary at a concentration of 100 mg/l. An overnight culture is used to inoculate a 30 ml culture to an OD600 of 0.2. After the culture has reached an OD600 of 4.5 to 5, 1,25 ml of a 50% glucose solution and 0.75 ml of a 2M MOPS (pH 6.9) are added and the culture is agitated for 1 hour.
35 Subsequently IPTG is added if necessary.

Extracellular metabolites are analyzed during the batch phase. Amino acids are quantified by HPLC after OPA/Fmoc derivatization and other relevant metabolites are analyzed using GC-MS after silylation.

Strain	Methionine (mmol/g Dw)	Isoleucine (mmol/g Dw)	CGS (mU/mg protein)	γ -elimination (mU/mg protein)
DmetBJ metA*11 pSB5	0.48	0.67	2468	307
DmetBJ metA*11 pSB3	0.26	0.13	6	<0.05

Table 2 Specific methionine production of strains harboring *E. coli* (pSB5) and *Arabidopsis* (pSB3) MetB and their corresponding Cystathionine- γ -synthase (CGS) and γ -elimination activities.

Production of methionine using METB from *A. thaliana* having low γ -eliminase activity significantly reduces the production of isoleucine through the γ -eliminase reaction, while methionine production is not strongly affected. This is in contrast to experiments in which exclusively the *E. coli* pathway is used and significant amounts of the by product isoleucine are produced via γ -elimination.

Example 5: Construction and evaluation of a strain producing methionine using the yeast MetB enzyme that accepts acetyl-homoserine and H₂S as substrates

In order to test the γ -eliminase activity of yeast *in vivo* and to evaluate its potential for the production of methionine the following strain was constructed. The homoserine acetyl transferase encoding the MET2 gene of *Saccharomyces cerevisiae* was amplified by PCR from genomic DNA using the following oligonucleotides:

Ptac-metAlevF (SEQ ID NO 36): tgctacagct ggagctgttg acaattaatc atcggctcgt ataattgtgtg
gaaggaggac agaccatgtc gcatactta aaatcgaaaa cgctccaaga gc

Ptac-metAlevR (SEQ ID NO 37): CGTACTGACG ACCGGGTCCT ACCAGTTGGT
AACTTCTTCG GCCTCACC

Subsequently the PCR fragment was cloned into the restriction sites *PvuII* and *DrdI* of the vector pACYC184, resulting in vector pSB7. The oligonucleotide Ptac-metAlevF harbors the pTAC promoter that drives the expression of the yeast MET2 gene from the vector pACYC184.

The yeast acetyl-homoserine sulfhydrylase (MET17) was amplified using the oligonucleotides metBlev and gapA-metBlevF. Simultaneously the *E. coli* gapA promoter was amplified using the oligonucleotides gapA-metBlevR and GapAF. Subsequently the MET17 gene was fused to the gapA promoter by fusion PCR using only oligonucleotides metBlev and GapAF (see above for details).

gapA-metBlevR (38-75 : 1860797-1860761; SEQ ID NO 38): GGCCGGCGTG
TAGTTGAACA GTATCGAAAT GAGATGGCAT ATATTCCACC AGCTATTTGT
TAGTGAATAA AAGG

gapA-metBlevF (1-38 : 1860761-1860797; (SEQ ID NO 39): ccttttattc actaacaat
5 agctggtgga atatatgcca tctcatttcg atactgttca actacacgcc ggcc

metBlev (SEQ ID NO 40): TAATCGCGGAT CCGCGTCATG GTTTTTGGCC AGCG

GapAF (1860639-1860661; SEQ ID NO 41): acgtcccggg caagcccaaa ggaagagtga ggc

The fusion fragment was cloned into the *Sma*I and *Bam*HI site of the vector pSB1 described in example 1, resulting in vector pSB8.

- 10 Both vectors pSB7 and pSB8 were transformed into the strain $\Delta metJ \Delta metB \Delta metA$ resulting in DmetBJ DmetC DmetA pSB7 pSB8. This strain grew after a lag period with a growth rate of 0.21 h⁻¹. Plasmids and mutations were verified and the amount of methionine quantified.

Strain	Methionine (mmol/g Dw)	Isoleucine (mmol/g Dw)	OSHS or OAHS (mU/mg protein)	γ -elimination (mU/mg protein)
$\Delta metBJ \Delta metC metA^{*11}$ pSB5	0.23	0.62	1704 ₁	307 ₁
$\Delta metBJ \Delta metA \Delta metC$ pSB7 pSB8	0.57	0.14	194	< 0.05

15

Table 3 Specific methionine production of strains harboring *E. coli* (pSB5) and yeast (pSB8) MetB and their corresponding O-succinyl homoserine sulphydrylase (OSHS), O-acetyl homoserine sulphydrylase (OAHS) and γ -elimination activities. ₁values were obtained in strain $\Delta metBJ metA^{*11}$ pSB5.

20

Table 3 shows that the amount of methionine produced is significantly increased in the presence of the yeast MET2 and MET17 gene. At the same time the amount of isoleucine produced was reduced. This was explained by the low γ -eliminase activity of the MET17 enzyme (The γ -eliminase activity of the yeast acetyl-homoserine sulphydrylase
25 was determined *in vitro* as described in example 1).

Example 6: Construction and evaluation of a methionine producing strain expressing archaeal *metB* from *Methanosarcina barkeri* (strain Fusaro)

- 30 As in plants, in archaea methionine biosynthesis is supposed to proceed via phosphohomoserine. Therefore we presume that similar to the plant enzyme, MetB from *Methanosarcina* may also have a low γ -eliminase activity. *Methanosarcina metB* is therefore PCR amplified using the oligonucleotides metBmethano and gapA-

metBmethanoR. As described previously the *gapA* promoter from *E. coli* is subsequently fused to the *metB* gene using the oligonucleotides gapA-metBmethanoF and GapAF for the amplification of the promoter and metBmethano and GapAF for the actual fusion. The resulting fragment is cloned into the restriction site *SmaI* of the vector pJB137, resulting in plasmid pSB9.

MetBmethano (SEQ ID NO 42): GTCCCCCGGG AATCTAGTCT AGATTAAATT
ACTTCAAGG GCCTGTTTGA GG

gapA-metBmethanoR (32-69 : 1860797-1860761) (SEQ ID NO 43): cacattttgt tgcaaaactc
acttctcttt ccatatattc caccagctat ttgttagtga ataaaagg

10 gapA-metBmethanoF (1-38 : 1860761-1860797) (SEQ ID NO 44): ccttttattc actaacaat
agctgggtgga atatatggaaa gagaagtga gttgcaaca aaatgtg

GapAF (1860639-1860661) (SEQ ID NO 45): acgtcccggg caagcccaaa ggaagagtga ggc

Plasmid pSB9 is introduced into strains $\Delta metJ$ *metA**11, $\Delta metJ$ $\Delta metA$ $\Delta metB$ and $\Delta metJ$ $\Delta metA$ $\Delta metB$ $\Delta thrC$ and the resulting strains are fermented as described in example
15 2. Methionine production is increased and isoleucine production decreased when compared to the strain $\Delta metJ$ *metA**11 harboring the plasmid pSB5 with the *E. coli metB* gene. This is most likely due to the low γ -eliminase activity of the archaeal *metB* enzyme.

20 **Example 7: Construction and evaluation of a strain expressing the metB gene of *Chloroflexus aurantiacus***

The alignment in figure 2 shows that the MetB enzyme of *Chloroflexus aurantiacus* harbors several amino acids at positions required for the recognition of phosphohomoserine as a substrate. Therefore we presume that this enzyme like plant METB may have a lower γ -eliminase activity compared to the *E. coli* enzyme. To
25 determine if the use of the *metB* enzyme confers an advantage in the production of methionine *Chloroflexus metB* is PCR amplified using the oligonucleotides metBchloro and gapA-metBchloroR. As described previously the *gapA* promoter from *E. coli* is subsequently fused to the *metB* gene using the oligonucleotides gapA-metBchloroF and GapAF for the amplification of the promoter and metBchloro and GapAF for the actual
30 fusion. The resulting fragment is cloned into the restriction sites *BamHI* and *SmaI* of the vector pACYC177 (Biolabs), resulting in vector pSB10.

metBchloro (SEQ ID NO 46): ACGTGGATCC GAATTCCTTA TTCGTCGGCA
AGAGCCTGTT GC

gapA-metBchloroR (33-70 : 1860797-1860761) (SEQ ID NO 47): ggccgtacgg gtccggtaaa
35 ctgatcgata gccatatatt ccaccagcta ttgttagtg aataaaagg

gapA-metBchloroF (1-38 : 1860761-1860797) (SEQ ID NO 48): ccttttattc actaacaat
agctgggtgga atatatggct atcgatcagt ttaccggacc cgtacggcc

GapAF (1860639-1860661) (SEQ ID NO 49): acgtcccggg caagcccaaa ggaagagtga ggc

To evaluate the enzyme in methionine biosynthesis the plasmid pSB10 is introduced into the strains $\Delta metJ metA^*11$, $\Delta metJ \Delta metA \Delta metB$ and $\Delta metJ \Delta metA \Delta metB \Delta thrC$ and the resulting strains are fermented as described in example 2. Methionine production is significantly increased when compared to the strain $\Delta metJ metA^*11$ harboring the plasmid pSB6 with the *E. coli* metB gene.

Example 8: Construction of a strain expressing yeast homoserine acetyltransferase and acetylhomoserine sulphydrylase *metY* from *Corynebacterium glutamicum*

In order to test the γ -eliminase activity of *C. glutamicum* *in vivo* and to evaluate its potential for the production of methionine the following strain is constructed.

The codon usage of the *C. glutamicum* acetyl-homoserine sulphydrylase gene, *metY*, is adapted to *E. coli* and it is synthesized *in vitro*. Simultaneously the *gapA* promoter of *E. coli* is added.

The fusion fragment is subsequently cloned into the *Sma*I and *Bam*HI site of the vector pSB1 described in example 1, resulting in vector pSB11.

Both vector pSB7 carrying the yeast homoserine acetyltransferase and pSB11 are transformed into the strains MG1655 $\Delta metJ metA^*11$ and $\Delta metJ \Delta metB \Delta metA$ and the amount of methionine produced determined. It can be shown that the amount of methionine produced is significantly increased in the presence of the yeast homoserine acetyltransferase and the codon adapted Corynebacterial acetylhomoserine sulphydrylase, *metY*, when grown with hydrogen sulfide or sulfate as sulfur source. At the same time the amount of isoleucine produced is significantly reduced. Alternatively a codon adapted homoserine transacetylase from *Corynebacterium* may be used instead of the yeast enzyme.

Example 9: Construction of a strain expressing an *E. coli* homoserine succinyltransferase with reduced γ -elimination activity

To assure that isoleucine is not produced via its classic biosynthesis pathway the gene *ilvA* and the operon *tdc*ABCDEFGH harbouring a second threonine deaminase were deleted.

The *ilvA* gene was deleted using the deletion strategy described above and the following oligonucleotides:

DilvAF (SEQ ID NO 50)

GgctgactcgcaaccctgtccggtgctccggaaggtgccgaatatttaagagcagtgctgcgcgcgccggtttacgaggTGT

AGGCTGGAGCTGCTTCG

with

- a region (lower case) homologous to the sequence (3952954-3953033) of the gene *ilvA* (reference sequence on the website <http://genolist.pasteur.fr/Colibri/>),

- a region (upper case) for the amplification of the chloramphenicol resistance cassette (reference sequence in Datsenko, K.A. & Wanner, B.L., 2000, PNAS, 97: 6640-6645),
DilvAR (SEQ ID NO 51)

cctgaacgccgggtattggttcgtcgtggcaatcgtagcccagctcattcagccgggttcgaaatccgggtcatggCATAT
5 GAATATCCTCCTTAG

with

- a region (lower case) homologous to the sequence 3954478-3954400) of the gene *ilvA*
- a region (upper case) for the amplification of the chloramphenicol resistance cassette.

The oligonucleotides DilvAR and DilvAF were used to amplify the
10 chloramphenicol resistance cassette from the plasmid pKD3. The PCR product obtained
was then introduced by electroporation into the strains MG1655 Δ metBJ *metA*11 (pKD46)
and Δ metJ *metA*11 (pKD46) in which the Red recombinase enzyme expressed permitted
the homologous recombination. The chloramphenicol resistant transformants were then
selected and the insertion of the resistance cassette was verified by a PCR analysis with the
15 oligonucleotides *ilvAR* and *ilvAF* defined below. The resulting strains was Δ metBJ
*metA**11 Δ *ilvA*.

ilvAR (3954693-3954670) (SEQ ID NO 52): gccccgaaccggtgcgtaaccgcg

ilvAF (3952775-3952795) (SEQ ID NO 53): ggtaagcgatgccgaactggc

20 In addition to *IlvA* the threonine dehydratase (*TdcB*) is known to catalyze the
deamination of threonine to α -ketobutyrate under anaerobic or microaerobic conditions. To
eliminate the possible contribution of this enzyme to α -ketobutyrate production the gene
was deleted from the genome of the strain Δ metBJ *metA**11 Δ *ilvA*. *TdcB* is part of the
operon *tdcABCDEFG* that was deleted in a similar way as described for previous mutants
25 using the four oligonucleotides described below. *DtdcGR* and *DtdcAF* were used to
amplify the cassette and *tdcGR* and *tdcGF* for the verification

DtdcGR (3255915-3255993) (SEQ ID NO 54)

gctgacagcaatgtcagccgcagaccactttaatggccagtcctccgcgtgatgttcgcggtattatcgttcatacCATATG
AATATCCTCCTTAG

30 *DtdcAF* (3264726-3264648) (SEQ ID NO 55)

GgtaattaacgtaggtcggtatgagcactattcttctccgaaaacgcagcacctggtagctttcaggaagtcattagTG TAGGCTGG
AGCTGCTTCG

tdcGR (3255616-3255640) (SEQ ID NO 56)

gcgtctgcaatgacgcctttattcg

35 *tdcAF* (3264922-3264899) (SEQ ID NO 57)

Cgccataaaatatggttatccccg

The resulting strain was called Δ metBJ *metA**11 Δ *ilvA* Δ *tdcABCDEFG*.

To decrease the γ -eliminase activity of *E. coli* cystathionine- γ -synthase/sulphydrylase (MetB), mutations were introduced into regions that are involved in the binding of the substrate cysteine. To this end *Escherichia coli metB* was PCR-amplified from genomic DNA using the oligonucleotides MetBF and MetBR (numbers in parentheses correspond to positions on the *E. coli* genome). The PCR fragment was

restricted by *Pst*I and *Hind*III and cloned into pUC18 into the same restriction sites. MetBF (4125957-4125982) (SEQ ID NO 58): ttagacagaa **ctgcagc**gcc gctccattca gccatgagat ac

MetBR (4127500-4127469) (SEQ ID NO 59): cgtaacgccc**a agctt**aaata acacttcaca tcagccagac tactgcc

Subsequently mutations were introduced into the *Escherichia coli* cystathionine- γ -synthase/sulphydrylase that result in the amino acid changes T335A/A337P, R49L and D45V.

The following pairs of oligonucleotides were used for the introduction of each mutation using site directed mutagenesis according to Stratagene's Quick changeTM site directed mutagenesis KIT. Restriction sites (bold) were introduced for verification.

metBT335A/A337PF (SEQ ID NO 60): cgcgcttctg gtgccatgcc **cggatgtgcc atgggtgcgg** cg

metBT335A/A337PR (SEQ ID NO 61): cgccgcaacc **atggcacatc cgggcatggc** accagaagcg cg

metBR49LF (SEQ ID NO 62): **gaacctcgagcgc**atgattactcgcgtctgggcaacccaacgcgcg

metBR49LR (SEQ ID NO 63): **cgcgcgttgggttgcccagacgcgagtaatcatgcgctcgagggtc**

metBD45VF (SEQ ID NO 64): **gaacctcgagcgc**atgtgtactcgcgtcgcggcaacccaacgcgcgat

metBD45VR (SEQ ID NO 65): atcgcgcgttgggttgccgcgacgcgagtagacatgcgctcgagggtc

The resulting modified *metB* sequences were verified by sequencing, restricted with *Pst*I and *Hind*III and cloned into the same sites of vector pSB1. The resulting plasmids were transformed into the strain $\Delta metBJ metA^{*11} \Delta ilvA \Delta tdcABCDEFG$. Strains were evaluated in small Erlenmeyer flask cultures as described above. The amount of isoleucine produced by γ -elimination was significantly reduced, whereas methionine synthesis was only slightly affected. This correlates with the low γ -elimination activity and the retention of a significant cystathionine- γ -synthase activity.

Strain	Meth (mmol/gD w)	Iso (mmol /gDw)	CGS (mU/mg protein)	γ -elim (mU/mg protein)
DmetBJ metA*11 DilvA DtdcABCDEFGF pSB5	0.96	0.88	3790	101
DmetBJ metA*11 DilvA DtdcABCDEFGF pSB1-metB**T335A/A337P	0.24	0	425	1
DmetBJ metA*11 DilvA DtdcABCDEFGF pSB1-metB**D45V	0.69	0.33	891	34
DmetBJ metA*11 DilvA DtdcABCDEFGF pSB1-metB**R49L	0.99	0.48	445	22

CLAIMS

- 1) A microorganism in which enzymes are expressed that have one or several of the following activities cystathionine- γ -synthases and/or phosphohomoserine
5 sulfhydrylase and/or acylhomoserine sulfhydrylases and that have at the same time low γ -elimination activity.
- 2) The microorganism of claim 1 wherein cystathionine- γ -synthase and/or phosphohomoserine sulfhydrylase and/or acylhomoserine sulfhydrylase have a γ -elimination activity that is at least two times inferior, preferentially at least 10 times
10 inferior when compared to the *E. coli* cystathionine- γ -synthase.
- 3) The microorganism of one of claims 1 or 2 wherein phosphohomoserine accepting cystathionine- γ -synthase/phosphohomoserine sulfhydrylase is expressed.
- 4) The microorganism of claim 3 wherein the cystathionine- γ -synthase and/or phosphohomoserine sulfhydrylase is the METB gene of plants, preferentially of
15 *Arabidopsis thaliana*.
- 5) The microorganism of one of claims 1 or 2 wherein the cystathionine- γ -synthase and/or O-acetyl homoserine sulfhydrylase is the METB gene of *Saccharomyces cerevisiae*.
- 6) The microorganism of one of claims 1 or 2 wherein the cystathionine- γ -
20 synthase and/or phosphohomoserine sulfhydrylase is derived from archaea, preferentially from *Methanosarcina barkeri*.
- 7) The microorganism of one of claims 1 or 2 wherein the cystathionine- γ -synthase and/or phosphohomoserine sulfhydrylase is derived from *Chloroflexaceae*, preferentially from *Chloroflexus aurantiacus*.
- 25 8) The microorganism of one of claims 1 or 2 wherein the enzyme has at least two of the following amino acids at positions 107E, 111Y, 165K, 403S.
- 9) The microorganism of one of claims 1 or 2 wherein the cystathionine- γ -synthase and/or acylhomoserine sulfhydrylase is the *metY* and/or the *metB* gene of gram-positive bacteria.
- 30 10) The microorganism of one of claims 1 to 9 wherein the native or heterologous cystathionine- γ -synthase and/or phosphohomoserine sulfhydrylase and/or acylhomoserine sulfhydrylase enzyme is optimized and as a consequence has a lower γ -eliminase activity and thus allows increased methionine selectivity at the expense of isoleucine .
- 35 11) The microorganism of claim 1 wherein an optimized cystathionine- γ -synthase and/or phosphohomoserine sulfhydrylase and/or acylhomoserine sulfhydrylase enzyme is expressed that has a proline at position 337.

12) The microorganism of claim 1 wherein an optimized cystathionine- γ -synthase and/or phosphohomoserine sulfhydrylase and/or acylhomoserine sulfhydrylase enzyme is expressed that has an alanine at position 335.

13) The microorganism of claim 1 wherein an optimized cystathionine- γ -synthase and/or phosphohomoserine sulfhydrylase and/or acylhomoserine sulfhydrylase enzyme is expressed that has an alanine at position 335 and a proline at position 337 .

14) The microorganism of one of claims 1 to 13 wherein the polynucleotide, which codes for the cystathionine- γ -synthase and/or phosphohomoserine sulfhydrylase and/or acylhomoserine sulfhydrylase is overexpressed.

15) The microorganism of one of claim 1 to 13 wherein the catalytic properties of the cystathionine- γ -synthase and/or the phosphohomoserine sulfhydrylase and/or acylhomoserine sulfhydrylase are improved.

16) The microorganisms of one of claims 1 to 15 wherein heterologous homoserine acyltransferases are introduced that permit the use of a corresponding acylhomoserine accepting cystathionine- γ -synthase and/or acylhomoserine sulfhydrylases.

17) The microorganism of one of claims 1 to 16 wherein several different hybrid pathways are actively producing homocysteine and/or cystathionine from homoserine.

18) The microorganism of one of claims 1 to 17 wherein further genes of the biosynthesis pathway of the desired amino acid to be produced are additionally enhanced.

19) The microorganism of one of claims 1 to 17 wherein the metabolic pathways that reduce the production of the desired amino acids are at least partially reduced.

20) A method for the fermentative preparation of an amino acid, in particular methionine its precursors or derivatives using a microorganism as described in one of claims 1 to 19 comprising the steps of:

- a) fermentation of the microorganism producing the desired amino acid
- b) Concentration of the desired product in the cells of the bacteria or in the medium and
- c) isolation of the desired amino acids/ constituents of the fermentation broth and/or the biomass optionally remaining in portions or in the total amount (0-100%) in the end product.

21) The method of claim 20 wherein the sulfur molecule/compound is transferred to any activated homoserine from cysteine.

22) The method of claim 20 wherein the sulfur molecule/compound is transferred directly from H_2S to any activated homoserine.

23) The method of claim 20 wherein the sulfur source in the medium is sulfate or a derivative.

- 24) The method of claim 20 wherein the sulfur source in the medium is thiosulfate.
- 25) The method of claim 20 wherein the sulfur source in the medium is H₂S.
- 26) The method of claim 20 wherein the sulfur source in the medium is
5 methylmercaptan.

Fig. 1

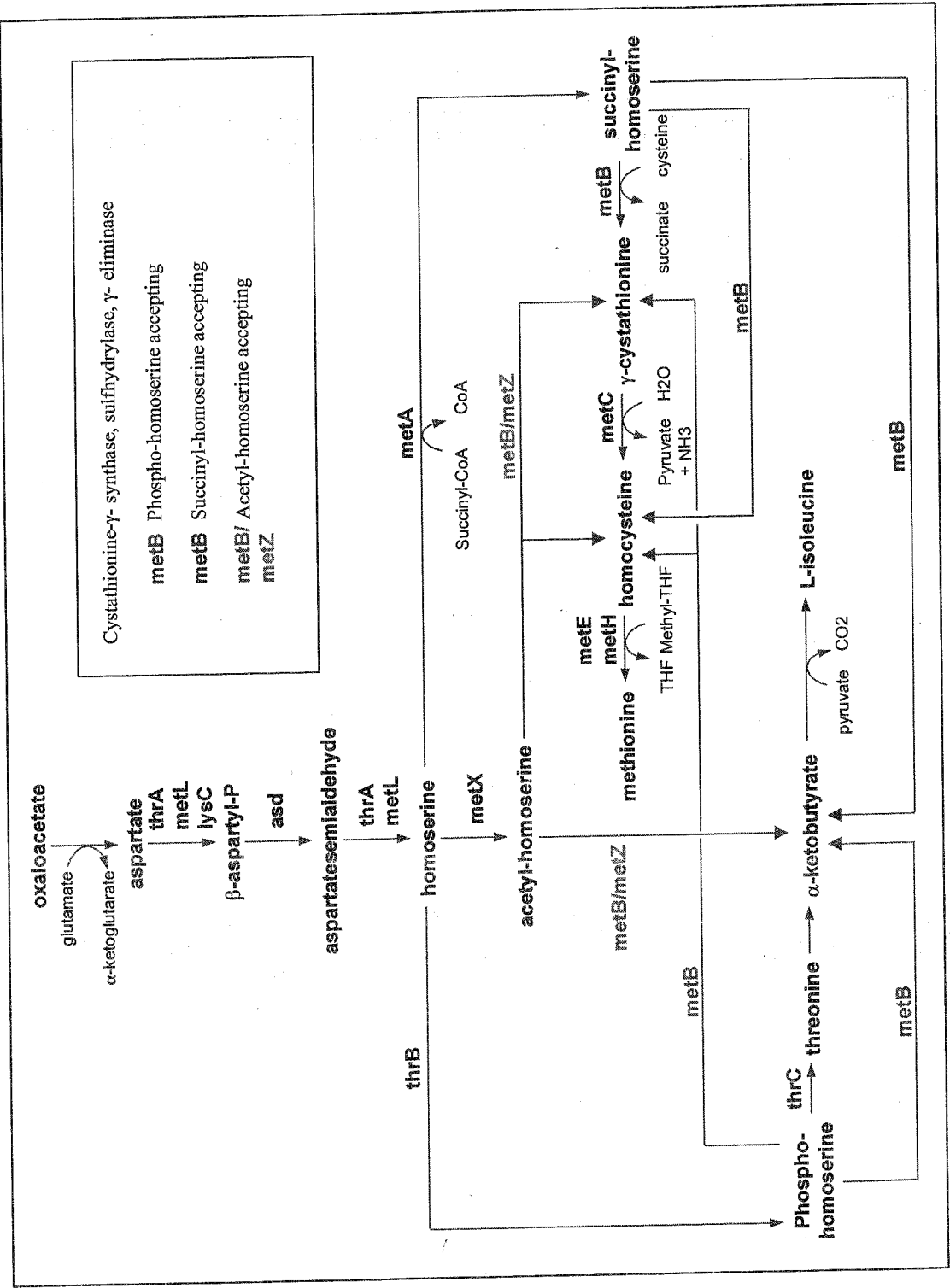


Fig. 2

gi 2852454	MAVSSFQCPT	IFSSSISGF	QCRSDPDLVG	SPVGGSSRRR	VHASAGISSS
gi 8439541	MAVSS-----	--YARAFPSF	ECRSEPDFSG	SLPHPKAGVR	FSGKYNSSGN
gi 4959932	MAVSS-----	--SHMRF-TF	ECRSDPDFSP	P-PPSFDNLR	RRNFRSSADS
gi 4322948					
gi 2198853				MATV	SLTPQAVFST
gi 1160283					
gi 5379875					
gi 4191271					
gi 3050421					
gi 6323333					
gi 4883842					
gi 2132341					
Consensus					
AFTGDAGLSSR	ILRFPPNFVR	QLSIKARRNC	SNIGVAQIVA	AKWSNNPSSA	LPSAAAAAAT
SRSQVYGLSSL	IFRFPPNFVR	QLSIKARRNC	SNIGVAQVVA	ASWSNNQAG-	-----PEFT
AGAAFHGICSL	ILRFPPNFQR	QLSTKARRNC	SNIGVAQIVA	ASWSNNSDN-	-----SPAA
A					MAKAVDAA
SEGGALASAT	ILRFPPNFVR	QLSTKARRNC	SNIGVAQIVA	AAWSDCPAAR	-----PHLGG
					GRRARGVA
.....					

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Fig. 2 (to be continued)

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APVAAAAPPVV LKSVDEEVVV AEEGIREKIG SVQLTD-SK- --HSFLSSDG SLTVHAGERL GRGIVTDAIT TPVVNTSAYF
SASAAVTSIG ITAGDEEVAV VENA-DCNDQ NVQITG-TGV KYASFNSDG SVAIHAGERL GRGIVTDAIT TPVVNTSAYF
TDAATVPLPV VVAANEDVVV SAAA-DENGA -VQLNS-SS- -YSSFLKSDA SKTIHAAERL GRGIETDGIT TPVVNTSAYF
AAAAAIAAPVD TTVVNEDVAL VENE-TCNDQ NVQFDSLPSM KYASFNSDG SVAIHAGERL GRGIVTDAIT TPVVNTSAYF
SHAAAAASAAA AASAAAEEVSA IPNAKVAQPS AVVLAE---- --RNLLGSDA SLAVHAGERL GRRIATDAIT TPVVNTSAYW
SAYW
MAIDQFTGPS TAAVHAGEPR QRAF--DAIT PPVVHSATYT
MTKTWRPA TQLVHGCTWR SEY---GETS EAIYLTQGFV
MTRKQA TIAVRSGLND DEQY--GCVV PPIHLSSTYN
MPSHFD TVQLHAGQEN PGDNAHRSRA VPIYATTSYV
--KSESLGVS TLAVHAGAKP --DPATGARS VPIYQTAAYV
--NADQWGFV TRSIHAGQSV --DAQTSARN LPIYQSTAFV
..... t..vhag.... .p!..t.a%.
MKDK NRNLNEKEGK ENRGSQTNES QTKESREKK-
MPKYDNS- .....
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FKKTAELIDF KEKRSVSFEY GRYGNPTTVV LEDKISALEG AES---TLVM ASGMCASVTM LLALVPAGGH IVTTTDCYRK
FNKTSIDLIDF KEKRRASFEEY GRYGNPTTVV LEEKISALEG AES---TLIV ASGMCASVTM FLALVPAGGH IVTTTDCYRK
FKKTAIDLIDF KENRQVSFEY GRYGNPTTVV LEEKISALEG AES---TVIM ASGMCASVVL FMALVPAGGH LVTTTDCYRK
FNKTSSELIDF KEKRRASFEEY GRYGNPTTVV LEEKISALEG AES---TLLM ASGMCASVTM LLALVPAGGH IVTTTDCYRK
FNNSQELIDF KEGRHASFEY GRYGNPTTEA LEKKMSALEK AES---TVFV ASGMYAAAM LSALVPAGGH IVTTTDCYRK
FNNSQELIDF KEGRHASFEY GRYGNPTTEA LEKKMSALEK AES---TVFV ASGMYASVAM LSALVPAGGH VVTTTDCYRK
FRDSAELIAF QSGDLEREY GRYGNPTVRA VEARLAALES PTAPAAALLC ASGMNALTTV MLALLPSGSH VILTTDDGYRR
YDTSEPAEAR FKGETDGFY ARYGSPTNDM FEKRMCALEG AED---ARAT ASGMAAVAAA VLCQVKAGDH IVAARALFGS
FTGFNE---- PRAHDY SRRGNPTRDV VQRALAELEG GAG---AVLT NTGMSAIHLV TTVFLKPGDL LVAPHDCYGG
FENSKHGSOL FGLEVPGYVY SRFQNPTSNV LEERIAALEG GAA---ALAV SSGQAQTLA IQGLAHTGDN IVSTSILYGG
FKDAEEAADL FGLRKEGNIY TRLMNPTDV FEKRIAALLEG GIG---ALAV ASGMAAITTA LLTFTTRPGDE IISGDKLYGG
FDSAETHAKQR FALEDLGPVY SRLTNPTVEA LENRIASLEG GVH---AVAF SSGQAATTNA ILNLGAGADH IVTSPLRYGG
F...s.e.... .RygnPT... l#.r.aaleg .....a.... asGm.A.t... .l.l..agdh i!.t.d.%g.
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Fig. 2 (to be continued)

TRIFMENFLP	KLGITVTVID	PADIAGLEAA	VNEFKVSLFF	TESPTNPFLR	CVDIELVSKI	CHKRGTLVCI	DGTFATP-LN
TRVFIETILP	KMGITATVID	PADMGALILT	LNQKKVDLFF	TESPTNPFLR	CVDIELVSKL	CREKGALVCI	DGTFATP-LN
TRIFIETFLP	KMGITTTVID	PADVGALESA	LEQHNVSLEFF	TESPTNPFLR	CVDIKLVSEL	CHKKGTLICI	DGTFATP-LN
TRIFIETILP	KMGITATVID	PADVGALELA	LNQKKVNLEFF	TESPTNPFLR	CVDIELVSKL	CHEKGALVCI	DGTFATP-LN
TRIYMETELP	KRGISMTVIR	PADMDALQNA	LDNNVSLFF	TETPTNPFLR	CIDIEHVSNM	CHSKGALLCI	DSTFASP-IN
TRIYMETELP	KTKSQMTVIR	PADMDALQNA	LDNNVSLFF	TETPTNPFLR	CIDIDLVS KM	CHSKGALLCI	DSTFASP-IN
TRQFVRTMLA	RLGVTHSVVA	AADPAIAAAA	IEPGRTRLIV	TEAPTNPYL R	VIDLAAVASI	AREHRIKTLI	DATFATP-YN
CRWVETLAP	KYGVECTLVD	GRDLANWEKA	IRP-NTKVFF	LESPTNP TLE	VVDISGVAKL	ANQVGAKLIV	DNVFATP-LF
SYRLFDSLAK	RGCYRVLFVD	QGDEQALRAA	LAE-KPKLVL	VESPSNPLLR	VVDIAKICHL	AREVGAVSVV	DNTFELSP-AL
TYNQFKISFK	RFGIEARFVE	G-DNPEEFK	VFDERTKAVY	LETIGNPKYN	VPDFEKIVAI	AHKHGIPVVV	DNTFGAGGYF
TYELFNYYTFF	KLGRTVKFVD	V-GKPEEFKN	AISEKTKAIY	VESIGNPGLD	IPDFEKLA EI	AHGAGIPFVV	DNTVSP--LI
TETFLITLN	RLGIDVSFVE	NPDDPESWQA	AVQPNTKAFF	GETFANPQAD	VLDIPAVAEV	AHRNSVPLII	DNTIATAALV
tr.....lp	k.g.....!d	..d.....a	tklff	.EsptNP.l r	v.Di..v....	ah..g.....!	Dntfa.p....
OKALALGADL	VVHSATKYIG	HNDVLAGCI	C-----	-----	-----	GSL	KLVSE-IRNL H
OKALALGADL	VVHSATKFLG	HNDVLAGCI	S-----	-----	-----	GPE	KLVSV-IRNL H
OKALALGADL	ILHSLTKYMG	HHDVLGGCI	S-----	-----	-----	GSI	KVVSQ-IRTL H
OKALALGADL	VLHSATKFLG	HNDVLAGCI	S-----	-----	-----	GPL	KLVSE-IRNL H
OKALT LGADL	VIHSATKYIA	HNDVIGCV	S-----	-----	-----	GRD	ELVSK-VRIY H
OKALT LGADL	VIHSATKYIA	HNDVIGCV	S-----	-----	-----	GRD	ELVSK-VRIY H
MRPLEYGIDL	VVHSCSKYLA	HNDLLAGVI	I-----	-----	-----	GRP	PIISA-LRET Q
QKPLELGAHI	VVYSATKHID	QGACLGCV	L-----	-----	-----	ADK	AWIDENLHDY F
QNPLALGADL	VLHSC TKYLN	HSDDVAGV	I-----	-----	-----	AKD	PDVVTELAWW A
CQPIKYGADI	VTHSATKWIG	HGTTIGGI	VDSGKFPW--	-KDYPEKFPQ	FSQPAEGYHG	TIYNEAYGNL	AYIVHVRTTEL L
LRPIDHGVDI	VVYSATKFIG	HGTSIGGI	VDSGNFYW--	-K--PEKFFE	ICEPDPGYHG	LKYKEAFGKA	AFIAKARVQF I
-RPLELGADV	VVASLTKFYT	NGSGLGVL	IDGKFDWTV	EKDGPVFPPY	FVTPDAAHYG	LKYAD-LGAP	AFGLKVRVGL L
q.pl.lGadl	VvhSatK.i.	h.d..gGvi				g	

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Fig. 2 (to be continued)

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HVLGGTLNP NAAVLIIRGM KTLHLRVQOQ NSTAFRMAEI LEAHPKVSHV YYPGLPSHPE HELAKRQ-MT GFGGVVSFEI
HILGGALNP NAAVLIIRGM KTLHLRVQOQ NSTALRMAEI LEAHPKVSHV YYPGLPSHPE YHLAKKQ-MT GFGGVVSFEV
HVLGGTLNP NAAVLIIRGM KTLHLRVQOQ NSTGMRMAKL LEAHPKVSHV YYPGLPSHPE HELAKRQ-MT GFGGVVSFEI
HILGGALNP NAAVLIIRGM KTLHLRVQOQ NSTALRMAEI LEAHPKVSHV YYPGLQSHPE HHIAKKQ-MT GFGGAVSFEV
HVVGVLNP NAAVLIIRGM KTLHLRVQOQ NNTALRMAQF LEEHLKIARV YYPGLPSHPE HHIAKSQ-MT GFGGVVSFEV
HVVGVLNP NAAVLIIRGM KTLHLRVQOQ NNTAMRMAQF LEEHPKIARV YYPGLPSHPE HHIAKSQ-MT GFGGVVSFEV
GILGGICDP HTAYLLGRGL KTFALRMRERH NHNGQAVAEF LARHPRVSRV HYPGLPEHPD HAVARAQ-MR GFGGVVSFEV
RHTGPAMSP FNAWTLKGI ETPLRVQOQ TENAAKIADF LAEQGKVAKV IYPGRKDHPQ AEIIAKQ-MT GGSTLVCFEL
NNIGVTGGA FDSYLLLRGL RTLVPRMELA QRNAQAIVKY LQTQPLVKKL YHPSLPENQG HEIAARQ-QK GFGAMLSFEL
RDLGPIMLNP FASFLLLQGV ETLSLRAERH GENALKLAKW LEQSPYVSWV SYPGLASHSH HENAKKYLSN GFGGVLSFGV
RDTGACISP FNSFLFTLGL ETPLRMKKH CDNALEIAKF LEKHPKVSWV SYPGLESHCS YELAKKYLS GYGALIGFGI
RDTGSTLSA FNAWAAVQGI DTLSRLRERH NENAIVKAEF LNNHEKVEKV NFAGLKDSPW YATKEKLGK YTGSVLTFEI
....G....p f.ayl.lrg. .Tl.lR.... n.na....a.f l..hpk!..v .ypgl...hp. he.a.kq.m. gfggv.sfe.

D---GDIETT IKF----- VDSLKIPYIA PSFGGCEISV DQPAIMSYWD LPQEERLKYG IKDNLVRFSF GVEDFEDVKA
D---GDLITT AKF----- VDALLRIPYIA PSFGGCEISV DQPAIMSYWD LSQSDRAKYG ILDNLVRFSF GVEDFEDVKA
D---GDLHTT IKF----- IDSLKIPYIA ASFGGCEISV DQPAILSYWD LPQSERAKYK IYDNLVRFSF GVEDFEDLKA
D---GDLITT AKF----- VDALKIPYIA PSFGGCEISV DQPAIMSYWD LSQSDRAKYG IMDNLVRFSF GVEDFDDLKA
A---GDFDAT RKF----- IDSVKIPYHA PSFGGCEISII DQPAIMSYWD -SKEQRDIYG IKDNLIRFSI GVEDFEDLKN
A---GDFDAT RRF----- IDSVKIPYHA PSFGGCEISII DQPAIMSYWD -SKEQREIYG IKDNLIRFSI GVEDFEDLKN
V---GDLQSA MAV----- VDRLRLPYIA PSFGGVESLV EQPAIMSYYE LSSEERLAVG IRDNLIRLSC GIEDVSDLLA
K---GGKEAA FAL----- QNALEIKIS NNLGDTKSLI THPATTTTHKN LTEEAREALG ISPGTVRLSA GIEDTDDLLIE
D---GDEQTL RRF----- LGGLSLFTLA ESLGVESLI SHAATMTHAG MAPEARAAAG ISETLLRIST GIEDGEDLLIA
KDLPNADKET DPFKLSGAQV VDNLKLASNL ANVGDAKTLV IAPYFTTHKQ LNDKEKLASG VTKDLIRVSV GIEFIDDIIA
K---GGAREC KKF----- IEGLEIFSHL ANIGDAKSLV IHPASTTHEQ LSKEEQIACG VTEDFIRLSI GIEDAKDLIS
K---GGKDEA WAF----- IDALKLHSNL ANIGDVRSIV VHPATTTTHSQ SDEAGLARAG VTQSTVRLSV GIETIDDIIA
....gd.... .f..... .d.l.i....a .s.Gg.esl! ..pa.mth.. l..e.r...G !...l.R.S. G!Ed..Dlia

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Fig. 2 (to be continued)

DILQALEAI
DVLQALDSI
DVLOALEAI
DILQALDSI
DLVQALEKI
DVVQALDKI
DLQQALADE
DFARGLTKVS A
DLENGFRAAN KG
DFQQSFETVF AGQP
DIKQALSEV
DLEGGFAAI
D...qal.....