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(54) Title: RECOMBINANT ENZYME WITH ALTERED FEEDBACK SENSITIVITY

(57) Abstract: The present invention relates to the use of recombinant homoserine transsuccinylase enzymes with altered feedback sensitivity (MetA*) and optionally, recombinant S-adenosyl methionine synthetase enzymes with reduced activity (MetK*) for the production of methionine, its precursors or derivatives thereof.



WO 2005/111202 A1

RECOMBINANT ENZYME WITH ALTERED FEEDBACK SENSITIVITY

DESCRIPTIONField of the invention

5 The present invention relates to the use of recombinant homoserine transsuccinylase enzymes with altered feedback sensitivity (MetA*) and eventually, recombinant S-adenosyl methionine synthetase enzymes with reduced activity (MetK*) for the production of methionine, its precursors or derivatives thereof.

10 Prior art

 Sulfur-containing compounds such as cysteine, homocysteine, methionine or S-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
15 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 directly used as a treatment for disorders like allergy and rheumatic fever. Nevertheless most of the methionine which is produced is added to animal feed.

 With the decreased use of animal-derived proteins as a result of BSE and chicken
20 flu, the demand for pure methionine has increased. Chemically D,L-methionine is commonly produced from acrolein, methyl mercaptan and hydrogen cyanide. Nevertheless the racemic mixture does not perform as well as pure L-methionine, as for example in chicken feed additives (Saunderson, C.L., (1985) British Journal of Nutrition 54, 621-633). Pure L-methionine can be produced from racemic methionine e.g. through
25 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 render microbial production of methionine attractive.

 Microorganisms have developed highly complex regulatory mechanisms that fine-tune the biosynthesis of cell components thus permitting maximum growth rates.
30 Consequently only the required amounts of metabolites, such as amino acids, are synthesized and can usually not be detected in the culture supernatant of wild-type strains. Bacteria control amino acid biosynthesis mainly by feedback inhibition of

enzymes, and repression or activation of gene transcription. Effectors for these regulatory pathways are in most cases the end products of the relevant pathways. Consequently, strategies for overproducing amino acids in microorganisms require the deregulation of these control mechanisms.

5 The pathway for L-methionine synthesis is well known in many microorganisms (Fig. 1A/B). Methionine 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
10 or 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
15 methyltransferase.

Methionine biosynthesis in *E. coli* is regulated by repression and activation of methionine biosynthetic genes via the MetJ and MetR proteins, respectively (reviewed in Neidhardt, F. C. (Ed. in Chief), R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger
20 (eds). 1996. *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology. American Society for Microbiology; Weissbach et al., 1991 Mol. Microbiol., 5, 1593-1597). MetJ uses S-adenosylmethionine as a corepressor, that is made from methionine by the enzyme S-adenosylmethionine synthetase (EC 2.5.1.6) encoded by the essential gene *metK*. *metA* encoding homoserine transsuccinylase (EC 2.3.1.46), the first enzyme
25 unique to the synthesis of methionine is another major control point for methionine production. Besides the transcriptional regulation of *metA* by MetJ and MetR the enzyme is also feedback regulated by the end-products methionine and S-adenosylmethionine (Lee, L.-W et al. (1966) Multimetabolite control of a biosynthetic pathway by sequential metabolites, JBC 241 (22), 5479-5780). Feedback inhibition by
30 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.

Amino acid analogues inhibit bacterial growth through the synthesis of abnormal polypeptides and by interfering with feedback inhibition, which lead to detrimental processes inside the cell. Analogue-resistant mutants have been obtained which show altered and deregulated enzymes leading to excess synthesis of the corresponding metabolites that can outcompete the analogue. Several groups have used the methionine analogues norleucine, ethionine, and α -methionine to generate microbial strains that overproduce methionine. It was shown that α -methionine is a potent inhibitor of the homoserine transsuccinylase enzyme MetA (Rowbury RJ, (1968) The inhibitory effect of α -methionine on *Escherichia coli*, J. gen. Microbiol., 52, 223-230). Feedback resistant mutants in *Salmonella typhimurium* that mapped to the *metA* locus were isolated (Lawrence D. A., (1972) Regulation of the methionine feedback-sensitive enzyme in mutants of *Salmonella typhimurium*; Lawrence, D.A., Smith, D.A., Rowbury, R.J. (1967) Regulation of methionine synthesis in *Salmonella typhimurium*: Mutants resistant to inhibition by analogues of methionine, Genetics 58, 473-492). Norleucine resistant mutants were shown to map to the *metK* locus. (Chattopadhyay, M.K., Ghosh, A. K. and Sengupta, S. (1991), Control of methionine biosynthesis in *Escherichia coli* K12: a closer study with analogue resistant mutants, Journal of General Microbiology, 137, 685-691). The same authors have reported the isolation of feedback resistant *metA* mutants and norleucine resistant mutants in *E. coli*, but the actual mutations in *metA* and *metK* have not been described.

The critical role of homoserine transsuccinylase for bacterial methionine production by fermentation has been demonstrated (Kumar, D. Garg, S. Bisaria V.S., Sreekrishnan, T.R. and Gomes, J. (2003) Production of methionine by a multi-analogue resistant mutant of *Corynebacterium lilium*, Process Biochemistry 38, 1165-1171). The patent application JP2000139471 describes a process for the production of L-methionine using mutants in the genes *metA* and *metK*. In this case the precise location of the mutations has been determined. The MetA mutant enzymes have partially lost the sensitivity to feed-back inhibition by methionine and S-adenosyl-methionine. Nevertheless their initial activities are decreased down to about 25% when compared to the wildtype enzyme, and at concentrations of 1 mM methionine for some mutants or 1 mM SAM for others another 25-90 % of the enzyme activity is lost. The *metK* mutants

were not characterized enzymatically but used in fermentations to increase the amount of methionine produced.

General disclosure of the invention

5 This invention relates to a method for the preparation of methionine, its precursors or products derived thereof in a fermentative process with a microorganism where L-homoserine is converted into O-succinyl-homoserine with a homoserine transsuccinylase, comprising the step of culturing the said microorganism on an appropriate medium and recovering methionine, its precursors or products derived
10 thereof once produced, wherein the homoserine transsuccinylase is a mutated homoserine transsuccinylase with reduced sensitivity for the feedback inhibitors S-adenosylmethionine and methionine.

 The invention also relates to the same method with microorganisms where the S-adenosyl-methionine synthetase enzyme activity is reduced.

15 The present invention also concerns the mutated enzymes, nucleotide sequences coding for the said enzymes with reduced sensitivity or activity and microorganisms comprising the said nucleotide sequences as disclosed above and below.

 The recombinant enzymes can be used together and can also be combined with several other changes in the corresponding microorganisms such as overexpression of
20 genes or their deletion. Preferentially the gene encoding aspartokinase/homoserine dehydrogenase (*metL*) is over expressed and the gene encoding the methionine repressor *metJ* is deleted.

 In the description of the present invention, genes and proteins are identified using the denominations of the corresponding genes in *E. coli*. However, and unless specified
25 otherwise, use of these denominations has a more general meaning according to the invention and covers all the corresponding genes and proteins in other organisms, more particularly microorganisms.

 PFAM (protein families database of alignments and hidden Markov models; <http://www.sanger.ac.uk/Software/Pfam/>) represents a large collection of protein
30 sequence alignments. 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.

COGs (clusters of orthologous groups of proteins; <http://www.ncbi.nlm.nih.gov/COG/>) are obtained by comparing protein sequences from 43 fully sequenced genomes representing 30 major phylogenic lines. Each COG is defined from at least three lines, which permits the identification of former conserved domains.

The means of identifying homologous sequences and their percentage homologies are well known to those skilled in the art, and include in particular the BLAST programs, which can be used from the website <http://www.ncbi.nlm.nih.gov/BLAST/> with the default parameters indicated on that website. The sequences obtained can then be exploited (e.g., aligned) using, for example, the programs CLUSTALW (<http://www.ebi.ac.uk/clustalw/>) or MULTALIN (<http://prodes.toulouse.inra.fr/multalin/cgi-bin/multalin.pl>), with the default parameters indicated on those websites.

Using the references given on GenBank for known genes, those skilled in the art are able to determine the equivalent genes in other organisms, bacterial strains, yeasts, fungi, mammals, plants, etc. This routine work is advantageously done using consensus sequences that can be determined by carrying out sequence alignments with genes derived from other microorganisms, and designing degenerate probes to clone the corresponding gene in another organism. These routine methods of molecular biology are well known to those skilled in the art, and are described, for example, in Sambrook *et al.* (1989 Molecular Cloning: a Laboratory Manual. 2nd ed. Cold Spring Harbor Lab., Cold Spring Harbor, New York.).

Detailed description of the invention

The modified homoserine succinyltransferases showing a decreased feed-back sensitivity to methionine and S-adenosylmethionine in comparison to the wild-type enzyme, according to the present invention, comprises at least one amino acid mutation when compared with the wild-type sequence. The mutation is preferentially selected in the conserved regions coding for amino acids 24 to 30 or in the region coding for amino

acids 58 to 65 or in the region coding for amino acids 292 to 298 with the first amino acid proline after the formylmethionine counting as number 1.

All references to amino acid positions are made based on the homoserine succinyltransferase encoded by the *metA* gene of *E. coli* represented in figure 2. The relative positions of corresponding conserved regions in other homoserine succinyltransferases from different organisms can be found by a person skilled in the art by simple sequence alignment as represented on figure 3 with the enzymes listed below:

- gi|20138683|sp|Q97I29|META *Clostridium acetobutylicum* Homoserine O-succinyltransferase
- 10 - gi|12230304|sp|Q9PLV2|META *Campylobacter jejuni* Homoserine O-succinyltransferase
- gi|12230277|sp|Q9KAK7|META *Bacillus halodurans* Homoserine O-succinyltransferase
- gi|20138686|sp|Q97PM9|META *Streptococcus pneumoniae* Homoserine O-succinyltransferase
- 15 - gi|20138715|sp|Q9CEC5|META *Lactococcus lactis* Homoserine O-succinyltransferase
- gi|20138656|sp|Q92L99|META *Sinorhizobium meliloti* Homoserine O-succinyltransferase
- 20 - gi|20138618|sp|Q8YBV5|META *Brucella melitensis* Homoserine O-succinyltransferase
- gi|20141549|sp|P37413|META *Salmonella typhimurium* Homoserine O-succinyltransferase
- gi|20138601|sp|Q8X610|META *Escherichia coli* O157:H7 Homoserine O-succinyltransferase
- 25 - gi|12231004|sp|P07623|META *Escherichia coli* Homoserine O-succinyltransferase
- gi|12230285|sp|Q9KRM5|META *Vibrio cholerae* Homoserine O-succinyltransferase
- gi|38258142|sp|Q8FWG9|META *Brucella melitensis* biovar suis Homoserine O-succinyltransferase
- 30 - gi|20138631|sp|Q8ZAR4|META *Yersinia pestis* Homoserine O-succinyltransferase
- gi|12231010|sp|P54167|META *Bacillus subtilis* Homoserine O-succinyltransferase

- gi|12230320|sp|Q9WZY3|META *Thermotoga maritima* Homoserine O-succinyltransferase
- gi|20138625|sp|Q8Z1W1|META *Salmonella typhi* Homoserine O-succinyltransferase
- 5 - >gi|31340217|sp|Q8D937|META *Vibrio vulnificus* Homoserine O-succinyltransferase
- gi|31340213|sp|Q87NW7|META *Vibrio parahaemolyticus* Homoserine O-succinyltransferase

10 The modified homoserine succinyltransferases preferentially exhibit a specific activity which is at least ten times that of the wild-type enzyme in the presence of 10 mM methionine and 1 mM S-adenosylmethionine and at least 80 times that of the wild-type enzyme in the presence of 10 mM methionine and 0,1 mM S-adenosylmethionine.

15 Preferentially, the protein sequence of the modified homoserine succinyltransferase according to the invention contains the amino acid mutation of at least one of the conserved regions sequences specified below.

20 Preferentially at least one mutation is present in the conserved region 1 comprising the amino acid sequence defined below, in the N-terminal part of the wild type homoserine transsuccinylases, corresponding to amino acid 24 to 30 in the amino acid sequence of *E. coli* MetA shown in SEQ ID NO. 2. This non mutated conserved region 1 has the following sequence:

X1-X2-X3-A-X4-X5-Q

In which

X1 represents E, D, T, S, L, preferentially T

X2 represents D, S, K, Q, E, A, R, preferentially S

25 X3 represents R, E, D, preferentially R

X4 represents Y, I, F, A, K, S, V, preferentially S

X5 represents H, S, N, G, T, R, preferentially G

In a preferred embodiment the unmodified homoserine succinyltransferase conserved region 1 has the following amino acid sequence: T-S-R-A-S-G-Q.

30 In another preferred embodiment of the invention, at least one mutation is present into the conserved region 2, also in the N-terminal part of the wild type

homoserine transsuccinylase, corresponding to amino acid 58 to 65 in the amino acid sequence of *E. coli* MetA shown in SEQ ID NO. 2. The non mutated conserved region 2 has the following formula:

X1-X2-X3-**P-L-Q**-X4-X5

5 In which

X1 represents G, A, S, preferentially S

X2 represents N, A, preferentially N

X3 represents S, T, preferentially S

X4 represents V, L, I, preferentially V

10 X5 represents N, E, H, D, preferentially D

In a preferred embodiment the unmodified homoserine succinyltransferase conserved region 2 has the following amino acid sequence: S-N-S-**P-L-Q**-V-D.

In a third embodiment of the invention the homoserine succinyltransferase comprises at least one mutation in a conserved region in its C-terminal part,
15 corresponding to amino acid 292 to 298 in the amino acid sequence of *E. coli* MetA shown in SEQ ID NO. 2. The non mutated conserved region 3 has the following formula:

X1-**Y-Q**-X2-**T-P**-X3

In which

20 X1 represents V, I, M, preferentially V

X2 represents E, K, G, I, Q, T, S, preferentially I

X3 represents F, Y, preferentially Y

In a preferred embodiment, the unmodified homoserine succinyltransferase conserved region 3 has the following amino acid sequence: V-**Y-Q**-I-**T-P**-Y.

25 In a preferred embodiment, the conserved alanine in conserved region 1 is replaced with another amino acid, more preferentially with a valine. The modified conserved region has most preferentially the following amino acid sequence: T-S-R-**V-S-G-Q**

In another preferred embodiment, the conserved amino acids L and/or Q in conserved
30 region 2 are replaced with other amino acids. Preferentially, leucine is replaced by

phenylalanine and/or glutamine is replaced with a glutamate. Most preferentially, the modified conserved region 2 has the following amino acid sequence: S-N-S-P-L-E-V-D

In a further preferred embodiment, the conserved amino acids L and/or Q in conserved region 3 are replaced with another amino acid.

5 In a preferred application the *metA* gene is the homoserine transsuccinylase enzyme of *E. coli* K12 represented by the SEQ ID NO 1 and sequences homologous to that sequence that have homoserine transsuccinylase activity and that share at least 80% homology, preferentially 90% homology with the amino acid sequence of SEQ ID NO 2.

10 Modified homoserine succinyltransferases may be obtained, for example, by selecting strains growing in the presence of methionine analogues such as α -methylmethionine, norleucine or ethionine. Preferentially these strains will be selected while growing in the presence of α -methylmethionine.

The present invention furthermore relates to nucleotide sequences, DNA or RNA sequences, which encode a mutated homoserine succinyltransferase according to the invention as defined above. In a preferred embodiment, the DNA sequence is
15 characterized by the fact that it comprises at least one mutation in the coding regions of the conserved regions 1 to 3 of the wild type *metA* gene, represented in SEQ ID NO 1, the said mutation being not a silent mutation.

These DNA sequences can be prepared, for example, from the strains growing in
20 the presence of methionine analogues. The starting DNA fragment encompassing the modified *metA* gene, is cloned in a vector using standard known techniques for preparing recombinant DNA.

These DNA sequences can also be prepared, for example, by non-specific or by targeted mutagenesis methods from strains harboring the DNA sequence encoding wild-
25 type homoserine succinyltransferase.

Non-specific mutations within the said DNA region may be produced, for example, by chemical agents (e.g. nitrosoguanidine, ethylmethanesulfonic acid and the like) and/or by physical methods and/or by PCR reactions, which are carried out under particular conditions.

Methods for introducing mutations at specific positions within a DNA fragment are known and are described in *Molecular Cloning: a Laboratory Manual*, 1989,. 2nd ed. Cold Spring Harbor Lab., Cold Spring Harbor, New York.

Using the afore mentioned methods, one or more mutations which cause the modified homoserine succinyltransferase to possess an amino acid sequence which leads to methionine and S-adenosylmethionine insensitivity can be introduced in the said DNA region of any *metA* gene. Preferentially, one or more nucleotides in the DNA sequence encoding homoserine succinyltransferase are changed such that the amino acid sequence that is encoded by the gene exhibits at least one mutation.

Another method of producing feedback-resistant *metA* alleles consists in combining different point mutations which lead to feedback resistance, thereby giving rise to multiple mutants possessing new properties.

The modified S-adenosylmethionine synthetase according to the invention has decreased activity in comparison to the wild-type enzyme, and has at least one mutation in its protein sequence when compared to the wild-type sequence.

The mutation is preferentially in one of the conserved regions defined below.

All references to amino acid positions are made based on the S-adenosylmethionine synthetase encoded by *metK* gene of *E. coli*. The relative positions of corresponding conserved regions in other S-adenosylmethionine synthetases from different organisms can be found by a person skilled in the art by simple sequence alignment as represented in figure 4 with enzymes listed below:

>gi|39574954|emb|CAE78795.1| methionine adenosyltransferase *Bdellovibrio bacteriovorus* HD100]

>gi|45657232|ref|YP_001318.1| s-adenosylmethionine synthetase protein *Leptospira interrogans* serovar Copenhageni str. Fiocruz L1-130

>gi|28378057|ref|NP_784949.1| methionine adenosyltransferase *Lactobacillus plantarum* WCFS1

>gi|26453553|dbj|BAC43885.1| S-adenosylmethionine synthetase *Mycoplasma penetrans*

- >gi|24212014|sp|Q9K5E4| S-adenosylmethionine synthetase *Corynebacterium glutamicum*
- >gi|18145842|dbj|BAB81883.1| S-adenosylmethionine synthetase *Clostridium perfringens* str. 13
- 5 >gi|13363290|dbj|BAB37241.1| methionine adenosyltransferase 1 *Escherichia coli* O157:H7
- >gi|45443250|ref|NP_994789.1| S-adenosylmethionine synthetase *Yersinia pestis* biovar Mediaevails str. 91001
- >gi|44888151|sp|Q7WQX8|METK S-adenosylmethionine synthetase *Borrelia burgdorferi*
- 10 >gi|44888141|sp|Q7U4S6| S-adenosylmethionine synthetase *Synechococcus* sp. WH8102
- >gi|44888135|sp|Q7MHK6| S-adenosylmethionine synthetase *Vibrio vulnificus* YJ016
- >gi|23466330|ref|NP_696933.1| S-adenosylmethionine synthetase *Bifidobacterium longum* NCC2705]
- 15 >gi|21219978|ref|NP_625757.1| S-adenosylmethionine synthetase *Streptomyces coelicolor* A3(2)]
- >gi|39937076|ref|NP_949352.1| methionine S-adenosyltransferase *Rhodopseudomonas palustris* CGA009
- 20 >gi|16766391|ref|NP_462006.1| methionine adenosyltransferase 1 *Salmonella typhimurium* LT2
- >gi|33594910|ref|NP_882553.1| S-adenosylmethionine synthetase *Bordetella parapertussis* 12822
- >gi|44888148|sp|Q7VRG5| S-adenosylmethionine synthetase *Candidatus Blochmannia floridanus*
- 25 >gi|44888147|sp|Q7VNG7|METK_HAEDU S-adenosylmethionine synthetase *Haemophilus ducreyi*
- >gi|44888146|sp|Q7VIFY5| S-adenosylmethionine synthetase *Helicobacter hepaticus*
- >gi|44888145|sp|Q7VDM7| S-adenosylmethionine synthetase *Prochlorococcus marinus*
- 30 >gi|44888142|sp|Q7URU7| S-adenosylmethionine synthetase *Pirellula spec.*
- >gi|44888138|sp|Q7NHG0| S-adenosylmethionine synthetase *Gloeobacter violaceus*

- >gi|44888137|sp|Q7N119| S-adenosylmethionine synthetase *Photorhabdus luminescens* subsp. *laumondii*
- >gi|44888136|sp|Q7MTQ0| S-adenosylmethionine synthetase *Porphyromonas gingivalis*
- >gi|39650934|emb|CAE29457.1| methionine S-adenosyltransferase *Rhodopseudomonas*
- 5 *palustris* CGA009
- >gi|15792421|ref|NP_282244.1| S-adenosylmethionine synthetase *Campylobacter jejuni* subsp. *jejuni* NCTC 11168
- >gi|39574954|emb|CAE78795.1| methionine adenosyltransferase *Bdellovibrio bacteriovorus* HD100
- 10 >gi|45657232|ref|YP_001318.1| s-adenosylmethionine synthetase protein *Leptospira interrogans* serovar Copenhageni str. Fiocruz L1-130
- >gi|28378057|ref|NP_784949.1| methionine adenosyltransferase *Lactobacillus plantarum* WCFS1
- >gi|45600470|gb|AAS69955.1| s-adenosylmethionine synthetase protein *Leptospira*
- 15 *interrogans* serovar Copenhageni str. Fiocruz L1-130
- >gi|26453553|dbj|BAC43885.1| S-adenosylmethionine synthetase *Mycoplasma penetrans*
- >gi|18145842|dbj|BAB81883.1| S-adenosylmethionine synthetase *Clostridium perfringens* str. 13
- 20 >gi|13363290|dbj|BAB37241.1| methionine adenosyltransferase 1 *Escherichia coli* O157:H7
- >gi|45443250|ref|NP_994789.1| S-adenosylmethionine synthetase *Yersinia pestis* biovar Mediaevails str. 91001
- >gi|44888153|sp|Q8CXS7| S-adenosylmethionine synthetase *Leptospira interrogans*
- 25 >gi|44888151|sp|Q7WQX8| S-adenosylmethionine synthetase *Bordetella bronchiseptica*
- >gi|44888150|sp|Q7W200| S-adenosylmethionine synthetase *Bordetella parapertussis*
- >gi|44888149|sp|Q7VUL5| S-adenosylmethionine synthetase *Bordetella pertussis*

The modified S-adenosylmethionine synthetase preferentially exhibits a specific activity which is at least five times less than that of the wild-type enzyme.

Preferentially, the protein sequence of a modified S-adenosylmethionine synthetase contains the amino acid mutation of at least one of the sequences specified below.

In one embodiment of the invention the amino acid changes concern the cysteine at position 89 or the cysteine at position 239 that both reduce the activity of MetK (Reczkowski and Markham, 1995, JBC 270, 31, 18484-18490)

In a preferred embodiment at least one mutation is present in the conserved region 1, corresponding to amino acid 54 to 59 in the amino acid sequence of *E. coli* MetK shown in SEQ ID NO. 4, the non mutated conserved region 1 comprising the amino acid sequence defined below:

G-E-X1-X2-X3-X4

wherein

X1 represents I, V, L, T preferentially I

X2 represents T, K, S, R, preferentially T

X3 represents T, S, G, preferentially T

X4 represents S, N, T, K, E, R, P, N, A preferentially S

Preferentially the unmodified S-adenosylmethionine synthetase conserved region 1 has the following amino acid sequence: **G-E-I-T-T-S**.

In another preferred embodiment at least one mutation is present in the conserved region 2, corresponding to amino acid 98 to 107 in the amino acid sequence of *E. coli* MetK shown in SEQ ID NO. 4, the non mutated conserved region 2 comprising the amino acid sequence defined below:

Q-S-X1-D-I-X2-X3-G-V-X4

wherein

X1 represents P, Q, A, S, preferentially P

X2 represents A, N, Q, S, F, preferentially N

X3 represents V, Q, Y, R, M, N preferentially Q

X4 represents K, D, N, T, S, A, E preferentially D

Preferentially the unmodified S-adenosylmethionine synthetase conserved region 2 has the following amino acid sequence: **Q-S-P-D-I-N-Q-G-V-D**.

In another preferred embodiment at least one mutation is present in the conserved region 3, corresponding to amino acid 114 to 121 in the amino acid sequence of *E. coli* MetK shown in SEQ ID NO. 4, the non mutated conserved region 3 comprising the amino acid sequence defined below

5 X1-G-A-G-D-Q-G-X2

wherein

X1 represents Q, A, I, V, E, T, preferentially Q

X2 represents L, I, S, V, M, preferentially L

Preferentially the unmodified S-adenosylmethionine synthetase conserved region
10 3 has the following amino acid sequence: Q-G-A-G-D-Q-G-L.

In another preferred embodiment at least one mutation is present in the conserved region 4, corresponding to amino acid 137 to 144 in the amino acid sequence of *E. coli* MetK shown in SEQ ID NO. 4, the non mutated conserved region 4 comprising the amino acid sequence defined below:

15 X1-I-X2-X3-X4-H-X5-X6

wherein

X1 represents S, P, T, A, preferentially P

X2 represents A, T, W, Y, F, S, N preferentially T

X3 represents M, Y, L, V preferentially Y

20 X4 represents S, A, preferentially A

X5 represents K, R, E, D, preferentially R

X6 represents L, I, preferentially L

Preferentially the unmodified S-adenosylmethionine synthetase conserved region
4 has the following amino acid sequence: P-I-T-Y-A-H-R-L

25 In another preferred embodiment at least one mutation is present in the conserved region 5, corresponding to amino acid 159 to 163 in the amino acid sequence of *E. coli* MetK shown in SEQ ID NO. 4, the non mutated conserved region 5 comprising the amino acid sequence defined below

X1-L-X2-X3-D

30 wherein

X1 represents W, F, Y, V, E preferentially W

X2 represents R, G, L, K, preferentially R

X3 represents P, L, H, V, preferentially P

Preferentially the unmodified S-adenosylmethionine synthetase conserved region 5 has the following amino acid sequence: **W-L-R-P-D**

5 Preferentially at least one mutation is present in the conserved region 6, corresponding to amino acid 183 to 189 in the amino acid sequence of *E. coli* MetK shown in SEQ ID NO. 4, the non mutated conserved region 6 comprising the amino acid sequence defined below.

X1-X2-X3-S-X4-Q-H

10 In which

X1 represents V, I, preferentially V

X2 represents V, L, I, preferentially V

X3 represents V, L, I, M, preferentially L

X4 represents T, V, A, S, H, preferentially T

15 In a preferred embodiment the unmodified S-adenosylmethionine synthetase conserved region 6 has the following amino acid sequence: **V-V-L-S-T-Q-H**.

Preferentially the mutations are introduced in the conserved region 7, corresponding to amino acid 224 to 230 in the amino acid sequence of *E. coli* MetK shown in SEQ ID NO. 4, the non mutated conserved region 7 comprising the amino acid sequence defined below:

20 **X1-N-P-X2-G-X3-F**

In which

X1 represents I, V preferentially I

X2 represents T, G, S, preferentially T

25 X3 represents R, T, Q, K, S, preferentially R

In a preferred embodiment the unmodified S-adenosylmethionine synthetase conserved region 7 has the following amino acid sequence: **I-N-P-T-G-R-F**

Preferentially the mutations are introduced into the conserved region 8, corresponding to amino acid 231 to 237 in the amino acid sequence of *E. coli* MetK shown in SEQ ID NO. 4, the non mutated conserved region 8 comprising the amino acid sequence defined below.

30

X1-X2-G-X3-P-X4-X5

In which

X1 represents T, V, I, Y, E, preferentially V

X2 represents V, I, L, N, preferentially I

5 X3 represents G, S, preferentially G

X4 represents M, I, Q, A, H, D preferentially M

X5 represents G, S, A, H preferentially G

In a preferred embodiment the unmodified S-adenosylmethionine synthetase conserved region 8 has the following amino acid sequence: **V-I-G-G-P-M-G**

10 Preferentially the mutations are introduced in the conserved region 9, corresponding to amino acid 246 to 253 in the amino acid sequence of *E. coli* MetK shown in SEQ ID NO. 4, the non mutated conserved region 9 comprising the amino acid sequence defined below.

X1-X2-D-T-Y-G-G

15 In which

X1 represents M, I, preferentially I

X2 represents V, I preferentially I

In a preferred embodiment the unmodified S-adenosylmethionine synthetase conserved region 9 has the following amino acid sequence: **I-V-D-T-Y-G-G**

20 Preferentially the mutations are introduced into the conserved region 10, corresponding to amino acid 269 to 275 in the amino acid sequence of *E. coli* MetK shown in SEQ ID NO. 4, the non mutated conserved region 10 comprising the amino acid sequence defined below.

K-V-D-R-S-X1-X2

25 In which

X1 represents A, G, preferentially A

X2 represents A, S, L, preferentially A

In a preferred embodiment the unmodified S-adenosylmethionine synthetase conserved region 10 has the following amino acid sequence: **K-V-D-R-S-A-A**.

In another preferred application of the invention at least one mutation is present in the conserved region 11. The unmodified S-adenosylmethionine synthetase harbors the conserved region in its C-terminal part with the following amino acid sequence:

X1-X2-Q-X3-X4-Y-A-I-G-X5-X6

5 In which

X1 represents L, E, I, Q, T preferentially E

X2 represents V, I, L preferentially I

X3 represents V, L, I preferentially V

X4 represents A, S preferentially S

10 X5 represents V, I, R, K, A preferentially V

X6 represents A, V, T, S, preferentially A

This region corresponds to amino acid 295 to 305 in the amino acid sequence of *E. coli* MetK shown in SEQ ID NO. 4.

15 In a preferred embodiment the unmodified S-adenosylmethionine synthetase conserved region 11 has the following amino acid sequence: E-I-Q-V-S-Y-A-I-G-V-A

In another preferred application of the invention the mutations are introduced into the N-terminus of the protein leading to a frameshift and changes in the last 6 amino acids.

20 In the S-adenosylmethionine synthetase with decreased activity preferentially the serine in conserved region 1 is replaced with another amino acid. In a preferred application of the invention the serine is replaced with a asparagine.

In a preferred embodiment the modified S-adenosylmethionine synthetase has the following amino acid sequence in conserved region 1: G-E-I-T-T-N.

25 In the S-adenosylmethionine synthetase with decreased activity preferentially the conserved glycine in conserved region 2 is replaced with another amino acid. In a preferred application of the invention the conserved glycine is replaced with a serine.

In a preferred embodiment the modified S-adenosylmethionine synthetase has the following amino acid sequence in conserved region 2: Q-S-P-D-I-N-Q-S-V-D

30 In the S-adenosylmethionine synthetase with decreased activity preferentially the conserved glycine in conserved region 3 is replaced with another amino acid. In a preferred application of the invention the conserved glycine is replaced with a serine.

In a preferred embodiment the modified S-adenosylmethionine synthetase has the following amino acid sequence in conserved region 3: **Q-S-A-G-D-Q-G-L**

In the S-adenosylmethionine synthetase with decreased activity preferentially the conserved histidine and/or the semi-conserved proline in conserved region 4 is/are replaced with other amino acids. In a preferred application of the invention the conserved histidine is replaced with a tyrosine and/or the semi-conserved proline is replaced with a leucine.

In a preferred embodiment the modified S-adenosylmethionine synthetase has one of the following amino acid sequences in conserved region 4:

10 **P-I-T-Y-A-Y-R-L,**
 L-I-T-Y-A-H-R-L,
 L-I-T-Y-A-Y-R-L.

In the S-adenosylmethionine synthetase with decreased activity preferentially the semi-conserved arginine in conserved region 5 is replaced with another amino acid. In a preferred application of the invention the arginine is replaced with a cysteine.

In a preferred embodiment the modified S-adenosylmethionine synthetase has the following amino acid sequence in conserved region 5: **W-L-C-P-D**

In the S-adenosylmethionine synthetase with decreased activity preferentially the conserved histidine or semi-conserved valine in conserved region 6 is replaced with another amino acid. In a preferred application of the invention the conserved histidine is replaced with a tyrosine and/or the valine with aspartate.

In a preferred embodiment the modified S-adenosylmethionine synthetase has one of the three following amino acid sequences in conserved region 6:

V-V-L-S-T-Q-Y
25 **V-D-L-S-T-Q-H**
 V-D-L-S-T-Q-Y

In the S-adenosylmethionine synthetase with decreased activity preferentially the semi-conserved threonine in conserved region 7 is replaced with another amino acid. In a preferred application of the invention the threonine is replaced with an isoleucine.

30 In a preferred embodiment the modified S-adenosylmethionine synthetase has the following amino acid sequence in conserved region 7: **I-N-P-I-G-R-F**

In the S-adenosylmethionine synthetase with decreased activity preferentially the conserved proline in conserved region 8 is replaced with another amino acid. In a preferred application of the invention the proline is replaced with a serine.

5 In a preferred embodiment the modified S-adenosylmethionine synthetase has the following amino acid sequence in conserved region 8: **V-I-G-G-S-M-G**

In the S-adenosylmethionine synthetase with decreased activity preferentially the second conserved glycine in conserved region 9 is replaced with another amino acid. In a preferred application of the invention the glycine is replaced with an aspartate.

10 In a preferred embodiment the modified S-adenosylmethionine synthetase has the following amino acid sequence in conserved region 9: **I-V-D-T-Y-G-D**

In the S-adenosylmethionine synthetase with decreased activity preferentially the conserved serine in conserved region 10 is replaced with another amino acid. In a preferred application of the invention the serine is replaced with a phenylalanine.

15 In a preferred embodiment the modified S-adenosylmethionine synthetase has the following amino acid sequence in conserved region 10: **K-V-D-R-F-A-A**

In the S-adenosylmethionine synthetase with decreased activity preferentially the conserved isoleucine in conserved region 11 is replaced with other amino acids. In a preferred application of the invention isoleucine is replaced by leucine.

20 In a preferred embodiment the modified S-adenosylmethionine synthetase has the following amino acid sequence in conserved region 11: **E-I-Q-V-S-Y-A-L-G-V-A**

25 The present invention furthermore relates to nucleotide sequences, DNA or RNA sequences, which encode a mutated S-adenosylmethionine synthetase according to the invention as defined above. In a preferred embodiment, these DNA sequences are characterized by the fact that they comprise at least one mutation in the coding DNA sequence regions for the conserved regions 1 to 11 of the wild type *metK* gene, represented as wild type in SEQ ID NO 3, the said mutation being not a silent mutation.

30 In a preferred application the *metK* gene is the S-adenosylmethionine synthetase of *E. coli* K12 represented by the SEQ ID NO 3 and sequences homologous to that sequence that have S-adenosylmethionine synthetase activity and that share at least 80% homology, preferentially 90% homology with the amino acid sequence of SEQ ID NO 4.

The mutated S-adenosylmethionine synthetase genes described above may be obtained by conventional techniques known to the person skilled in the art disclosed above and below, including random or targeted mutagenesis or synthetic DNA construction.

5 The *metA* gene encoding modified homoserine succinyltransferase and/or the *metK* gene encoding modified S-adenosylmethionine synthetase may be encoded chromosomally or extrachromosomally. Chromosomally there may be one or several copies on the genome that can be introduced by methods of recombination known to the expert in the field. Extrachromosomally both genes may be carried by different types of
10 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 *metA* and/or *metK* gene may be expressed using promoters with different
15 strength that need or need not to be induced by inducer molecules. Examples are the promoter pTrc, pTac, pLac, the lambda promoter cI or other promoters known to the expert in the field.

 MetA and/or MetK expression may be boosted or reduced by elements stabilizing or destabilizing the corresponding messenger RNA (Carrier and Keasling
20 (1998) Biotechnol. Prog. 15, 58-64) or the protein (e.g. GST tags, Amersham Biosciences)

 The present invention also relates to microorganisms which contain a feedback-resistant *metA* allele according to the invention and eventually a *metK* allele with reduced activity according to the invention.

25 Such strains are characterized by the fact that they possess a methionine metabolism which is deregulated by at least one feedback-resistant *metA* allele and/or by a reduced production of SAM caused by a MetK enzyme with reduced activity.

 Novel strains may be prepared from any microorganism in which methionine metabolism proceeds by the same metabolic pathway.

30 Gram-negative bacteria, in particular *E. coli*, are especially suitable.

For the purpose of expressing the modified homoserine succinyltransferase enzyme and S-adenosylmethionine synthetase, the feedback-resistant *metA* alleles and *metK* alleles with reduced activity are transformed in a host strain using customary methods. The screening for strains possessing modified homoserine succinyltransferase and S-adenosylmethionine synthetase properties is, for example, realized using enzymatic tests.

For example, the homoserine succinyltransferase activity can be determined in an enzymatic test with homoserine and succinyl-CoA as substrates. The reaction is started by adding the protein extract containing the homoserine succinyltransferase enzyme, and the formation of O-succinylhomoserine is monitored by GC-MS after protein precipitation and derivatization with a silylating reagent. Feedback inhibition is tested in the presence of methionine and S-adenosylmethionine in the reaction mixture.

S-adenosylmethionine synthetase activity can be determined in an enzymatic test with methionine and ATP as substrates. The reaction is started by adding the protein extract containing the S-adenosylmethionine synthetase enzyme, and the formation of S-adenosylmethionine is monitored by FIA-MS/MS.

Preferentially, use is made of *E. coli* strains in which the endogenous *metA* and *metK* genes are inactivated and complemented by novel recombinant genes.

The feedback-resistant *metA* alleles and the *metK* alleles with reduced activity render it possible to abolish the control at important biosynthetic control points, thereby amplifying the production of a large number of compounds which are situated downstream of aspartate. These include, in particular, homoserine, O-succinylhomoserine, cystathionine, homocysteine, methionine and S-adenosylmethionine.

In particular the invention relates to the preparation of L-methionine, its precursors or compounds derived thereof, by means of cultivating novel microorganisms. The above-described products are classified below as compound (I).

An increase in the production of compound (I) can be achieved by changing the expression levels or deleting the following genes implicated in the production of aspartate, a precursor of compound (I).

	<i>Gene</i>	<i>genbank entry name</i>	
	<i>ackA</i>	g1788633	acetate kinase
	<i>pta</i>	g1788635	phosphotransacetylase
	<i>acs</i>	g1790505	acetate synthase
5	<i>aceA</i>	g1790445	isocitrate lyase
	<i>aceB</i>	g1790444	malate synthase
	<i>aceE</i>	g1786304	pyruvate dehydrogenase E1
	<i>aceF</i>	g1786305	pyruvate dehydrogenase E2
	<i>lpd</i>	g1786307	pyruvate dehydrogenase E3
10	<i>aceK</i>	g1790446	isocitrate dehydrogenase kinase/phosphatase
	<i>sucC</i>	g1786948	succinyl-CoA synthetase, beta subunit
	<i>sucD</i>	g1786949	succinyl-CoA synthetase, alpha subunit
	<i>ppc</i>	g1790393	phosphoenolpyruvate carboxylase
	<i>pck</i>	g1789807	phosphoenolpyruvate carboxykinase
15	<i>pykA</i>	g1788160	pyruvate kinase II
	<i>pykF</i>	g1787965	pyruvate kinase I
	<i>poxB</i>	g1787096	pyruvate oxidase
	<i>pps</i>	g1787994	phosphoenolpyruvate synthase
	<i>ilvB</i>	g1790104	acetohydroxy acid synthase I, large subunit
20	<i>ilvN</i>	g1790103	acetohydroxy acid synthase I, small subunit
	<i>ilvG</i>	g1790202	acetohydroxy acid synthase II, large subunit
		g1790203	
	<i>ilvM</i>	g1790204	acetohydroxy acid synthase II, small subunit
	<i>ilvI</i>	g1786265	acetohydroxy acid synthase III, large subunit
25	<i>ilvH</i>	g1786266	acetohydroxy acid synthase III, small subunit
	<i>aroF</i>	g1788953	DAHP synthetase
	<i>aroG</i>	g1786969	DAHP synthetase
	<i>aroH</i>	g1787996	DAHP synthetase
	<i>aspC</i>	g1787159	aspartate aminotransferase
30	In addition pyruvate carboxylase from <i>Rhizobium etli</i> (accession number U51439) may be introduced by genetic engineering into <i>E. coli</i> and overexpressed.		

An additional increase in the production of compound (I) can be achieved by overexpressing genes of the lysine/methionine/pathway, such as the homoserine synthesizing enzymes encoded by the genes *thrA* (homoserine dehydrogenase/aspartokinase, g1786183) or *metL* (homoserine dehydrogenase/aspartokinase, g1790376) or *lysC* (aspartokinase, g1790455) or *asd* (aspartate semialdehyde dehydrogenase, g1789841) or a combination thereof.

A further increase in the production of (I) is possible by overexpressing genes involved in sulfate assimilation and production of cysteine. 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 Colyer 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 Microbiol 133 515-25). The following genes need to be overexpressed.

15	<i>CysA</i>	g1788761	sulfate permease
	<i>CysU</i>	g1788764	cysteine transport system
	<i>CysW</i>	g1788762	membrans bound sulphate transport system
	<i>CysZ</i>	g1788753	ORF upstream of <i>cysK</i>
	<i>cysN</i>	g1789108	ATP sulfurylase
20	<i>cysD</i>	g1789109	sulfate adenylyltransferase
	<i>cysC</i>	g1789107	adenylylsulfate kinase
	<i>cysH</i>	g1789121	adenylylsulfate reductase
	<i>cysI</i>	g1789122	sulfite reductase, alpha subunit
	<i>cysJ</i>	g1789123	sulfite reductase, beta subunit
25	<i>cysE</i>	g1790035	serine acetyltransferase
	<i>cysK</i>	g1788754	cysteine synthase
	<i>cysM</i>	g2367138	O-acetyl-sulfhydrylase

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

30	<i>serA</i>	g1789279	phosphoglycerate dehydrogenase
	<i>serB</i>	g1790849	phosphoserine phosphatase

<i>serC</i>	g1787136	phosphoserine aminotransferase
<i>glyA</i>	g1788902	serine hydroxymethyltransferase
<i>metF</i>	g1790377	5,10-Methylenetetrahydrofolate reductase

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

<i>metB</i>	g1790375	Cystathionine gamma-synthase	
<i>metC</i>	g1789383	Cystathionine beta-lyase	
<i>metH</i>	g1790450	B12-dependent	homocysteine-N5-
		methyltetrahydrofolate	
		transmethylase	
<i>metE</i>	g2367304	Tetrahydropteroyltriglutamate methyltransferase	
<i>metF</i>	g1790377	5,10-Methylenetetrahydrofolate reductase	
<i>metR</i>	g1790262	Positive regulatory gene for <i>metE</i> and <i>metH</i> and	
		autogenous	
		regulation	

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>	g1786311	S-Adenosylmethionine decarboxylase
<i>speC</i>	g1789337	Ornithine decarboxylase
20 <i>thrB</i>	g1786184	Homoserine kinase
<i>astA</i>	g1788043	Arginine succinyltransferase
<i>dapA</i>	g1788823	Dihydrodipicolinate synthase

A further increase in the production of (I) is possible by means of deleting the
25 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
g1790373).

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

The *metA* and *metK* alleles described above may be used in eukaryotes or prokaryotes. Preferentially the organism used is a prokaryote. In a preferred application the organism is either *E. coli* or *C. glutamicum*.

The invention also concerns the process for the production of compound (I).

5 Compound (I) is usually prepared by fermentation of the designed bacterial strain.

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.

10 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 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.

15 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 a 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*.

20 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 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* 25 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 Laboratory Press, Cold Spring Harbor, New York) or a medium such as defined by Schaefer *et al.* (1999, *Anal. Biochem.* 270: 88-96).

30 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. Microbiol. Biotechnol. **3**: 573-583). The media can be supplemented to compensate for auxotrophies introduced by mutations.

After fermentation compound (I) is recovered and purified if necessary. The methods for the recovery and purification of compound (I) such as methionine in the culture media are well known to those skilled in the art.

- **Figure 1A/B** : Methionine metabolism in *Escherichia coli*.
- **Figure 2** : Alignment of wildtype and recombinant *metA* genes obtained upon selection on α -methyl-methionine. Conserved residues are represented by light grey boxes and mutated residues are indicated by white boxes.
- **Figure 3** : Alignment of MetA sequences from different microorganisms.
- **Figure 4** : Alignment of MetK sequences from different microorganisms.
- **Figure 5** : Amino acid replacements in the *E. coli* MetK protein. Amino acids above the coherent lines indicate the position and the replacing amino acid.

EXAMPLES

Example 1

Isolation of *E. coli* mutants containing homoserine transsuccinylase enzymes which show decreased feedback-sensitivity towards methionine and S-adenosylmethionine

Isolation of E. coli strains growing on α -methylmethionine

α -methylmethionine is a growth-inhibitory analogue of methionine producing an immediate effect on the growth rate of *E. coli* at very low concentrations (minimal inhibitory concentration of 1 μ g/ml and minimal concentration for a maximal inhibition 5 μ g/ml, Rowbury *et al.*, 1968). Analogues can mimic methionine in the feedback inhibition of homoserine transsuccinylase and interfere with protein synthesis without being metabolized. Only mutant strains are able to grow in a medium containing the analogue.

E. coli was routinely grown aerobically at 37°C in LB supplemented when needed with the appropriate antibiotic. The medium used for the α -methyl-methionine analogue resistant was a minimal medium containing (per liter): K₂HPO₄ 8g, Na₂HPO₄ 2g, (NH₄)₂SO₄ 0.75g, (NH₄)₂HPO₄ 8g, NH₄Cl 0.13g, citric acid 6.55g, MgSO₄ 2.05g,

CaCl₂ 40mg, FeSO₄ 40mg, MnSO₄ 20mg, CoCl₂ 8 mg, ZnSO₄ 4mg, (NH₄)₂Mo₂O₇ 2.8mg, CuCl₂ 2mg, H₃BO₃ 1mg. The pH was adjusted to 6.7 and the medium sterilized. Before use, glucose 10g/l and thiamine 15ml/l were added.

The α -methylmethionine powder commercialized by Sigma contains methionine traces. An overnight liquid culture of an *E. coli* strain (MG1655 $\Delta metE$) unable to grow without methionine addition was carried out to eliminate the methionine (coming from α -methylmethionine) from the minimal medium. The culture was centrifuged 10 minutes at 7000rpm and the supernatant was filtered (Sartorius 0.2 μ m). Agar 15g/l was added to this minimal medium.

1*10⁸ cells / ml from an overnight culture in minimal medium of the wild type strain (MG1655) were spread onto plates with minimal medium and α -methylmethionine after 4 washing steps in sterile water. The plates were incubated at 37°C until colonies appeared.

Evidence of mutations in the coding sequence of the metA gene coding for the homoserine transsuccinylase enzyme

Genomic DNA was prepared from 4 clones that grew on α -methyl-methionine at 4mg/ml after cultivation in LB medium. The cell pellet was washed once with sterile water and resuspended in 30 μ l of sterile water. Cells were broken by heating 5 minutes at 95°C and the debris were pelleted. The *metA* gene was PCR-amplified using Taq polymerase and the following primers:

MetAF (SEQ ID NO 5): tcacctcaacatgcaggctcgacattggc (4211759-4211788)

MetAR (SEQ ID NO 6): ataaaaaaggcaccgaaggtgcctgaggt (4212857-4212828)

In three clones point mutations were detected which led to amino acid substitutions. In clone *metA*11 CAG was exchanged for a GAG leading to the replacement of Q by E. In *metA**13 TTG was exchanged for a TTT leading to the replacement of L by F. In *metA**14 GCG was exchanged for GTG leading to the replacement of A by V (see fig.2).

As can be seen from the alignment shown in Fig. 3 all three amino acid that are replaced are highly conserved in MetA proteins from various species.

The mutated homoserine transsuccinylase enzymes show decreased feedback-sensitivity towards methionine and S-adenosylmethionine

The activity of homoserine transsuccinylase was determined *in vitro*. *E. coli* strains carrying either wild-type or mutant enzymes were cultured in rich medium with 2.5 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 30 minutes at 25°C with 30 mM homoserine and 4 mM succinyl-CoA. Methionine and/or S-adenosylmethionine were added as indicated. The succinylhomoserine produced by homoserine transsuccinylase enzymes was quantified by GC-MS after derivatization with tert-butyltrimethylsilyltrifluoroacetamide (TBDMS-TFA). L-Serine[1-¹³C] was included as an internal standard.

Results of homoserine transsuccinylase activities are reported in the table 1 below:

Strain	Methionine mM	S-adenosylmethionine mM	Specific activity mUI/mg proteins
metA	0	0	36.7 (100%)
	0	0.1	27.9 (76%)
	0	1	4.7 (12.8%)
	10	0	0.4 (1.1%)
	10	0.1	0.2 (0.5%)
	10	1	0.1 (0.3%)
metA*11	0	0	19.2 (100%)
	0	0.1	21.7 (113%)
	0	1	18.5 (96%)
	10	0	18.7 (97%)
	10	0.1	16.9 (88%)
	10	1	2.7 (14.1%)
metA*13	0	0	10.9 (100%)
	0	1	12.7 (117%)

	10	0	13.7 (126%)
	10	1	1.0 (9.2%)
metA*14	0	0	18.0 (100%)
	0	1	15.1 (83.9%)
	10	0	20.8 (116%)
	10	1	2.0 (11.1%)

Table 1. Specific activities of wild-type and mutant homoserine transsuccinylase enzymes upon the addition of inhibitors L-methionine and S-adenosyl methionine.

5 The mutated homoserine transsuccinylase enzymes thus show decreased feedback-sensitivity towards methionine and S-adenosylmethionine.

Example 2

Isolation of *E. coli* mutants containing S-adenosylmethionine synthetase enzymes with reduced activity

10 *Isolation of E. coli strains growing on norleucine*

Norleucine is a growth-inhibitory analogue of methionine. At higher concentrations (50 mg/l) only mutant strains are able to grow in a medium containing the analogue. Most of these mutations map to the *metK* and *metJ* loci.

15 *E. coli* was routinely grown aerobically at 37°C in LB supplemented when needed with the appropriate antibiotic. The medium used for the norleucine analogue resistant was a minimal medium containing (per liter): K₂HPO₄ 8g, Na₂HPO₄ 2g, (NH₄)₂SO₄ 0.75g, (NH₄)₂HPO₄ 8g, NH₄Cl 0.13g, citric acid 6.55g, MgSO₄ 2.05g, CaCl₂ 40mg, FeSO₄ 40mg, MnSO₄ 20mg, CoCl₂ 8 mg, ZnSO₄ 4mg, (NH₄)₂MoO₇ 2.8mg, CuCl₂ 2mg, H₃BO₃ 1mg. The pH was adjusted to 6.7 and the medium sterilized. Before
20 use, glucose 10g/l and thiamine 15ml/l were added.

1*10⁸ cells / ml from an overnight culture in minimal medium of the wild type strain (MG1655) were spread onto plates with minimal medium and norleucine (50 to 200 g/l) after 4 washing steps in sterile water. The plates were incubated at 37°C until colonies appeared.

Evidence of mutations in the coding sequence of the metK gene coding for the S-adenosylmethionine synthetase enzyme

Genomic DNA was prepared from cultures grown in LB liquid medium. The cell pellet was washed once with sterile water and resuspended in 30µl of sterile water. Cells

5 were broken by heating 5 minutes at 95°C and the debris was extracted.

DNA was PCR-amplified with Taq polymerase using the following primers:

MetKpF : cccggctggaagtggcaacacg (3084372-3084393) SEQ ID NO 07

MetKR : gccggatgcggcgtgaacgcctatcc (3085956-3085931) SEQ ID NO 08

The *metK* gene was sequenced. In 10 clones point mutations were detected
 10 which led to amino acid substitutions. In clone *metK**59/105 AGC was exchanged for AAC leading to the replacement of S by N (position 59) and GGC was exchanged for AGC leading to the replacement of G by S (position 105). In clone *metK**105 GGC was exchanged for AGC leading to the replacement of G by S. In *metK**115 GGC was exchanged for AGC leading to the replacement of G by S. In *metK**137 CCT was
 15 replaced by CTT leading to the replacement of P by L. In *metK**142 CAC was replaced by TAC leading to the replacement of H by Y. In *metK**161/235 CGC was replaced by TGC leading to the replacement of R by C and CCA was replaced by TCA leading to the replacement of P by S. In *metK**189 CAC was replaced by TAC leading to the replacement of H by Y. In *metK**227 ACC was replaced by ATC leading to the
 20 replacement of T by I. In *metK**253 GGC was replaced by GAC leading to the replacement of G by D. In *metK**273 TCC was replaced by TTC leading to the replacement of S by F (see fig.5).

As can be seen from the alignment shown in fig. 4 all amino acid that are replaced are conserved in MetK proteins from various species.

25 *Recombinant S-adenosylmethionine synthetase enzymes with decreased activity*

The activity of S-adenosylmethionine synthetase was determined *in vitro*. *E. coli* strains carrying either wild-type or mutant enzymes 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
 30 centrifugation, proteins contained in the supernatants were quantified (Bradford, 1976).

One hundred μ L of the extracts were incubated for 30 minutes at 37°C with 10 mM methionine and 10 mM ATP. Potassium chloride was included to activate the enzymes. The adenosylmethionine produced by methionine adenosyltransferase enzymes was quantified by FIA-MS/MS.

5 Results of S-adenosylmethionine synthetase activities are reported in Table 2 below:

strain	Mutations	MAT mUI/mg prot
WT	-	7.5
MetK*	H189Y	0.6
MetK*	S273F	2.2
MetK*	T227I	4.9
MetK*	G115S	0.4
MetK*	G105S	2.8
MetK*	H142Y	2.5
MetK*	R161C + P235S	0.7
MetK*	S59N + G105S	2.9
MetK*	P137L	1.2
MetK*	G253D	0.2

Table 2: S-adenosylmethionine synthetase activities (MAT) of WT and MetK* mutants.

Example 3

Construction of *E. coli* strains for the production of O-succinylhomoserine or methionine by overexpressing altered homoserine transsuccinylase and other enzymes of the methionine biosynthesis pathway.

15 For the construction of *E. coli* strains that allow the production of O-succinylhomoserine, a plasmid overexpressing the *metL* gene, coding for homoserine dehydrogenase and aspartokinase was introduced into strains harboring different alleles of *metA*. The plasmid overexpressing *metL* was constructed as follows:

The following two oligonucleotides were used:

20 - MetLF with 32 bases (SEQ ID NO 13):

TATTCatgagtgtgattgcgcaggcaggggcg

with

- a region (lower case) homologous to the sequence (4127415 to 4127441) of the gene *metL* (sequence 4127415 to 4129847, reference sequence on the website <http://genolist.pasteur.fr/Colibri/>),

- a region (upper case) that together with the sequence pertaining to *metL* forms a restriction site for the enzyme *Bsp*HI (underlined),

- MetBLAR with 38 bases (SEQ ID NO 14):

TATAAAGCTTccataaacccgaaaacatgagtaccgggc

with

- a region (lower case) homologous to the sequence (4129894 to 4129866) of the gene *metL*

- a region (upper case) that harbors the restriction site *Hind*III.

The gene *metL* was amplified by PCR using the oligonucleotides MetLF and MetBLAR and the restriction sites *Bsp*HI and *Hind*III were introduced at the N- and C-terminus of the *metL* gene, respectively. The resulting PCR fragment was restricted by *Bsp*HI and *Hind*III and cloned into the vector pTrc99A (Stratagene) previously cut by *Nco*I and *Hind*III.

Plasmid preparations were examined for the presence of inserts of the correct size. The DNA sequence of *metL* was verified and the resulting plasmid pTrc::*metL* transformed into the strains harboring the alleles *metA**11 and *metA**13.

The activity of Homoserine dehydrogenase II (HDH) was determined *in vitro*. *E. coli* strains were cultured in minimal medium with 10 g.l⁻¹ 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).

Thirty µl of the extracts were incubated at 30°C in a spectrophotometer, with 25 mM homoserine and 1 mM NADP⁺. Potassium chloride was included to activate the enzyme. Since *E. coli* harbors a second gene encoding a homoserine dehydrogenase activity (*thrA*), threonine was added which inhibits this activity. The appearance of NADPH was monitored for 30 minutes at 340 nm.

Expression of *metL* from the pTrc promoter increases drastically the HDH activity when compared to a similar strain with the plasmid.

Strain	HDH in mUI/mg protein
MG1655 <i>metA</i> *11	11
MG1655 <i>metA</i> *11 pTrc:: <i>metL</i>	117

5 Tab. 3 Homoserine dehydrogenase activities of the MetL protein in the MG1655 *E. coli* strain harbouring the *metA**11 mutation with or without overexpressing *metL* from the pTrc promoter.

10 In another application the methionine regulatory gene *metJ* was deleted in the strains harboring the *metA**11, *metA**13 and *metA**14 alleles. To inactivate the *metJ* gene the homologous recombination strategy described by Datsenko & Wanner (2000) was used. This strategy allows the insertion of a chloramphenicol resistance cassette, while deleting most of the gene concerned. For this purpose 2 oligonucleotides were used:

- DmetJF with 100 bases (SEQ ID NO 15):

15 CaggcaccagagtaaacattgtgtaatggacgtcaatacatctggacatctaaactctttgcgtatagattgagcaaaCAT
ATGAATATCCTCCTTAG

with

- a region (lower case) homologous to the sequence (4126216 to 4126137) of the gene *metJ* (sequence 4125658 to 4125975 , reference sequence on the website
20 <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),

- DmetJR with 100 bases (SEQ ID NO 16):

25 tgacgtaggcctgataagcgtagcgcacatcaggcgattccactccgcgccgctctttttgcttagtattcccacgtctcTGTA
GGCTGGAGCTGCTTCG

with

- a region (lower case) homologous to the sequence (4125596 to 4125675) of the gene *metJ*

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

The oligonucleotides DmetJR and DmetJF 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 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 oligonucleotides MetJR and MetJF defined below. The strain retained is designated MG1655 ($\Delta metJ::Cm$) *metA**.

MetJR (SEQ ID NO 17): ggtacagaaaccagcaggctgaggatcagc (homologous to the sequence from 4125431 to 4125460).

MetBR (SEQ ID NO 18): ttcgtcgtcatttaaccgctacgcactgc (homologous to the sequence from 4126305 to 4126276).

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. The strains retained were designated MG1655 ($\Delta metJ$) *metA**.

Subsequently the plasmid pTrc::metL harbouring the *metL* gene was introduced into these strains giving rise to $\Delta metJ metA^*11$ pTrc::*metL*, $\Delta metJ metA^*13$ pTrc::*metL* and $\Delta metJ metA^*14$ pTrc::*metL*.

Example 4

Construction of *E. coli* strains for the production of O-succinyl homoserine or methionine by combining feed-back resistant MetA alleles with MetK alleles with decreased activity.

In order to transfer the recombinant *metK* alleles into strains harboring feedback resistant *metA* alleles, a kanamycin resistance cassette was introduced between the *metK* and *galP* gene.

To introduce the cassette the homologous recombination strategy described by Datsenko & Wanner (2000) is used. This strategy allows the insertion of a kanamycin

resistance cassette, while deleting most of the gene concerned. For this purpose 2 oligonucleotides are used:

- DMetKFscr with 100 bases (SEQ ID NO 9):

ccgccccgcacaataacatcattcttctgatcacgtttcaccgcagattatcatcacaaactgaaaccgattacaccaaccTGTA

5 GGCTGGAGCTGCTTCG

with

- a region (lower case) homologous to the sequence of the region between *metK* and *galP* (sequence 3085964 to 3086043, reference sequence on the website <http://genolist.pasteur.fr/Colibri/>),

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

- DmetKRscr with 100 bases (SEQ ID NO 10):

gagttatatcatcatagattaaacgctgttatctgcaattaagactttactgaaaagaaatgtaacaactgtgaaaaccgCATA
TGAATATCCTCCTTAG

15 with

- a region (lower case) homologous to the region between the gene *metK* and *galP* (3086162 to 3086083)

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

The oligonucleotides DMetKFscr and DmetKRscr are used to amplify the
20 kanamycin resistance cassette from the plasmid pKD4. The PCR product obtained is then introduced by electroporation into the strain MG1655 (pKD46) in which the Red recombinase enzyme expressed allows the homologous recombination. The kanamycin resistant transformants are then selected and the insertion of the resistance cassette is verified by a PCR analysis with the oligonucleotides MetKFscr and MetKRscr defined
25 below. The strain retained is designated MG1655 ($\Delta metK$, Km).

MetKFscr (SEQ ID NO 11): gcgcccatacgggtctgattcagatgctgg (homologous to the sequence from 3085732 to 3085760).

MetKRscr (SEQ ID NO 12) gcgccagcaattacaccgatatccaggcc (homologous to the sequence from 3086418 to 3086390).

30 To transfer the *metK* alleles, the method of phage P1 transduction is used. The protocol followed is implemented in 2 steps with the preparation of the phage lysate of

the strain MG1655 (*metK*, Km) and then transduction into strain MG1655 $\Delta metJ$ *metA**11 pTrc::*metL*.

The construction of the strain (*metK*, Km) is described above.

Preparation of phage lysate P1:

- 5 - Inoculation with 100 μ l of an overnight culture of the strain MG1655 ($\Delta metK$, Km) of 10 ml of LB + Km 50 μ g/ml + glucose 0.2% + CaCl₂ 5 mM.
- Incubation for 30 min at 37°C with shaking.
- Addition of 100 μ l of phage lysate P1 prepared on the wild strain MG1655 (about 1.10⁹ phage/ml)
- 10 - Shaking at 37°C for 3 hours until all the cells were lysed.
- Addition of 200 μ l chloroform and vortexing.
- Centrifugation for 10 min at 4500 g to eliminate cell debris.
- Transfer of supernatant to a sterile tube and addition of 200 μ l chloroform.
- Storage of lysate at 4°C.

15 Transduction

- Centrifuging for 10 min at 1500 g of 5 ml of an overnight culture of the strain MG1655 $\Delta metJ$ *metA**11 pTrc::*metL* in LB medium.
- Suspension of the cell pellet in 2.5 ml of 10 mM MgSO₄, 5 mM CaCl₂
- Control tubes: 100 μ l cells
- 20 100 μ l phages P1 of strain MG1655 ($\Delta metK$, Km)
- Test tube: 100 μ l of cells + 100 μ l of phages P1 of the strain MG1655 ($\Delta metK$, Km)
- Incubation for 30 min at 30°C without shaking.
- Addition of 100 μ l of 1 M sodium citrate in each tube and vortexing.
- Addition of 1 ml of LB
- 25 - Incubation for 1 hour at 37°C with shaking
- Spreading on dishes LB + Km 50 μ g/ml after centrifuging of tubes for 3 min at 7000 rpm.
- Incubation at 37°C overnight.

Verification of the strain

- 30 The kanamycin resistant transformants are then selected and the insertion of the region containing (*metK*, Km) is verified by a PCR analysis with the oligonucleotides

MetKFscr and MetKRscr. The strain retained is designated MG1655 $\Delta metJ$ *metA**11 pTrc::*metL metK**.

The kanamycin resistance cassette can then be eliminated. The plasmid pCP20 carrying FLP recombinase acting at the FRT sites of the kanamycin resistance cassette is then introduced into the recombinant sites by electroporation. After a series of cultures at 42°C, the loss of the kanamycin resistance cassette is verified by a PCR analysis with the same oligonucleotides as used previously (MetKFscr and MetKRscr).

Example 5

10 Construction of an *E. coli* strain overexpressing the *metA**11 allele

For overexpression the *metA**11 allele was cloned into the vector pTc99A (Stratagene).

The following two oligonucleotides were used:

- MetA-NcoI with 49 bases (SEQ ID NO 19):

15 TATTAAATTACCCatggcaccgattcgtgtgccggacgagctacccgccg
with

- a region (lower case) homologous to the sequence (4211862 to 4211892) of the gene *metA* (sequence 4211859 to 4212788 , reference sequence on the website <http://genolist.pasteur.fr/Colibri/>),

20 - a region (upper case) that together with the sequence pertaining to *metA* forms a restriction site for the enzyme *NcoI* (underlined),

- MetA-EcoRI with 47 bases (SEQ ID NO 20):

TATTAAATTAGGaattccgactatcacagaagattaatccagcgttgg
with

25 - a region (lower case) homologous to the sequence (4212804 to 42127774) of the gene *metA*

- a region (upper case) that harbors the restriction site *EcoRI*.

The gene *metA* was amplified by PCR using oligonucleotides MetAF and MetAR and the restriction sites *NcoI* and *EcoRI* were introduced at the N- and C-terminus of the *metA* gene, respectively. The resulting PCR fragment was restricted by *NcoI* and *EcoRI* and cloned into the vector pTrc99A (Stratagene) previously cut by *NcoI* and *EcoRI*.

Plasmid preparations were examined for the presence of inserts of the correct size. The DNA sequence of *metA* was verified and the plasmid transformed into the strains harboring the allele *metA**11.

5 Example 6

Fermentation of *E. coli* production strains and analysis of yield

Production strains were initially analyzed in small Erlenmeyer flask cultures using modified M9 medium (Anderson, 1946, *Proc. Natl. Acad. Sci. USA* **32**:120-128) that was supplemented with 5 g/l MOPS and 5 g/l glucose. Carbenicillin was added if
10 necessary at a concentration of 100 mg/l. An overnight culture was used to inoculate a 30 ml culture to an OD600 of 0.2. After the culture had 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) were added and culture was agitated for 1 hour. Subsequently IPTG was added if necessary.

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

The following results were obtained for the strains MG1655, MG1655 pTrc::*metL* MG1655 *metA**11 pTrc::*metL*, MG1655 *metA**13 pTrc::*metL*, MG1655 *metA**11 Δ *metJ* pTrc::*metL*.

Product (mM) strain	MG1655	MG1655 pTrc:: <i>metL</i>	MG1655 <i>metA</i> *11 pTrc:: <i>metL</i>	MG1655 <i>metA</i> *13 pTrc:: <i>metL</i>	MG1655 <i>metA</i> *11 Δ <i>metJ</i> pTrc:: <i>metL</i>	MG1655 <i>metA</i> *11 Δ <i>metJ</i> pTrc:: <i>metL</i> <i>metK</i> *142
OSH	0.03	0.08	7.80	4.99	0.10	In progress
HOMO	0.02	13.05	0.19	0.43	N.D.	In progress
THR	0.06	3.76	9.16	8.37	0.55	In progress
MET	0.01	0.06	0.82	0.76	1.18	In progress

20 Tab. 4 Concentrations of extracellular metabolites (mM) after batch fermentation of strains MG1655, MG1655 pTrc::*metL*, MG1655 *metA**11 pTrc::*metL*, MG1655

*metA**13 pTrc::*metL*, MG1655 *metA**11 Δ *metJ* pTrc::*metL*, MG1655 *metA**11 Δ *metJ* pTrc::*metL* *metK**142. Abbreviations: OSH O-succinylhomoserine, HOMO homoserine, THR threonine, MET methionine, ND not detected.

Strains that produced substantial amounts of metabolites of interest were subsequently tested under production conditions in 300 ml fermentors (DASGIP) using a fed batch protocol.

For this purpose the fermentor was filled with 145 ml of modified minimal medium and inoculated with 5 ml of preculture to an optical density (OD_{600nm}) between 0.5 and 1.2.

The temperature of the culture was maintained constant at 37 °C and the pH was permanently adjusted to values between 6.5 and 8 using an NH₄OH solution. The agitation rate was maintained between 200 and 300 rpm during the batch phase and was increased to up to 1000 rpm at the end of the fed-batch phase. The concentration of dissolved oxygen was maintained at values between 30 and 40% saturation by using a gas controller. When the optical density reached a value between three and five the fed-batch was started with an initial flow rate between 0.3 and 0.5 ml/h and a progressive increase up to flow rate values between 2.5 and 3.5 ml/h. At this point the flow rate was maintained constant for 24 to 48 hours. The media of the fed was based on minimal media containing glucose at concentrations between 300 and 500 g/l.

When the concentration of biomass reached values between 30 and 60 g/l the fermentation was stopped and the extracellular methionine concentration was determined using HPLC.

strain	Methionine titer (mM)
Δ <i>metJ</i> <i>metA</i> *11 pTrc:: <i>metL</i>	22.9
Δ <i>metJ</i> <i>metA</i> *11 pTrc:: <i>metL</i> <i>metK</i> *142	In progress

Tab 5 Methionine titer of strains Δ *metJ* *metA**11 pTrc::*metL* after fed batch fermentation.

What is claimed is:

1. A method for the preparation of methionine, its precursors or products derived thereof, in a fermentative process with a microorganism where L-homoserine is converted into O-succinylhomoserine with a homoserine transsuccinylase, comprising
 5 the step of culturing the said microorganism on an appropriate medium and recovering methionine, its precursors or products derived thereof once produced, wherein the homoserine transsuccinylase is a mutated homoserine transsuccinylase with reduced sensitivity for the feedback inhibitors S-adenosylmethionine and methionine.

2. The method as claimed in claim 1, wherein the modified homoserine succinyltransferases exhibit a specific activity which is at least ten times that of the wild-type enzyme in the presence of 10 mM methionine and 1 mM S-adenosylmethionine and at least 80 times that of the wild-type enzyme in the presence of 10 mM methionine and 0.1 mM S-adenosylmethionine.

3. The method as claimed in one of claims 1 or 2, wherein the modified
 15 homoserine succinyltransferases comprise at least one amino acid mutation when compared with the wild-type sequence in one of the following non mutated conserved regions and combinations thereof:

conserved region 1 comprising the following sequence:

X1-X2-X3-A-X4-X5-Q

20 In which

X1 represents E, D, T, S, L, preferentially T

X2 represents D, S, K, Q, E, A, R, preferentially S

X3 represents R, E, D, preferentially R

X4 represents Y, I, F, A, K, S, V, preferentially S

25 X5 represents H, S, N, G, T, R, preferentially G

conserved region 2 comprising the following sequence:

X1-X2-X3-P-L-Q-X4-X5

In which

X1 represents G, A, S, preferentially S

30 X2 represents N, A, preferentially N

X3 represents S, T, preferentially S

X4 represents V, L, I, preferentially V

X5 represents N, E, H, D, preferentially D

conserved region 3 comprising the following sequence:

X1-Y-Q-X2-T-P-X3

5 In which

X1 represents V, I, M, preferentially V

X2 represents E, K, G, I, Q, T, S, preferentially I

X3 represents F, Y, preferentially Y

10 4. The method as claimed in claim 3, wherein the modified homoserine succinyltransferases comprise at least one of the following mutations and combinations thereof:

conserved alanine in conserved region 1 is replaced with a valine,

conserved amino acids L and/or Q in conserved region 2 are replaced with other amino acids, preferentially leucine is replaced by phenylalanine and/or glutamine is replaced

15 with a glutamate, and

conserved amino acids L and/or Q in conserved region 3 are replaced with another amino acid.

20 5. The method as claimed in claim 4, wherein the modified conserved region 1 comprises the amino acid sequence **T-S-R-V-S-G-Q**, and/or the modified conserved region 2 comprises the amino acid sequence selected among **S-N-S-P-L-E-V-D**, **S-N-S-P-F-Q-V-D** and **S-N-S-P-F-E-V-D**.

6. The method as claimed in any one of claims 1 to 5, wherein the microorganisms comprise a S-adenosylmethionine synthetase enzyme with reduced S-adenosylmethionine synthetase enzymatic activity.

25 7. The method as claimed in claim 6, wherein the modified S-adenosylmethionine synthetase contains at least one amino acid mutation of at least one of the sequences specified below:

cystein at position 89 and/or cystein at position 239

region 1, the non mutated region 1 comprising the amino acid sequence defined below:

30 **G-E-X1-X2-X3-X4**

wherein

X1 represents I, V, L, T preferentially I

X2 represents T, K, S, R, preferentially T

X3 represents T, S, G, preferentially T

X4 represents S, N, T, K, E, R, P, N, A preferentially S

- 5 conserved region 2, the non mutated conserved region 2 comprising the amino acid sequence defined below:

Q-S-X1-D-I-X2-X3-G-V-X4

wherein

X1 represents P, Q, A, S, preferentially P

- 10 X2 represents A, N, Q, S, F, preferentially N

X3 represents V, Q, Y, R, M, N, preferentially Q

X4 represents K, D, A, N, T, S, E, preferentially D

conserved region 3, the non mutated conserved region 3 comprising the amino acid sequence defined below

- 15 **X1-G-A-G-D-Q-G-X2**

wherein

X1 represents Q, A, I, V, E, T, preferentially Q

X2 represents L, I, S, V, M, preferentially L

conserved region 4, the non mutated conserved region 4 comprising the amino acid sequence defined below:

- 20 **X1-I-X2-X3-X4-H-X5-X6**

wherein

X1 represents S, P, T, A, preferentially P

X2 represents A, T, W, Y, F, S, N preferentially T

- 25 X3 represents M, Y, L, V preferentially Y

X4 represents S, A, preferentially A

X5 represents K, R, E, D, preferentially R

X6 represents L, I, preferentially L

conserved region 5, the non mutated conserved region 5 comprising the amino acid sequence defined below

- 30 **X1-L-X2-X3-D**

wherein

X1 represents W, F, Y, V, E preferentially W

X2 represents R, G, L, K, preferentially R

X3 represents P, L, H, V, preferentially P

- 5 conserved region 6, the non mutated conserved region 6 comprising the amino acid sequence defined below.

X1-X2-X3-S-X4-Q-H

In which

X1 represents V, I, preferentially V

- 10 X2 represents V, L, I, preferentially V

X3 represents V, L, I, M, preferentially L

X4 represents T, V, A, S, H, preferentially T

conserved region 7, the non mutated conserved region 7 comprising the amino acid sequence defined below:

- 15 **X1-N-P-X2-G-X3-F**

In which

X1 represents V, I, preferentially I

X2 represents T, G, S, preferentially T

X3 represents R, T, Q, K, S, preferentially R

- 20 conserved region 8, the non mutated conserved region 8 comprising the amino acid sequence defined below.

X1-X2-G-X3-P-X4-X5

In which

X1 represents T, V, I, Y, E, preferentially V

- 25 X2 represents V, I, L, N, preferentially I

X3 represents G, S, preferentially G

X4 represents M, I, Q, A, H, D preferentially M

X5 represents G, S, A, H preferentially G

- 30 conserved region 9, the non mutated conserved region 9 comprising the amino acid sequence defined below.

X1-X2-D-T-Y-G-G

In which

X1 represents M, I, preferentially I

X2 represents V, I, preferentially I

- 5 conserved region 10, the non mutated conserved region 10 comprising the amino acid sequence defined below.

K-V-D-R-S-X1-X2

In which

X1 represents A, G, preferentially A

- 10 X2 represents A, S, L, preferentially A

conserved region 11, the non modified conserved region 11 comprising the amino acid sequence defined below:

X1-X2-Q-X3-X4-Y-A-I-G-X5-X6

In which

- 15 X1 represents L, E, I, Q, T preferentially E

X2 represents V, I, L preferentially I

X3 represents V, L, I preferentially V

X4 represents A, S preferentially S

X5 represents V, I, R, K, A preferentially V

- 20 X6 represents A, V, T, S, preferentially A

8. The method as claimed in claim 7, wherein the modified S-adenosylmethionine synthetase with decreased activity comprises at least one of the following mutations and combinations thereof:

- the conserved serine in conserved region 1 is replaced with another amino acid,
 25 preferentially with a asparagine,
 the conserved glycine in conserved region 2 is replaced with another amino acid,
 preferentially with a serine,
 the conserved glycine in conserved region 3 is replaced with another amino acid,
 preferentially with a serine,

the conserved histidine and/or semiconserved proline in conserved region 4 is replaced with another amino acid, preferentially the conserved histidine with a tyrosine and/ or the conserved proline with a leucine,

the semi-conserved arginine in conserved region 5 is replaced with another amino acid, preferentially with a cysteine,

the conserved histidine and/or semi-conserved valine in conserved region 6 is replaced with another amino acid, preferentially the conserved histidine is replaced with a tyrosine and/or the valine with aspartate,

the semi-conserved threonine in conserved region 7 is replaced with another amino acid, preferentially with an isoleucine,

the conserved proline in conserved region 8 is replaced with another amino acid, preferentially with a serine,

the second conserved glycine in conserved region 9 is replaced with another amino acid, preferentially with a aspartate,

the conserved serine in conserved region 10 is replaced with another amino acid, preferentially with a phenylalanine, and

the conserved isoleucine in conserved region 11 is replaced with other amino acids, preferentially with a leucine.

9. The method as claimed in claim 8, wherein the modified region 1 comprises the amino acid sequence **G-E-I-T-T-N** and/or the modified conserved region 2 comprises the amino acid sequence **Q-S-P-D-I-N-Q-S-V-D** and/or the modified conserved region 3 comprises the amino acid sequence **Q-S-A-G-D-Q-G-L** and/or the modified conserved region 4 comprises the amino acid sequence selected among **P-I-T-Y-A-Y-R-L**, **L-I-T-Y-A-H-R-L** and **L-I-T-Y-A-Y-R-L**, and/or the modified conserved region 5 comprises the amino acid sequence **W-L-C-P-D** and/or the modified conserved region 6 comprises the amino acid sequence selected among **V-V-L-S-T-Q-Y**, **V-D-L-S-T-Q-H** and **V-D-L-S-T-Q-Y**, and/or the modified conserved region 7 comprises the amino acid sequence **I-N-P-I-G-R-F** and/or the modified conserved region 8 comprises the amino acid sequence **V-I-G-G-S-M-G** and/or the modified conserved region 9 comprises the amino acid sequence **I-V-D-T-Y-G-D** and/or the modified conserved

region 10 comprises the amino acid sequence **K-V-D-R-F-A-A** and/or the modified conserved region 11 comprises the amino acid sequence **E-I-Q-V-S-Y-A-L-G-V-A**

10. The method as claimed in any one of claims 1 to 9, wherein the modified microorganism is selected among prokaryotes and eukaryotes, preferentially under
5 prokaryotes and more specifically *Escherichia coli* or *Corynebacterium glutamicum*.

11. A mutated homoserine transsuccinylase with reduced sensitivity for the feedback inhibitors S-adenosylmethionine and methionine as defined in any one of claims 2 to 5.

12. A nucleotide sequence encoding a mutated homoserine transsuccinylase
10 with reduced sensitivity for the feedback inhibitors S-adenosylmethionine and methionine as defined in claim 11

13. A mutated S-adenosylmethionine synthetase as defined in one of claims 6 to 9.

14. A nucleotide sequence encoding a mutated S-adenosylmethionine
15 synthetase as defined in claim 13.

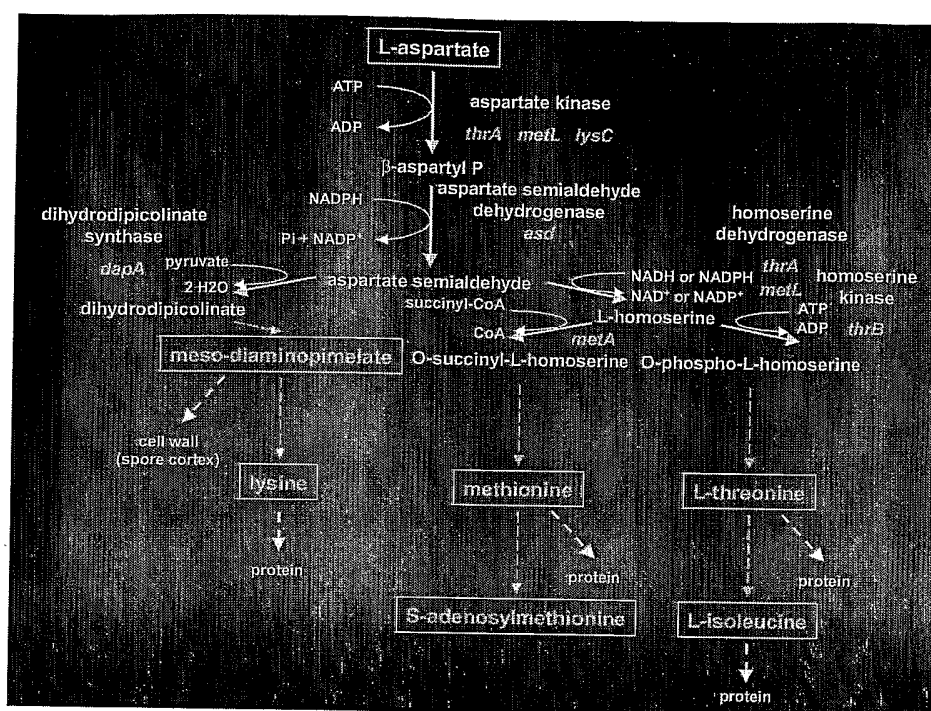
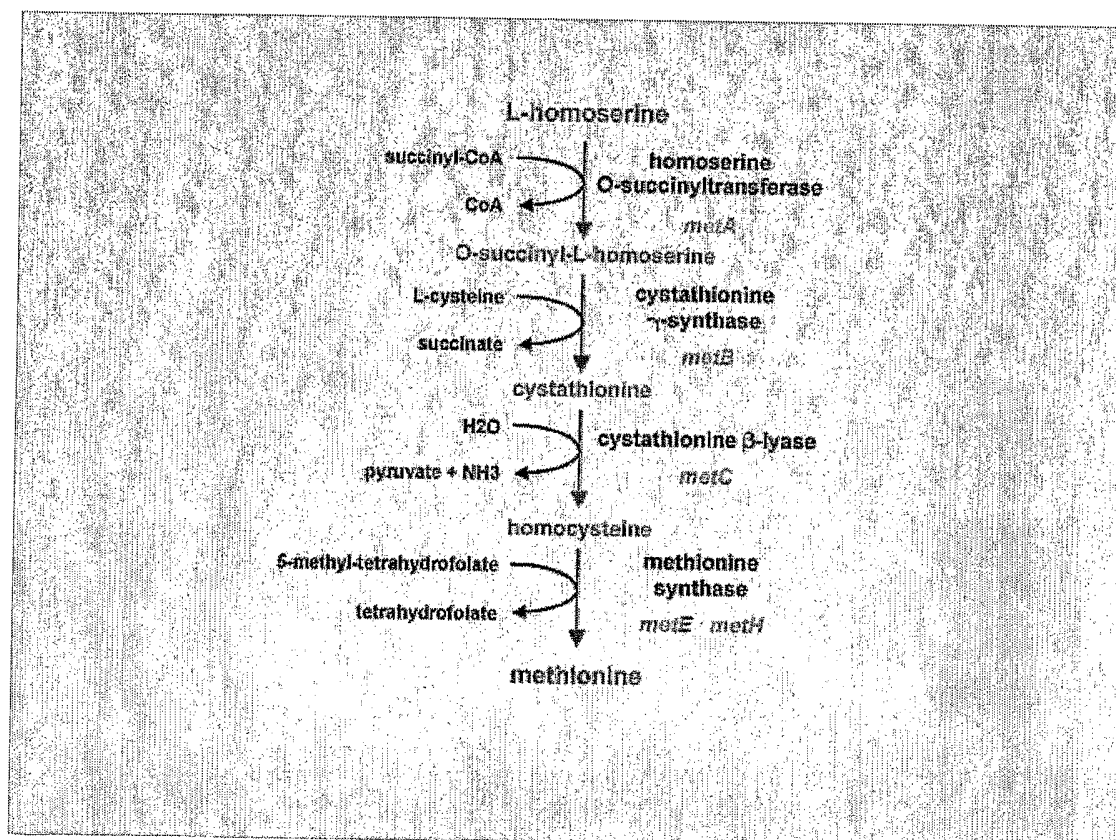
15. A microorganism comprising a DNA sequence coding for a mutated homoserine transsuccinylase with reduced sensitivity for the feedback inhibitors S-adenosylmethionine and methionine as defined in claim 14.

16. The microorganism as claimed in claim 15, further comprising a
20 nucleotide sequence encoding a mutated S-adenosylmethionine synthetase as defined in claim 13.

17. The microorganism of claim 15 or 16, wherein the microorganism is selected among prokaryotes and eukaryotes, preferentially under prokaryotes and more specifically *Escherichia coli* or *Corynebacterium glutamicum*.

25 18. The microorganism of one of claims 15 to 17, wherein the gene encoding aspartokinase/homoserine dehydrogenase is over expressed and/or the gene encoding methionine repressor metJ is deleted.

1/9

**Fig.1A/B**

2/9

		1		50
metA	(1)	MPIRVPDELPAVNILREENVFVMTTSRASGOEIRPLKVLILNLMPKKIET		
metA11/metA12	(1)	MPIRVPDELPAVNILREENVFVMTTSRASGOEIRPLKVLILNLMPKKIET		
metA13	(1)	MPIRVPDELPAVNILREENVFVMTTSRASGOEIRPLKVLILNLMPKKIET		
metA14	(1)	MPIRVPDELPAVNILREENVFVMTTSRVSGOEIRPLKVLILNLMPKKIET		
Consensus	(1)	MPIRVPDELPAVNILREENVFVMTTSRASGOEIRPLKVLILNLMPKKIET		
		51		100
metA	(51)	ENQFLRLLSNSPLQVDIQLLRIDSRESRNTPAEHLNNFYCNFEDIQDQNF		
metA11/metA12	(51)	ENQFLRLLSNSPLEVDIQLLRIDSRESRNTPAEHLNNFYCNFEDIQDQNF		
metA13	(51)	ENQFLRLLSNSPFQVDIQLLRIDSRESRNTPAEHLNNFYCNFEDIQDQNF		
metA14	(51)	ENQFLRLLSNSPLQVDIQLLRIDSRESRNTPAEHLNNFYCNFEDIQDQNF		
Consensus	(51)	ENQFLRLLSNSPLQVDIQLLRIDSRESRNTPAEHLNNFYCNFEDIQDQNF		
		101		150
metA	(101)	DGLIVTGAPLGLVEFNDVAYWPQIKOVLEWSKDHVTSTLFVCWAVQAALN		
metA11/metA12	(101)	DGLIVTGAPLGLVEFNDVAYWPQIKOVLEWSKDHVTSTLFVCWAVQAALN		
metA13	(101)	DGLIVTGAPLGLVEFNDVAYWPQIKOVLEWSKDHVTSTLFVCWAVQAALN		
metA14	(101)	DGLIVTGAPLGLVEFNDVAYWPQIKOVLEWSKDHVTSTLFVCWAVQAALN		
Consensus	(101)	DGLIVTGAPLGLVEFNDVAYWPQIKOVLEWSKDHVTSTLFVCWAVQAALN		
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metA11/metA12	(151)	ILYGIPKQTRTEKLSGVYEHHTLHPHALLTRGFDDSFAPHSRYADFPAA		
metA13	(151)	ILYGIPKQTRTEKLSGVYEHHTLHPHALLTRGFDDSFAPHSRYADFPAA		
metA14	(151)	ILYGIPKQTRTEKLSGVYEHHTLHPHALLTRGFDDSFAPHSRYADFPAA		
Consensus	(151)	ILYGIPKQTRTEKLSGVYEHHTLHPHALLTRGFDDSFAPHSRYADFPAA		
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metA	(201)	LIRDYTDLELLAEETEGDAYLFASKDKRIAFVTGHPEYDAQTLAQEFFRD		
metA11/metA12	(201)	LIRDYTDLELLAEETEGDAYLFASKDKRIAFVTGHPEYDAQTLAQEFFRD		
metA13	(201)	LIRDYTDLELLAEETEGDAYLFASKDKRIAFVTGHPEYDAQTLAQEFFRD		
metA14	(201)	LIRDYTDLELLAEETEGDAYLFASKDKRIAFVTGHPEYDAQTLAQEFFRD		
Consensus	(201)	LIRDYTDLELLAEETEGDAYLFASKDKRIAFVTGHPEYDAQTLAQEFFRD		
		251		300
metA	(251)	VEAGLDPDVPYNYFPHNDPQNTPRASWRSHGNLLFTNWLNYVYQITPYD		
metA11/metA12	(251)	VEAGLDPDVPYNYFPHNDPQNTPRASWRSHGNLLFTNWLNYVYQITPYD		
metA13	(251)	VEAGLDPDVPYNYFPHNDPQNTPRASWRSHGNLLFTNWLNYVYQITPYD		
metA14	(251)	VEAGLDPDVPYNYFPHNDPQNTPRASWRSHGNLLFTNWLNYVYQITPYD		
Consensus	(251)	VEAGLDPDVPYNYFPHNDPQNTPRASWRSHGNLLFTNWLNYVYQITPYD		
		301		
metA	(301)	LRHMNPTLD-		
metA11/metA12	(301)	LRHMNPTLD-		
metA13	(301)	LRHMNPTLD-		
metA14	(301)	LRHMNPTLD-		
Consensus	(301)	LRHMNPTLD		

Fig. 2

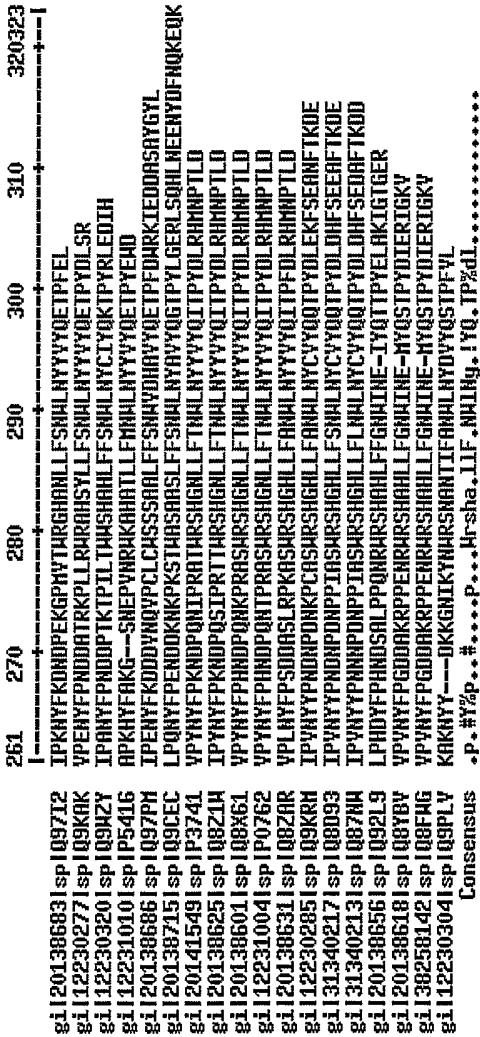


Fig. 3 (end)

5/9

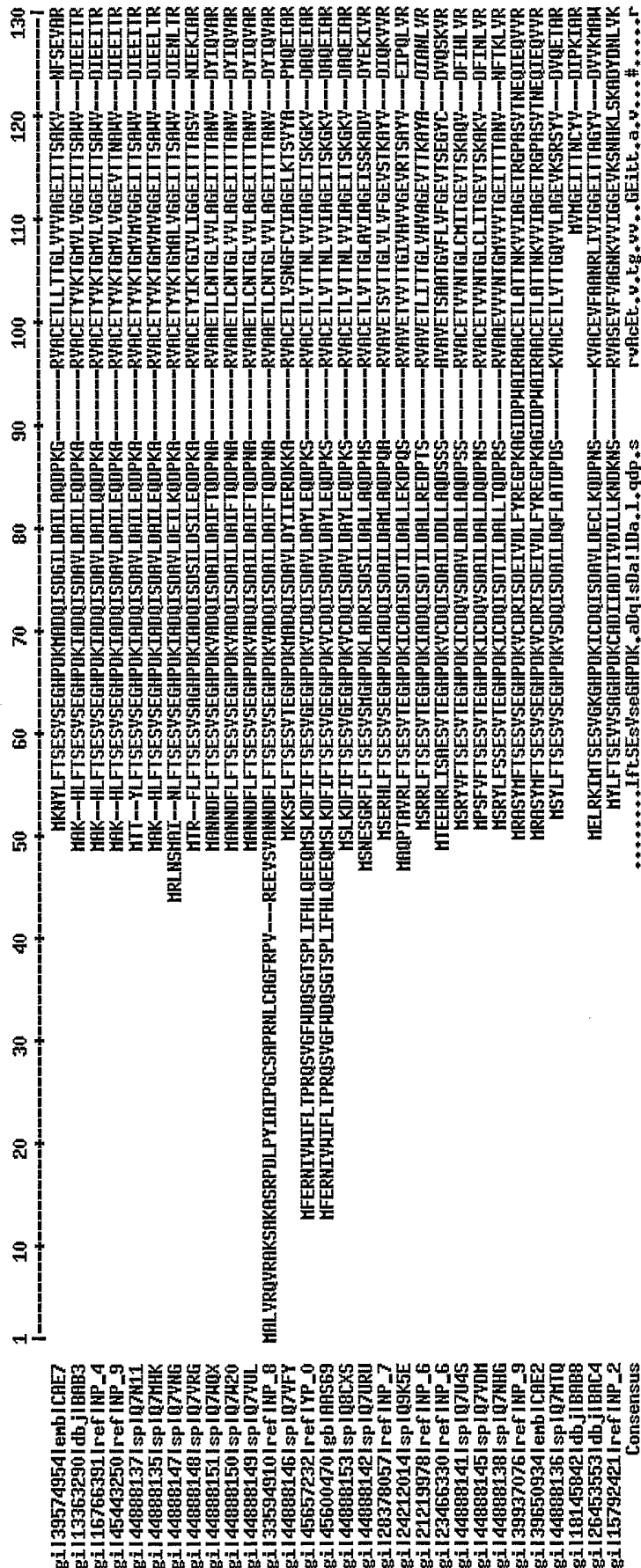


Fig. 4

[illegible]

Fig. 4 (cont.)

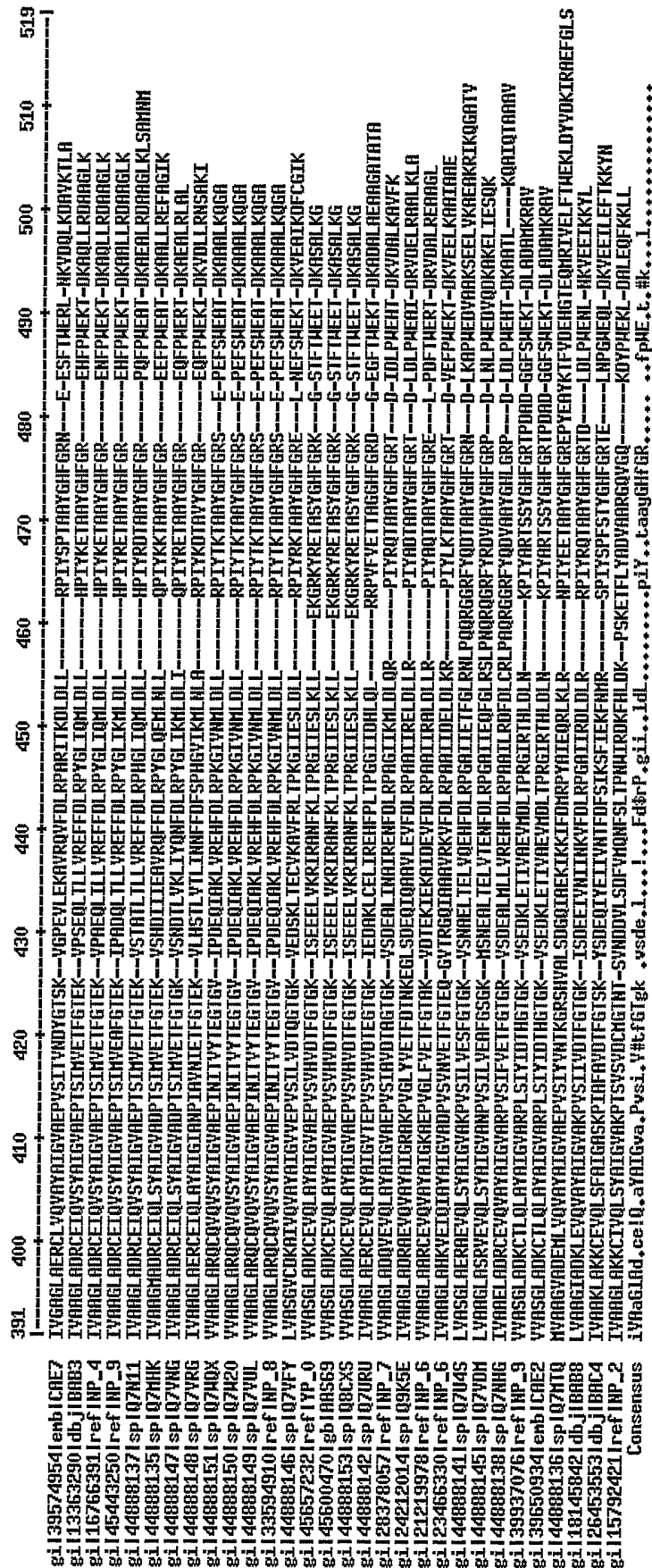


Fig. 4 (end)

9/9

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S S L Y
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C D Y
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I S A
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D F
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Fig. 5

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Ile Arg Pro Leu Lys Val Leu Ile Leu Asn Leu Met Pro Lys Lys Ile	
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MetA.ST25.txt

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Val	Glu	Phe	Asn	Asp	Val	Ala	Tyr	Trp	Pro	Gln	Ile	Lys	Gln	Val	Leu	
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Val Asp Ile Gln Leu Leu Arg Ile Asp Ser Arg Glu Ser Arg Asn Thr
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Pro Ala Glu His Leu Asn Asn Phe Tyr Cys Asn Phe Glu Asp Ile Gln
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Meta.ST25.txt

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 245 250 255

Arg His Gly Gly Gly Ala Phe Ser Gly Lys Asp Pro Ser Lys Val Asp
 260 265 270

Arg Ser Ala Ala Tyr Ala Ala Arg Tyr Val Ala Lys Asn Ile Val Ala
 275 280 285

Ala Gly Leu Ala Asp Arg Cys Glu Ile Gln Val Ser Tyr Ala Ile Gly
 290 295 300

Val Ala Glu Pro Thr Ser Ile Met Val Glu Thr Phe Gly Thr Glu Lys
 305 310 315 320

Val Pro Ser Glu Gln Leu Thr Leu Leu Val Arg Glu Phe Phe Asp Leu
 325 330 335

Arg Pro Tyr Gly Leu Ile Gln Met Leu Asp Leu Leu His Pro Ile Tyr
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Lys Glu Thr Ala Ala Tyr Gly His Phe Gly Arg Glu His Phe Pro Trp
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48

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB2004/001901

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/00

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)
IPC 7 C12N

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 practical, search terms used)

EPO-Internal, PAJ, Sequence Search, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 2000, no. 08, 6 October 2000 (2000-10-06) & JP 2000 139471 A (AJINOMOTO CO INC), 23 May 2000 (2000-05-23) cited in the application abstract	1-4, 6-12, 15-17
X	WO 2004/038013 A (LEONHARTSBERGER SUSANNE ; BAUER BRIGITTE (DE); CONSORTIUM ELEKTROCHEM) 6 May 2004 (2004-05-06) the whole document ----- -/--	1-4, 10-12,15

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

12 January 2005

Date of mailing of the international search report

19.04.05

Name and mailing address of the ISA

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB2004/001901

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHATTOPADHYAY M K ET AL: "CONTROL OF METHIONINE BIOSYNTHESIS IN ESCHERICHIA-COLI K12 A CLOSER STUDY WITH ANALOGUE-RESISTANT MUTANTS" JOURNAL OF GENERAL MICROBIOLOGY, vol. 137, no. 3, 1991, pages 685-692, XP009041956 ISSN: 0022-1287 cited in the application the whole document -----	1
X	DATABASE USPTO Proteins 'Online! 18 December 2003 (2003-12-18), "Sequence 8623 from patent US 6610836." XP002313076 retrieved from EBI accession no. USPOP:AAR48906 Database accession no. AAR48906 the sequence -----	11,12
X	DATABASE Geneseq 'Online! 20 November 2003 (2003-11-20), "Photorhabdus luminescens protein sequence #2067." XP002313077 retrieved from EBI accession no. GSN:ABM68970 Database accession no. ABM68970 the sequence -----	11,12
A	KUMAR D ET AL: "Production of methionine by a multi-analogue resistant mutant of Corynebacterium lilium." PROCESS BIOCHEMISTRY, vol. 38, no. 8, 28 March 2003 (2003-03-28), pages 1165-1171, XP002313075 ISSN: 1359-5113 cited in the application -----	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2004/001901

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-5, 11, 12, 15 completely; 6-10, 16-18 partially

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-5, 11, 12, 15 completely; 6-10, 16-18 partially

Feedback resistant mutants of the metA gene encoding homoserine transsuccinylase and methods for producing methionine using the same

1.1. claims: 1,2,6-10, 11, 12, 15-18 partially

Any such mutant outside regions 1 to 3 identified in claim 3

1.2. claims: 1-10,11,12,15-18 partially

Mutants of the conserved region 1 identified in claim 3

1.3. claims: 1-10,11,12,15-18 partially

Mutants of the conserved region 2 identified in claim 3

1.4. claims: 1-10,11,12,15-18 partially

Mutants of the conserved region 3 identified in claim 3

2. claims: 13,14 completely; claims 6-10, 16-18 partially

Mutants of the metK gene encoding a S-adenosylmethionine transferase with reduced S-adenosylmethionine transferase activity and methods for the production of methionine using the same

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB2004/001901

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 2000139471 A	23-05-2000	NONE	
WO 2004038013 A	06-05-2004	DE 10249642 A1 WO 2004038013 A2	13-05-2004 06-05-2004