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(54) **ERGOTHIONEINE PRODUCTION METHOD**

VERFAHREN ZUR HERSTELLUNG VON ERGOTHIONIN

PROCÉDÉ DE FABRICATION D'ERGOTHIONÉINE

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**WO-A1-2016/121285** **WO-A1-2017/150304**
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- **DATABASE UniProt [Online] 25 October 2017 (2017-10-25), JOHNSON, T.J. ET AL.: "Full=L-carnitine/gamma-butyrobetaine antiporter", XP055847416, Database accession no. A1A790**





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- **JONES, GARY W. et al.: "The evolutionary history of the genes involved in the biosynthesis of the antioxidant ergothioneine", Gene, vol. 549, 2014, pages 161-170, XP029044015, DOI: doi:10.1016/j.gene.2014.07.065**


Remarks:

The complete document including Reference Table(s) and the Sequence Listing(s) can be downloaded from the EPO website

## Description

TECHNICAL FIELD

**[0001]** The present invention relates to a method for producing ergothioneine or a related substance thereof, or a mixture thereof, using a bacterium belonging to the family *Enterobacteriaceae.* The present invention enhances the ergothioneine-producing ability of a bacterium belonging to the family *Enterobacteriaceae* having said ability by modification thereof to reduce the activity of a given protein comprising a core sequence region comprising 5 amino acid residues: Ser-Arg-Gly-Arg-Thr (SEQ ID NO:7) as a part thereof.

BACKGROUND ART

**[0002]** Ergothioneine is a kind of sulfur-containing amino acid and is known to have various physiological activities such as antioxidant ability. It is also suggested that its antioxidant ability is higher than that of vitamin C, vitamin E, cysteine and glutathione. It has also been shown that ergothioneine has an ultraviolet absorption effect, a melanin production inhibitory activity, a scavenging ability to reactive oxygen species, an elastase activity inhibitory activity that suppresses the formation of wrinkles and sagging, and a tyrosinase activity inhibitory activity that suppresses the formation of spots. Therefore, ergothioneine is one of the compounds that receive a lot of attention especially in the field of cosmetic and food industries.

**[0003]** Ergothioneine is abundant in some microorganisms, especially basidiomycetes, and is also present in trace amounts in plants and animals. Although mammals cannot biosynthesize ergothioneine, it is suggested that they can take up ergothioneine into the body by feeding mushrooms including basidiomycetes and that ergothioneine accumulates in many organs such as epidermis, brain, liver, kidney, spinal cord and eyes to protect cells. For production of ergothioneine, a method for culture and extraction of microorganisms such as basidiomycetes and a method for organic synthesis are known (cf. Patent references 1 and 2). However, the method for extraction from basidiomycetes has not yet come into widespread use due to various reasons such as its low productivity, a long period of time for culturing microorganisms, a complicated manufacturing process for extraction from within the cells due to its accumulation within the cells, and difficulty in improving the microorganisms. Also, the organic synthetic method has not yet come into widespread use due to various reasons such as complexity of multi-step reaction, possible use of a harmful raw material, and a high cost due to the use of an expensive raw material. A fermentation production method using a microorganism overexpressing ergothioneine biosynthesis genes or a microorganism having the ergothioneine-producing ability in which the histidine ammonia lyase activity is reduced or eliminated or the expression of histidine ammonia lyase gene is reduced or eliminated has been developed (Patent reference 3). Further, a fermentation method for producing ergothioneine has been developed, which comprises culturing, in a culture medium, a bacterium belonging to the family *Enterobacteriaceae* having ergothioneine-producing ability, and collecting the ergothioneine from the culture medium or from the cells obtained by culture (Patent reference 4). For both methods, a higher efficiency for producing ergothioneine is desirable.

[Prior Art]

[Patent reference]

**[0004]**

Patent reference 1: JP 2006-160748
Patent reference 2: JP 2007-300916
Patent reference 3: WO2017150304
Patent reference 4: WO2014100752

DISCLOSURE OF THE INVENTION

(Technical Problem to be Solved by the Invention)

**[0005]** As described above, under existing conditions, ergothioneine is expensive and its supply is problematic. Therefore, there is a need for a method for producing ergothioneine at a low cost in large quantities.

(Means for Solving the Problems)

**[0006]** The present inventors have earnestly studied to solve the above problems, and as a result, surprisingly have

found that a production level of ergothioneine can significantly increase by modifying bacteria belonging to the family *Enterobacteriaceae* having the ergothioneine-producing ability so as to reduce the activity of a given protein comprising a core sequence region comprising 5 amino acid residues: Ser-Arg-Gly-Arg-Thr (SEQ ID NO:7) as a part thereof to thereby complete the present invention.

**[0007]** Namely, the present invention provides a method for producing ergothioneine (hereinafter also referred to as "EGT"), or an oxidized or alkylated EGT, or selenoneine, or a mixture thereof, using a bacterium belonging to the family *Enterobacteriaceae* and enhances the ergothioneine-producing ability of a bacterium belonging to the family *Enterobacteriaceae* having said ability by modification thereof to reduce the activity of a given protein comprising a core sequence region comprising 5 amino acid residues: Ser-Arg-Gly-Arg-Thr (SEQ ID NO:7) as a part thereof.

**[0008]** Thus, the present invention provides the followings. A method for producing ergothioneine, or an oxidized or alkylated ergothioneine, or selenoneine, or a mixture thereof, comprising culturing a bacterium belonging to the family *Enterobacteriaceae* having the ergothioneine-producing ability in a culture medium, and collecting the ergothioneine, or the oxidized or alkylated ergothioneine, or the selenoneine, or the mixture thereof, from the culture medium or from the cells obtained by culture, wherein said bacterium is the one which is modified so as to have a reduced activity of a protein comprising a core sequence region comprising 5 amino acid residues: Ser-Arg-Gly-Arg-Thr (SEQ ID NO:7) as a part thereof.

**[0009]** Said method wherein the core sequence region has the sequence: Phe-X15aa-Ala-Arg-X16aa-Ser-Arg-Gly-Arg-Thr-X17aa-Arg
wherein X15aa, X16aa and X17aa are arbitrary amino acid residues (SEQ ID NO: 8).

**[0010]** Said method wherein the core sequence region is the sequence having the amino acid residues selected from the following (1) to (3) :

(1) X15aa is Leu or Ile;
(2) X16aa is Ile or Val;
(3) X17aa is Ile or Val.

**[0011]** Said method wherein the protein comprising a core sequence region is either a BetT protein or a CaiT protein, or both, wherein the BetT protein is the protein of (a), (b), or (c) below and wherein the CaiT protein is the protein of (d), (e), or (f) below:

(a) a protein comprising the amino acid sequence shown in SEQ ID NO: 10;
(b) a protein comprising the amino acid sequence shown in SEQ ID NO: 10 wherein one to two, three, four, five, six, seven, eight, nine or ten amino acid residue(s) is/are substituted, deleted, inserted or added;
(c) a protein comprising an amino acid sequence having 90% or more identity to the amino acid sequence shown in SEQ ID NO: 10;
(d) a protein comprising the amino acid sequence shown in SEQ ID NO: 11;
(e) a protein comprising the amino acid sequence shown in SEQ ID NO: 11 wherein one to two, three, four, five, six, seven, eight, nine or ten amino acid residue(s) is/are substituted, deleted, inserted or added;
(f) a protein comprising an amino acid sequence having 90% or more identity to the amino acid sequence shown in SEQ ID NO: 11.

**[0012]** Said method wherein the bacterium belonging to the family *Enterobacteriaceae* having the ergothioneine-producing ability is a bacterium modified so as to enhance the expression of ergothioneine biosynthesis genes.

**[0013]** Said method wherein the ergothioneine biosynthesis genes comprise any one or more of a gene corresponding to egtA, egtB, egtC, egtD or egtE of *Mycobacterium smegmatis,* a gene corresponding to Egt1 or Egt2 of *Shizosaccaromyces pombe,* a gene corresponding to Egt1 or Egt2 of *Neurospora crassa,* a gene corresponding to eanA or eanB of *Chlorobium limicola.*

**[0014]** Said method wherein the bacterium belonging to the family *Enterobacteriaceae* having the ergothioneine-producing ability is a bacterium belonging to the genus *Escherichia.*

**[0015]** Said method wherein the bacterium belonging to the family *Enterobacteriaceae* having the ergothioneine-producing ability is *Escherichia coli.*

**[0016]** A bacterium belonging to the family *Enterobacteriaceae* having the ergothioneine-producing ability modified so as to have a reduced activity of a protein comprising a core sequence region comprising 5 amino acid residues: Ser-Arg-Gly-Arg-Thr (SEQ ID NO:7) as a part thereof.

**[0017]** Said bacterium wherein the core sequence region has the sequence: Phe-X15aa-Ala-Arg-X16aa-Ser-Arg-Gly-Arg-Thr-X17aa-Arg
wherein X15aa, X16aa and X17aa are arbitrary amino acid residues (SEQ ID NO: 8).

**[0018]** Said bacterium wherein the core sequence region is the sequence having the amino acid residue selected from

the following (1) to (3) :

(1) X15aa is Leu or Ile;
(2) X16aa is Ile or Val;
(3) X17aa is Ile or Val.

**[0019]** Said bacterium wherein the protein comprising a core sequence region is either a BetT protein or a CaiT protein, or both, wherein the BetT protein is the protein of (a), (b), or (c) below and wherein the CaiT protein is the protein of (d), (e), or (f) below:

(a) a protein comprising the amino acid sequence shown in SEQ ID NO: 10;
(b) a protein comprising the amino acid sequence shown in SEQ ID NO: 10 wherein one to two, three, four, five, six, seven, eight, nine or ten amino acid residue(s) is/are substituted, deleted, inserted or added;
(c) a protein comprising an amino acid sequence having 90% or more identity to the amino acid sequence shown in SEQ ID NO: 10;
(d) a protein comprising the amino acid sequence shown in SEQ ID NO: 11;
(e) a protein comprising the amino acid sequence shown in SEQ ID NO: 11 wherein one to two, three, four, five, six, seven, eight, nine or ten amino acid residue(s) is/are substituted, deleted, inserted or added;
(f) a protein comprising an amino acid sequence having 90% or more identity to the amino acid sequence shown in SEQ ID NO: 11.

**[0020]** Said bacterium wherein the bacterium is a bacterium modified so as to enhance the expression of ergothioneine biosynthesis genes.

**[0021]** Said bacterium wherein the ergothioneine biosynthesis genes comprise any one or more of a gene corresponding to egtA, egtB, egtC, egtD or egtE of *Mycobacterium smegmatis,* a gene corresponding to Egt1 or Egt2 of *Shizosaccaromyces pombe,* a gene corresponding to Egt1 or Egt2 of *Neurospora crassa,* a gene corresponding to eanA or eanB of *Chlorobium limicola.*

**[0022]** Said bacterium wherein the bacterium is a bacterium belonging to the genus *Escherichia.*

**[0023]** Said bacterium wherein the bacterium is *Escherichia coli*.

## EFFECTS OF THE INVENTION

**[0024]** The present invention has made it possible to produce ergothioneine having various physiological activities such as a potent antioxidant activity at a low cost in large quantities.

## BRIEF DESCRIPTION OF DRAWINGS

**[0025]**

Fig. 1 is a graph showing the results (after 72 h of culture) of a test for ergothioneine fermentation production using an ergothioneine-producing *Escherichia coli,* MG1655/pACG-EGT. "out" and "in" indicate the EGT content in the culture medium and within the cells, respectively.
Fig. 2 is a graph showing the results (after 96 h of culture) of a test for ergothioneine fermentation production using a betT gene-disrupted strain and a caiT gene-disrupted strain, into which pACG-EGT was introduced. "out" and "in" indicate the EGT content in the culture medium and within the cells, respectively.
Fig. 3 is a graph showing the results (time course of EGT production level in culture medium) of a test for ergothioneine fermentation production using a betT gene-disrupted strain and a caiT gene-disrupted strain, into which pACG-EGT was introduced.
Fig. 4 is a graph showing the results of a test for ergothioneine fermentation production using a betT gene-disrupted strain and a caiT gene-disrupted strain, into which pACG_egtD-eanB was introduced, showing EGT production level accumulated in culture (48-hour culture) of MG1655, ΔbetT, and ΔcaiT strains transformed with pACG_egtD-eanB.

## BEST MODE FOR CARRYING OUT THE INVENTION

(Detailed Explanation of the Invention)

**[0026]** In the first aspect, the present invention relates to a method for producing ergothioneine (hereinafter also referred to as "EGT"), or an oxidized or alkylated ergothioneine, or selenoneine, or a mixture thereof, comprising culturing

a bacterium belonging to the family *Enterobacteriaceae* having the EGT-producing ability in a culture medium, and collecting the EGT or the oxidized or alkylated ergothioneine, or the selenoneine, or the mixture thereof, from the culture medium or from the cells obtained by culture, wherein said bacterium is the one which is modified so as to have a reduced activity of a given protein comprising a core sequence region comprising 5 amino acid residues: Ser-Arg-Gly-Arg-Thr (SEQ ID NO:7) as a part thereof. In accordance with the present invention, by making modification resulting in reduction in the activity of the given protein in a bacterium belonging to the family *Enterobacteriaceae* having the EGT-producing ability, EGT production level can dramatically increase.

**[0027]** In accordance with the present invention, modification is made to a bacterium belonging to the family *Enterobacteriaceae* having the EGT-producing ability so as to increase the EGT production level in the culture of said bacterium. Specifically, the modification is made so that the bacterium has a reduced activity of a protein comprising a core sequence region comprising 5 amino acid residues: Ser-Arg-Gly-Arg-Thr (SEQ ID NO:7) as a part thereof.

**[0028]** The protein comprising a core sequence region as mentioned above may be a protein comprising the sequence:
Phe-X15aa-Ala-Arg-X16aa-Ser-Arg-Gly-Arg-Thr-X17aa-Arg
wherein X15aa, X16aa and X17aa are arbitrary amino acid residues (SEQ ID NO: 8) as a core sequence region. A core sequence region is preferably the sequence having the amino acid residue selected from the following (1) to (3) :

(1) X15aa is Leu or Ile;
(2) X16aa is Ile or Val;
(3) X17aa is Ile or Val.

**[0029]** Furthermore, the protein comprising a core sequence region as mentioned above may be a protein comprising the sequence: X1aa-Trp-Thr-X2aa-X3aa-X4aa-Trp-X5aa-Trp-Trp-X6aa-X7aa-X8aa-X9aa-X10aa-X11aa-X12aa-X13aa-X14aa-Phe-X15aa-Ala-Arg-X16aa-Ser-Arg-Gly-Arg-Thr-X17aa-Arg-X18aa-X19aa
wherein X1aa, X2aa, X3aa, X4aa, X5aa, X6aa, X7aa, X8aa, X9aa, X10aa, X11aa, X12aa, X13aa, X14aa, X15aa, X16aa, X17aa, X18aa and X19aa are arbitrary amino acid residues (SEQ ID NO: 9) as a core sequence region. A core sequence region is preferably a sequence having the amino acid residue selected from the following (1) to (19):

(1) X1aa is Glu, Ser or Gly;
(2) X2aa is Leu, Val or Ser;
(3) X3aa is Phe or Leu;
(4) X4aa is Phe or Tyr;
(5) X5aa is Ala or Gly;
(6) X6aa is Val, Leu or Ile;
(7) X7aa is Ala, Ser or Ile;
(8) X8aa is Trp or Tyr;
(9) X9aa is Ser or Ala;
(10) X10aa is Pro or Ile;
(11) X11aa is Phe or Gln;
(12) X12aa is Val or Met;
(13) X13aa is Gly or Ser;
(14) X14aa is Leu, Met or Ile;
(15) X15aa is Leu or Ile;
(16) X16aa is Ile or Val;
(17) X17aa is Ile or Val;
(18) X18aa is Gln or Glu;
(19) X19aa is Phe or Leu.

**[0030]** Specific examples of the protein comprising a core sequence region include a BetT protein and a CaiT protein wherein the BetT protein is the protein of (a), (b), or (c) below and wherein the CaiT protein is the protein of (d), (e), or (f) below:

(a) a protein comprising the amino acid sequence shown in SEQ ID NO: 10;
(b) a protein comprising the amino acid sequence shown in SEQ ID NO: 10 wherein one to two, three, four, five, six, seven, eight, nine or ten amino acid residue(s) is/are substituted, deleted, inserted or added;
(c) a protein comprising an amino acid sequence having 90% or more identity to the amino acid sequence shown in SEQ ID NO: 10;
(d) a protein comprising the amino acid sequence shown in SEQ ID NO: 11;
(e) a protein comprising the amino acid sequence shown in SEQ ID NO: 11 wherein one to two, three, four, five,

six, seven, eight, nine or ten amino acid residue(s) is/are substituted, deleted, inserted or added;
(f) a protein comprising an amino acid sequence having 90% or more identity to the amino acid sequence shown in SEQ ID NO: 11.

**[0031]** The protein comprising a core sequence region is preferably a BetT protein comprising an amino acid sequence having 90% or more, 95% or more, or 98% or more identity to the amino acid sequence shown in SEQ ID NO: 10, a CaiT protein comprising an amino acid sequence having 90% or more, 95% or more, or 98% or more identity to the amino acid sequence shown in SEQ ID NO: 11.

**[0032]** As set forth above, "one to two, three, four, five, six, seven, eight, nine or ten amino acid residue(s) is/are substituted, deleted, inserted or added" means that 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid residue(s) is/are substituted, deleted, inserted or added, preferably 1, 2, 3, 4, 5 or 6 amino acid residue (s) is/are substituted, deleted, inserted or added, more preferably 1, 2, 3 or 4 amino acid residue(s) is/are substituted, deleted, inserted or added.

**[0033]** With the method of the invention also EGT analogs can be prepared. Such an EGT analog includes an oxidized EGT, and an alkylated EGT. Specific examples of oxidized EGT include Ergothioneine sulfenic acid, Ergothioneine sulfinic acid, Ergothioneine sulfonic acid, disulfide bound EGT, mixed disulfide EGT and Hercynine. Examples of an alkylated EGT include S-Methyl-Ergothioneine. It has been reported that an oxidized EGT and an alkylated EGT as mentioned above are generated from ergothioneine via an intracellular metabolic reaction or an oxidative reaction with oxygen or an oxidant in the culture medium (described in L. Servillo et al., Free. Radic. Biol. Med., 2015, 79, 228-36, and R. M. Y. Tang, et al., Sci. Rep., 2018, 8(1), 1601). Also, an EGT analog includes selenoneine.

**[0034]** By making the above-mentioned modification to a bacterium belonging to the family *Enterobacteriaceae* having the EGT-producing ability, i.e. the modification allowing for reduction in the activity of a protein comprising the given core sequence region, it becomes possible to dramatically increase the EGT production level.

**[0035]** A bacterium belonging to the family *Enterobacteriaceae* having the EGT-producing ability for use in the present invention is the one modified so as to have a reduced activity of a protein comprising a core sequence region comprising 5 amino acid residues: Ser-Arg-Gly-Arg-Thr (SEQ ID NO:7) as a part thereof, the one modified so as to have a reduced activity of a protein comprising the sequence: Phe-X15aa-Ala-Arg-X16aa-Ser-Arg-Gly-Arg-Thr-X17aa-Arg

wherein X15aa, X16aa and X17aa are arbitrary amino acid residues (SEQ ID NO: 8) as a core sequence region, or the one modified so as to have a reduced activity of a protein comprising the sequence: X1aa-Trp-Thr-X2aa-X3aa-X4aa-Trp-X5aa-Trp-Trp-X6aa-X7aa-X8aa-X9aa-X10aa-X11aa-X12aa-X13aa-X14aa-Phe-X15aa-Ala-Arg-X16aa-Ser-Arg-Gly-Arg-Thr-X17aa-Arg-X18aa-X19aa
wherein X1aa, X2aa, X3aa, X4aa, X5aa, X6aa, X7aa, X8aa, X9aa, X10aa, X11aa, X12aa, X13aa, X14aa, X15aa, X16aa, X17aa, X18aa and X19aa are arbitrary amino acid residues (SEQ ID NO: 9) as a core sequence region, preferably the one modified so as to have a reduced activity of a BetT protein or a CaiT protein or a protein comprising an amino acid sequence having 90% or more identity to the amino acid sequence of a BetT protein or a CaiT protein. The rate of reduction of the activity of a BetT protein or a CaiT protein is preferably 50% or less, more preferably 25% or less, even more preferably 10% or less than its parent strain. The bacterium having the eliminated activity of a BetT protein or a CaiT protein is most preferable. The bacterium having the eliminated activity of a BetT protein or a CaiT protein is a bacterium in which the activity of a BetT protein or a CaiT protein is not detected at all. In other words, the bacterium having the reduced or eliminated activity of a BetT protein or a CaiT protein is a bacterium in which the expression of a BetT protein or CaiT protein gene is reduced or eliminated. The rate of reduction of the expression of a BetT protein or CaiT protein gene is preferably 50% or less, more preferably 25% or less, even more preferably 10% or less than its parent strain. The bacterium having the eliminated expression of a BetT protein or CaiT protein gene is most preferable. The bacterium having the eliminated expression of a BetT protein or CaiT protein gene is a bacterium in which the expression of a BetT protein or CaiT protein gene is not detected at all. The activity of a BetT protein or a CaiT protein and the expression of a BetT protein or CaiT protein gene can be measured by known methods such as Northern blotting, RT-PCR, real-time PCR, and Western blotting.

**[0036]** A method for reducing or eliminating the activity of a BetT protein or a CaiT protein and a method for reducing or eliminating the expression of a BetT protein or CaiT protein gene are also known and include e.g. knocking-out or knocking-down of a gene of a BetT protein or a CaiT protein by homologous recombination, mutation treatment, genome editing , administering a substance that inhibits a BetT protein or a CaiT protein to bacteria. Also, the bacterium to be used in the present invention may be the one in which the activity of a BetT protein or a CaiT protein is reduced or eliminated as a result of natural mutation.

**[0037]** A bacterium belonging to the family *Enterobacteriaceae* having the EGT-producing ability for use in the present invention is preferably a bacterium modified so as to enhance the expression of ergothioneine biosynthesis genes.

**[0038]** An EGT biosynthesis gene is a gene encoding an enzyme involved in EGT biosynthesis. Enzymes involved in EGT biosynthesis and EGT biosynthesis genes are known and, for some of the genes, their nucleotide sequences are

also known. Those skilled in the art can appropriately select such EGT biosynthesis genes. The EGT biosynthesis genes to be enhanced include e.g. one or more of the genes corresponding to egtA, egtB, egtC, egtD or egtE of *Mycobacterium smegmatis,* either or both of the genes corresponding to Egt1 or Egt2 of *Schizosaccharomyces pombe,* either or both of the genes corresponding to Egt1 or Egt2 of *Neurospora crassa,* or either or both of the genes corresponding to eanA or eanB of *Chlorobium limicola.* With respect to the EGT biosynthesis genes of *Mycobacterium*, F. P. Seebeck, J. Am. Chem. Soc., 2010, 132, 6632-6633 can be referred to. With respect to the EGT biosynthesis genes of *Schizosaccharomyces*, T. Pluskal et al, PLOS ONE., 2014, 9(5), e97774 can be referred to. With respect to the EGT biosynthesis genes of *Neurospora*, W. Hu et al., Org. Lett., 2014, 16, 5382-5385 can be referred to. Also, with respect to the EGT biosynthesis genes of *Streptomyces*, S. Nakajima et al., J. Biosci. Bioeng., 2015, 120, 294-298 can be referred to. With respect to the EGT biosynthesis genes of *Chlorobium,* R. Burn, et al., Angew. Chem. Int. Edit., 2017, 56, 12508-12511 can be referred to. With respect to the EGT biosynthesis genes of various microorganisms, G. W. Jones et al, Gene, 2014, 549, 161-170 can be referred to.

**[0039]** "To enhance the expression of a gene" means that an expression level of a given gene is higher than that of a parent strain. The expression level is preferably twice or more, more preferably five times or more, even more preferably ten times or more higher than that of a parent strain or a wild strain. Quantification of gene expression can be performed by a known method such as Northern blotting or real-time PCR.

**[0040]** A "corresponding gene" is, with regard to a gene that encodes an enzyme that catalyzes a reaction in the EGT biosynthetic pathway of a given microorganism, a gene of another microorganism that encodes an enzyme that catalyzes a reaction in the EGT biosynthetic pathway that is the same as or similar to that in the given microorganism. A given gene and a corresponding gene may have the same nucleotide sequence or different nucleotide sequences. For instance, a given gene and a corresponding gene may have a nucleotide sequence identity of 50% or more, 70% or more, 80% or more, or 90% or more. Furthermore, an enzyme protein encoded by a given gene and an enzyme protein encoded by a corresponding gene may have the same amino acid sequence or different amino acid sequences. For instance, an enzyme protein encoded by a given gene and an enzyme protein encoded by a corresponding gene may have an amino acid sequence identity of 50% or more, 70% or more, 80% or more, or 90% or more.

**[0041]** The expression of EGT biosynthesis genes can be enhanced by using a known method for enhancing gene expression. The most commonly used method is to place EGT biosynthesis genes under the control of a promoter having potent expression. For instance, the expression of EGT biosynthesis genes may be enhanced by introducing into a microorganism an expression vector which incorporates a promoter suitable for the expression of EGT biosynthesis genes and EGT biosynthesis genes, or by incorporating EGT biosynthesis genes into the host genome by homologous recombination etc. For the vector, a plasmid vector or a virus vector can be used. When constructing the vector, constituent elements of the vector such as a promoter, a terminator, a marker gene such as a drug resistance gene or a metabolic enzyme gene can be appropriately selected and used. Methods for introducing genes into bacteria are also known. The gene to be introduced can be introduced into bacteria directly or after incorporation into a vector. Examples of a method for introducing a gene include lipofection, calcium phosphate, the use of polymer, electroporation and particle gun and these methods may be appropriately selected depending on the type of bacteria to which a gene is introduced and the gene to be introduced. In order to enhance the expression of EGT biosynthesis genes, the expression of endogenous EGT biosynthesis genes may also be induced by deleting a specific gene or region on the genome.

**[0042]** The method for enhancing the expression of EGT biosynthesis genes is not limited to the above methods. In addition, the bacterium used in the present invention may be the one having the enhanced expression of EGT biosynthesis genes as a result of natural mutation. The enhanced expression of EGT biosynthesis genes can be confirmed by a known method such as Northern blotting or real-time PCR. Alternatively, the enhanced expression of EGT biosynthesis genes may be confirmed by actually culturing the bacteria and quantifying EGT produced inside or outside (in the culture solution) the cells.

**[0043]** EGT biosynthesis genes to be enhanced for its expression in bacteria may be of one type or two or more types. A set of genes encoding multiple enzymes involved in EGT biosynthesis may be introduced into bacteria as a cluster. EGT biosynthesis genes from microorganisms of a different genus or species may be introduced to enhance the expression, or EGT biosynthesis genes from microorganisms of the same genus or species may be introduced to enhance the expression. It is preferable to introduce EGT biosynthesis genes from the same genus or a related genus to enhance the expression. It is preferable to introduce EGT biosynthesis genes from the same species or a related species to enhance the expression. It is more preferable to introduce EGT biosynthesis genes from the same species to enhance the expression.

**[0044]** A bacterium belonging to the family *Enterobacteriaceae* having the EGT-producing ability for use in the present invention includes a bacterium of *Escherichiai, Enterobacter*, *Pantoea*, *Klebsiella*, and *Salmonella*. A particularly preferable enterobacterium includes those of *Escherichia* such as *Escherichia coli*, *Pantoea* such as *Pantoea ananatis.*

**[0045]** The culture of a bacterium belonging to the family *Enterobacteriaceae* in the method for producing EGT of the present invention can be carried out by an ordinary method. Specifically, LB medium, 2×YT medium, NZY medium, M9 medium, SOC medium, YPD medium or the like can be used. The above media can be used to produce EGT, but the

media to be used are not limited thereto. The produced EGT may be accumulated inside the cells or may be secreted and accumulated outside the cells (in the culture solution).

**[0046]** EGT inside the cells or EGT released from the cells can be collected by a known method. For instance, the culture can be subjected to solid-liquid separation such as centrifugation or filtration, and the EGT extract can be obtained from inside the cells by solvent extraction, hot water extraction or crushing treatment. From the EGT extract or the culture supernatant, EGT can be obtained by known chromatography such as ion exchange chromatography, hydrophobic chromatography and gel filtration chromatography.

**[0047]** In the second aspect, the present invention relates to a bacterium belonging to the family *Enterobacteriaceae* having the EGT-producing ability, which is modified according to the present invention so as to have a reduced activity of a given protein comprising a core sequence region comprising 5 amino acid residues: Ser-Arg-Gly-Arg-Thr (SEQ ID NO:7) as a part thereof.

**[0048]** A bacterium belonging to the family *Enterobacteriaceae* having the EGT-producing ability, which is modified according to the present invention so as to have a reduced activity of a given protein, is a bacterium belonging to the family *Enterobacteriaceae* having the EGT-producing ability, which is modified so as to have a reduced activity of a protein comprising a core sequence region comprising 5 amino acid residues: Ser-Arg-Gly-Arg-Thr (SEQ ID NO:7) as a part thereof, a bacterium belonging to the family *Enterobacteriaceae* having the EGT-producing ability, which is modified so as to have a reduced activity of a protein comprising the sequence: Phe-X15aa-Ala-Arg-X16aa-Ser-Arg-Gly-Arg-Thr-X17aa-Arg wherein X15aa, X16aa and X17aa are arbitrary amino acid residues (SEQ ID NO: 8) as a core sequence region, or a bacterium belonging to the family *Enterobacteriaceae* having the EGT-producing ability, which is modified so as to have a reduced activity of a protein comprising the sequence: X1aa-Trp-Thr-X2aa-X3aa-X4aa-Trp-X5aa-Trp-Trp-X6aa-X7aa-X8aa-X9aa-X10aa-X11aa-X12aa-X13aa-X14aa-Phe-X15aa-Ala-Arg-X16aa-Ser-Arg-Gly-Arg-Thr-X17aa-Arg-X18aa-X19aa
wherein X1aa, X2aa, X3aa, X4aa, X5aa, X6aa, X7aa, X8aa, X9aa, X10aa, X11aa, X12aa, X13aa, X14aa, X15aa, X16aa, X17aa, X18aa and X19aa are arbitrary amino acid residues (SEQ ID NO: 9) as a core sequence region, preferably a bacterium belonging to the family *Enterobacteriaceae* having the EGT-producing ability, which is modified so as to have a reduced activity of a BetT protein or a CaiT protein or a protein comprising an amino acid sequence having 90% or more identity to the amino acid sequence of a BetT protein or a CaiT protein. The reduction of the activity of a BetT protein or a CaiT protein is as described above.

**[0049]** A bacterium belonging to the family *Enterobacteriaceae* having the EGT-producing ability, which is modified according to the present invention so as to have a reduced activity of the given protein, is preferably a bacterium modified so as to enhance the expression of ergothioneine biosynthesis genes.

**[0050]** A bacterium belonging to the family *Enterobacteriaceae* having the EGT-producing ability, which is modified according to the present invention so as to have a reduced activity of the given protein and is modified so as to enhance the expression of EGT biosynthesis genes, is preferably a bacterium modified so as to enhance the expression of EGT biosynthesis genes by enhancing one or more of the genes corresponding to egtA, egtB, egtC, egtD or egtE of *Mycobacterium smegmatis,* either or both of the genes corresponding to Egt1 or Egt2 of *Schizosaccharomyces pombe,* or either or both of the genes corresponding to Egt1 or Egt2 of *Neurospora crassa.*

**[0051]** A bacterium belonging to the family *Enterobacteriaceae* having the EGT-producing ability, which is modified according to the present invention so as to have a reduced activity of the given protein, may be a bacterium of *Escherichia*, *Enterobacter*, *Pantoea, Klebsiella*, and *Salmonella*, and most preferably *Escherichia* such as *Escherichia coli*, *Pantoea* such as *Pantoea ananatis*.

**[0052]** The present invention is explained in more detail and specifically with the following Examples. Unless otherwise specified, experiments were carried out using the methods described in a collection of standard protocols for molecular biology and applied microbiology, or modified or altered methods thereof. In addition, "%" represents "w/v%" unless otherwise specified.

[Example 1]

Construction of vector for EGT production (egt1-2):

**[0053]** In order to produce EGT in *Escherichia coli,* the following vector for EGT production was constructed. As EGT biosynthesis genes, egt1 (SPBC1604.01: SEQ ID NO: 1) and egt2 (SPBC660.12c: SEQ ID NO: 2) from *Schizosaccharomyces pombe* were used. Using as a template a vector containing the EGT biosynthesis genes egt1 and egt2 shown in the sequence listing, egt1 and egt2 were amplified using the primers shown in Table 1.

Table 1

| Primer | Sequence |
|---|---|
| Sp. egt1-Fw | 5' -AAATAGCTGGTGAATGACCGAAATCGAAAACAT-3' (SEQ ID NO:3) |
| Sp. egt1-Rv | 5' -AGCCCTCCTGGATCCTCAGTTTTTAACCAGACGAG-3' (SEQ ID NO:4) |
| Sp. egt2-Fw | 5' -GGATCCAGGAGGGCTAGCATGGCTGAA-3' (SEQ ID NO:5) |
| Sp. egt2-Rv | 5' -CGACTCTAGAGGATCTCACAGAGAGCAGAAGTCTT-3' (SEQ ID NO:6) |

**[0054]** As an expression vector, pACG was used in which the promoter of an expression vector having the p15A replication origin was modified to the promoter of the gapA gene from *Escherichia coli,* and the drug resistance marker was changed to the tetracycline resistance gene. Using In-Fusion HD cloning Kit (Takara Bio Inc.), the PCR products of egt1 and egt2 were ligated to the HindIII-BamHI site immediately downstream the promoter of pACG to construct pACG-EGT as a vector for EGT production. The In-Fusion reaction was performed according to the protocol provided by Takara Bio Inc.

**[0055]** MG1655 strain (ATCC deposit number: 700926) was used as a wild strain of *Escherichia coli,* and transformation was performed using the constructed pACG-EGT to give an EGT-producing strain (MG1655/pACG-EGT).

[Example 2]

Test for EGT production in *Escherichia coli*:

**[0056]** Using the EGT production strain MG1655/pACG-EGT constructed in Example 1, a test for EGT fermentation production was conducted. MG1655/pACG obtained by introducing the empty vector pACG into MG1655 strain was used as a control strain. A respective glycerol stock of MG1655/pACG and MG1655/pACG-EGT was added to a test tube containing tetracycline (final concentration of 10 ug/ml) in 3 ml of LB medium (polypeptone 10 g, yeast extract 5 g, NaCl 10 g in medium 1 1), which was subjected to shaking culture at 37 °C at 200 rpm for 15-18 h until reaching a stationary phase to give a preculture solution. Furthermore, the preculture solution was added to 50 ml of 2 × TY medium (polypeptone 16 g, yeast extract 10 g, NaCl 5 g, Glucose 5%, L-Histidine 5 mM, L-Cystine 2.5 mM, L-Methionine 5 mM, tetracycline 10 ug/ml in medium 1 l) in a 500 ml baffled flask to give OD=0.4, which was subjected to main culture by shaking at 37 °C at 160 rpm for 96 h.

**[0057]** The quantification of EGT was performed as described below. During the main culture, 1 ml of the culture solution was collected at 24, 48, 72, and 96 h after initiation of culture to evaluate the culture samples. After measuring the microbial cell turbidity (OD600) of the collected culture solution, centrifugation was performed at 14000 rpm for 10 min to separate the extracellular culture solution and the precipitate (cells). The precipitate was subjected to hot water extraction (60 °C, 10 min) to extract intracellular components for use as an intracellular sample. The EGT content in the intracellular sample and in the separated extracellular culture solution (extracellular sample) was measured by HPLC. The conditions of HPLC measurement were as shown in Table 2 below and the quantification was performed based on the EGT peak generated at a retention time of around 10 min.

Table 2

| | |
|---|---|
| Column | Inertsil HILIC (5 μM; 4.6 ϕmm×250 mm) manufactured by GL Sciences Inc. |
| Solvent | 100 mM ammonium acetate/acetonitrile/ isopropanol/water (2:82:6:10) |
| Gradient condition | Isocratic |
| Detection wavelength | 265 nm |
| Flow rate | 1 ml/min |
| temperature of column | 40 °C |

**[0058]** As a result of the culture test, after 72 h of culture, EGT production was not confirmed from the control strain MG1655/pACG, but the peak of peak of EGT could be confirmed in the MG1655/pACG-EGT culture solution and the intracellular extract to confirm EGT synthesis gene-dependent EGT production (Fig. 1).

[Example 3]

Test for EGT production in ΔbetT and ΔcaiT:

**[0059]** Next, using the respective gene-disrupted strains of betT and caiT, a test for EGT fermentation production was conducted. The betT gene-disrupted strain (ΔbetT) and the caiT gene-disrupted strain (ΔcaiT) were constructed by a known method of homologous recombination using a counter selection vector (including sacB gene and drug resistance gene) containing upstream and downstream sequences of the betT gene and the caiT gene, respectively, to disrupt the respective genes in the *Escherichia coli* MG1655 strain. For the construction of the above-mentioned disrupted strains, M. S. Donnenberg and J. S. Kaper, Infect. Immun., 1991, 4310-4317, H. Mizoguchi et al., Biosci. Biotechnol. Biochem., 2007, 71 (12), 2905-2911 can be referred to. The constructed ΔbetT and ΔcaiT were transformed with pACG-EGT to give ΔbetT/pACG-EGT and ΔcaiT/pACG-EGT. As a control strain, MG1655/pACG-EGT constructed in Example 2 was used in the following culture test.

**[0060]** The culture and the EGT analysis were performed as described in Example 2. As a result of the culture test, the EGT production level in the culture solution 96 h after initiation of the culture resulted in surprisingly drastic increase both intracellularly and extracellularly in both gene-disrupted strains, ΔbetT/pACG-EGT by about 8.6 times (12.3 times intracellularly) and ΔcaiT/pACG-EGT by about 4.6 times (3.75 times intracellularly), as compared to the wild strain (Figs. 2 and 3).

[Example 4]

Construction of vector for EGT production (egtD-eanB):

**[0061]** A vector for production was constructed using EGT synthesis genes from anaerobic bacteria. In addition to the enzyme encoded by the egtD gene, the expression in *Escherichia coli* of the enzyme encoded by the eanB gene found in anaerobic bacteria allows for biosynthesis of EGT utilizing the sulfur from thiosulfate ion. For the egtD gene, the nucleotide sequence (SEQ ID NO: 12) obtained by codon-optimizing the amino acid sequence from *Streptomyces coelicolor*, which was classified into the same *Actinomycetales* as *Mycobacterium smegmatis,* was used, and for the eanB gene, the nucleotide sequence (SEQ ID NO: 13) obtained by codon-optimizing the amino acid sequence from the anaerobic bacterium *Chlorobium limicola* was used. The above two genes were artificially synthesized. For the vector, pACG-EGT constructed in Example 1 was used. The vector pACG-EGT was treated with restriction enzymes HindIII-BamHI to cleave the egt1 gene and the artificially synthesized egtD gene was linked to the HindIII-BamHI site by ligation. The vector cloned for the egtD gene was further treated with NheI-XbaI to cleave the egt2 and the eanB gene was linked to the NheI-XbaI site by ligation to construct pACG_ egtD-eanB in which egtD-eanB was cloned downstream the gapA promoter.

[Example 5]

Test for EGT production using vector for EGT production (egtD-eanB) :

**[0062]** Three *Escherichia coli* strains, MG1655, and ΔbetT and ΔcaiT prepared in Example 3, were transformed with pACG_egtD-eanB constructed in Example 4, and the obtained strains were used in the following test for EGT production.

**[0063]** A production test using the prepared three strains was carried out using 10 mM sodium thiosulfate instead of 2.5 mM Cystine under the main culture conditions as described in Example 2. As a result, even when the egtD-eanB genes were used in the vector for EGT production, the EGT productivity was significantly improved in the ΔbetT and ΔcaiT strains at 48 h after initiation of culture as compared to the wild strain (Fig. 4).

**[0064]**

SEQ ID NO: 1 is the nucleotide sequence of the egt1 gene (SPBC1604.01) from *Schizosaccharomyces pombe.*
SEQ ID NO: 2 is the nucleotide sequence of the egt2 gene (SPBC660.12c) from *Schizosaccharomyces pombe.*
SEQ ID NO: 3 is the nucleotide sequence of a forward primer for amplifying the egt1 gene (SPBC1604.01) from *Schizosaccharomyces pombe.*
SEQ ID NO: 4 is the nucleotide sequence of a reverse primer for amplifying the egt1 gene (SPBC1604.01) from *Schizosaccharomyces pombe.*
SEQ ID NO: 5 is the nucleotide sequence of a forward primer for amplifying the egt2 gene (SPBC660.12c) from *Schizosaccharomyces pombe.*
SEQ ID NO: 6 is the nucleotide sequence of a reverse primer for amplifying the egt2 gene (SPBC660.12c) from *Schizosaccharomyces pombe.*

SEQ ID NO: 7 is the amino acid sequence common to core sequence regions.
SEQ ID NO: 8 is the amino acid sequence of a core sequence region.
SEQ ID NO: 9 is the amino acid sequence of a core sequence region.
SEQ ID NO: 10 is the amino acid sequence of a BetT protein.
SEQ ID NO: 11 is the amino acid sequence of a CaiT protein.
SEQ ID NO: 12 is the nucleotide sequence obtained by codon-optimizing the amino acid sequence of an EgtD protein from Streptomyces coelicolor for *Escherichia coli.*
SEQ ID NO: 13 is the nucleotide sequence obtained by codon-optimizing the amino acid sequence of an EanB protein from *Chlorobium limicola* for *Escherichia coli.*

INDUSTRIAL APPLICABILITY

**[0065]** A method for producing ergothioneine, or an oxidized or alkylated ergothioneine, or selenoneine, or a mixture thereof, of the present invention allows for producing ergothioneine having various physiological activities such as a potent antioxidant activity at a low cost in large quantities.

## Claims

1. A method for producing ergothioneine, or an oxidized or alkylated ergothioneine, or selenoneine, or a mixture thereof, comprising culturing a bacterium belonging to the family *Enterobacteriaceae* having the ergothioneine-producing ability in a culture medium, and collecting the ergothioneine, or the oxidized or alkylated ergothioneine, or the selenoneine, or the mixture thereof, from the culture medium or from the cells obtained by culture, wherein said bacterium is the one which is modified so as to have a reduced activity of a protein comprising a core sequence region comprising 5 amino acid residues: Ser-Arg-Gly-Arg-Thr (SEQ ID NO:7) as a part thereof.

2. The method of claim 1 wherein the core sequence region has the sequence: Phe-X15aa-Ala-Arg-X16aa-Ser-Arg-Gly-Arg-Thr-X17aa-Arg
wherein X15aa, X16aa and X17aa are arbitrary amino acid residues (SEQ ID NO: 8).

3. The method of claim 2 wherein the core sequence region is the sequence having the amino acid residues selected from the following (1) to (3):

   (1) X15aa is Leu or Ile;
   (2) X16aa is lie or Val;
   (3) X17aa is lie or Val.

4. The method of claim 1 wherein the protein comprising a core sequence region is either a BetT protein or a CaiT protein, or both, wherein the BetT protein is the protein of (a), (b), or (c) below, and wherein the CaiT protein is the protein of (d), (e), or (f) below:

   (a) a protein comprising the amino acid sequence shown in SEQ ID NO: 10;
   (b) a protein comprising the amino acid sequence shown in SEQ ID NO: 10 wherein one to two, three, four, five, six, seven, eight, nine or ten amino acid residue(s) is/are substituted, deleted, inserted or added;
   (c) a protein comprising an amino acid sequence having 90% or more identity to the amino acid sequence shown in SEQ ID NO: 10;
   (d) a protein comprising the amino acid sequence shown in SEQ ID NO: 11;
   (e) a protein comprising the amino acid sequence shown in SEQ ID NO: 11 wherein one to two, three, four, five, six, seven, eight, nine or ten amino acid residue(s) is/are substituted, deleted, inserted or added;
   (f) a protein comprising an amino acid sequence having 90% or more identity to the amino acid sequence shown in SEQ ID NO: 11.

5. The method of any one of claims 1 to 4 wherein the bacterium belonging to the family *Enterobacteriaceae* having the ergothioneine-producing ability is a bacterium modified so as to enhance the expression of ergothioneine biosynthesis genes.

6. The method of claim 5 wherein the ergothioneine biosynthesis genes comprise any one or more of a gene corresponding to egtA, egtB, egtC, egtD or egtE of *Mycobacterium smegmatis,* a gene corresponding to Egt1 or Egt2 of

*Shizosaccaromyces pombe,* a gene corresponding to Egt1 or Egt2 of *Neurospora crassa,* a gene corresponding to eanA or eanB of *Chlorobium limicola*.

**7.** The method of any one of claims 1 to 6 wherein the bacterium belonging to the family *Enterobacteriaceae* having the ergothioneine-producing ability is a bacterium belonging to the genus *Escherichia.*

**8.** The method of claim 7 wherein the bacterium belonging to the family *Enterobacteriaceae* having the ergothioneine-producing ability is *Escherichia coli*.

**9.** A bacterium belonging to the family *Enterobacteriaceae* having the ergothioneine-producing ability modified so as to have a reduced activity of a protein comprising a core sequence region comprising 5 amino acid residues: Ser-Arg-Gly-Arg-Thr (SEQ ID NO:7) as a part thereof.

**10.** The bacterium of claim 9 wherein the core sequence region has the sequence: Phe-X15aa-Ala-Arg-X16aa-Ser-Arg-Gly-Arg-Thr-X17aa-Arg
wherein X15aa, X16aa and X17aa are arbitrary amino acid residues (SEQ ID NO: 8).

**11.** The bacterium of claim 10 wherein the core sequence region is the sequence having the amino acid residue selected from the following (1) to (3):

(1) X15aa is Leu or Ile;
(2) X16aa is lie or Val;
(3) X17aa is lie or Val.

**12.** The bacterium of claim 9 wherein the protein comprising a core sequence region is either a BetT protein or a CaiT protein, or both, wherein the BetT protein is the protein of (a), (b), or (c) below and wherein the CaiT protein is the protein of (d), (e), or (f) below:

(a) a protein comprising the amino acid sequence shown in SEQ ID NO: 10;
(b) a protein comprising the amino acid sequence shown in SEQ ID NO: 10 wherein one to two, three, four, five, six, seven, eight, nine or ten amino acid residue(s) is/are substituted, deleted, inserted or added;
(c) a protein comprising an amino acid sequence having 90% or more identity to the amino acid sequence shown in SEQ ID NO: 10;
(d) a protein comprising the amino acid sequence shown in SEQ ID NO: 11;
(e) a protein comprising the amino acid sequence shown in SEQ ID NO: 11 wherein one to two, three, four, five, six, seven, eight, nine or ten amino acid residue(s) is/are substituted, deleted, inserted or added;
(f) a protein comprising an amino acid sequence having 90% or more identity to the amino acid sequence shown in SEQ ID NO: 11.

**13.** The bacterium of any one of claims 9 to 12 wherein the bacterium is a bacterium modified so as to enhance the expression of ergothioneine biosynthesis genes.

**14.** The bacterium of claim 13 wherein the ergothioneine biosynthesis genes comprise any one or more of a gene corresponding to egtA, egtB, egtC, egtD or egtE of *Mycobacterium smegmatis,* a gene corresponding to Egt1 or Egt2 of *Shizosaccaromyces pombe,* a gene corresponding to Egt1 or Egt2 of *Neurospora crassa,* a gene corresponding to eanA or eanB of *Chlorobium limicola*.

**15.** The bacterium of any one of claims 9 to 14 wherein the bacterium is a bacterium belonging to the genus *Escherichia,* preferably the bacterium is *Escherichia coli.*

**Patentansprüche**

**1.** Verfahren zur Herstellung von Ergothionein oder eines oxidierten oder alkylierten Ergothioneins oder von Selenonein oder eines Gemischs davon, umfassend das Kultivieren eines Bakteriums, das zur Familie *Enterobacteriaceae* gehört und die Fähigkeit aufweist, Ergothionein zu produzieren, in einem Kulturmedium und das Gewinnen des Ergothioneins oder des oxidierten oder alkylierten Ergothioneins oder des Selenoneins oder des Gemischs davon aus dem Kulturmedium oder aus den durch die Kultur erhaltenen Zellen, wobei das Bakterium so modifiziert ist,

dass es eine reduzierte Aktivität eines Proteins aufweist, das einen Kernsequenzbereich umfasst, der die 5 Aminosäurereste Ser-Arg-Gly-Arg-Thr (SEQ ID Nr. 7) als Teil davon umfasst.

**2.** Verfahren gemäß Anspruch 1, wobei der Kernsequenzbereich die folgende Sequenz aufweist: Phe-X15aa-Ala-Arg-X16aa-Ser-Arg-Gly-Arg-Thr-X17aa-Arg,
wobei X15aa, X16aa und X17aa willkürliche Aminosäurereste sind (SEQ ID Nr. 8).

**3.** Verfahren gemäß Anspruch 2, wobei der Kernsequenzbereich der Sequenz entspricht, die die Aminosäurereste aufweist, die aus den folgenden (1) bis (3) ausgewählt sind:

(1) X15aa ist Leu oder Ile;
(2) X16aa ist Ile oder Val;
(3) X17aa ist Ile oder Val.

**4.** Verfahren gemäß Anspruch 1, wobei das Protein, das einen Kernsequenzbereich umfasst, entweder ein BetT-Protein oder ein CaiT-Protein oder beides ist, wobei das BetT-Protein das Protein gemäß der folgenden Punkte (a), (b) oder (c) ist und wobei das CaiT-Protein das Protein gemäß der folgenden Punkte (d), (e) oder (f) ist:

(a) ein Protein, das die in SEQ ID Nr. 10 gezeigte Aminosäuresequenz umfasst;
(b) ein Protein, das die in SEQ ID Nr. 10 gezeigte Aminosäuresequenz umfasst, bei der ein bis zwei, drei, vier, fünf, sechs, sieben, acht, neun oder zehn Aminosäurereste substituiert, deletiert, inseriert oder addiert ist/ sind;
(c) ein Protein, das eine Aminosäuresequenz umfasst, die 90% oder mehr Identität mit der in SEQ ID Nr. 10 gezeigten Aminosäuresequenz aufweist;
(d) ein Protein, das die in SEQ ID Nr. 11 gezeigte Aminosäuresequenz umfasst;
(e) ein Protein, das die in SEQ ID Nr. 11 gezeigte Aminosäuresequenz umfasst, bei der ein bis zwei, drei, vier, fünf, sechs, sieben, acht, neun oder zehn Aminosäurereste substituiert, deletiert, inseriert oder addiert ist/ sind;
(f) ein Protein, das eine Aminosäuresequenz umfasst, die 90% oder mehr Identität mit der in SEQ ID Nr. 11 gezeigten Aminosäuresequenz aufweist.

**5.** Verfahren gemäß einem der Ansprüche 1 bis 4, wobei das Bakterium, das zur Familie *Enterobacteriaceae* gehört und die Fähigkeit aufweist, Ergothionein zu produzieren, ein Bakterium ist, das so modifiziert ist, dass die Expression von Ergothionein-Biosynthese-Genen verstärkt ist.

**6.** Verfahren gemäß Anspruch 5, wobei die Ergothionein-Biosynthese-Gene eines oder mehrere aus einem Gen, das egtA, egtB, egtC, egtD oder egtE von *Mycobacterium smegmatis* entspricht, einem Gen, das Egt1 oder Egt2 von *Schizosaccharomyces pombe* entspricht, einem Gen, das Egt1 oder Egt2 von *Neurospora crassa* entspricht, einem Gen, das eanA oder eanB von *Chlorobium limicola* entspricht, umfassen.

**7.** Verfahren gemäß einem der Ansprüche 1 bis 6, wobei das Bakterium, das zur Familie *Enterobacteriaceae* gehört und die Fähigkeit aufweist, Ergothionein zu produzieren, ein Bakterium ist, das zur Gattung *Escherichia* gehört.

**8.** Verfahren gemäß Anspruch 7, wobei es sich bei dem Bakterium, das zur Familie *Enterobacteriaceae* gehört und die Fähigkeit aufweist, Ergothionein zu produzieren, um *Escherichia coli* handelt.

**9.** Bakterium, das zur Familie *Enterobacteriaceae* gehört und die Fähigkeit aufweist, Ergothionein zu produzieren, und so modifiziert ist, dass es eine reduzierte Aktivität eines Proteins aufweist, das einen Kernsequenzbereich umfasst, der die 5 Aminosäurereste Ser-Arg-Gly-Arg-Thr (SEQ ID Nr. 7) als Teil davon umfasst.

**10.** Bakterium gemäß Anspruch 9, wobei der Kernsequenzbereich die folgende Sequenz aufweist: Phe-X15aa-Ala-Arg-X16aa-Ser-Arg-Gly-Arg-Thr-X17aa-Arg,
wobei X15aa, X16aa und X17aa willkürliche Aminosäurereste sind (SEQ ID Nr. 8).

**11.** Bakterium gemäß Anspruch 10, wobei der Kernsequenzbereich der Sequenz entspricht, die den Aminosäurerest aufweist, der aus den folgenden (1) bis (3) ausgewählt ist:

(1) X15aa ist Leu oder Ile;
(2) X16aa ist Ile oder Val;
(3) X17aa ist Ile oder Val.

**12.** Bakterium gemäß Anspruch 9, wobei das Protein, das einen Kernsequenzbereich umfasst, entweder ein BetT-Protein oder ein CaiT-Protein oder beides ist, wobei das BetT-Protein das Protein gemäß der folgenden Punkte (a), (b) oder (c) ist und wobei das CaiT-Protein das Protein gemäß der folgenden Punkte (d), (e) oder (f) ist:

(a) ein Protein, das die in SEQ ID Nr. 10 gezeigte Aminosäuresequenz umfasst;
(b) ein Protein, das die in SEQ ID Nr. 10 gezeigte Aminosäuresequenz umfasst, bei der ein bis zwei, drei, vier, fünf, sechs, sieben, acht, neun oder zehn Aminosäurereste substituiert, deletiert, inseriert oder addiert ist/ sind;
(c) ein Protein, das eine Aminosäuresequenz umfasst, die 90% oder mehr Identität mit der in SEQ ID Nr. 10 gezeigten Aminosäuresequenz aufweist;
(d) ein Protein, das die in SEQ ID Nr. 11 gezeigte Aminosäuresequenz umfasst;
(e) ein Protein, das die in SEQ ID Nr. 11 gezeigte Aminosäuresequenz umfasst, bei der ein bis zwei, drei, vier, fünf, sechs, sieben, acht, neun oder zehn Aminosäurereste substituiert, deletiert, inseriert oder addiert ist/ sind;
(f) ein Protein, das eine Aminosäuresequenz umfasst, die 90% oder mehr Identität mit der in SEQ ID Nr. 11 gezeigten Aminosäuresequenz aufweist.

**13.** Bakterium gemäß einem der Ansprüche 9 bis 12, wobei das Bakterium ein Bakterium ist, das so modifiziert ist, dass die Expression von Ergothionein-Biosynthese-Genen verstärkt ist.

**14.** Bakterium gemäß Anspruch 13, wobei die Ergothionein-Biosynthese-Gene eines oder mehrere aus einem Gen, das egtA, egtB, egtC, egtD oder egtE von *Mycobacterium smegmatis* entspricht, einem Gen, das Egt1 oder Egt2 von *Schizosaccharomyces pombe* entspricht, einem Gen, das Egt1 oder Egt2 von *Neurospora crassa* entspricht, einem Gen, das eanA oder eanB von *Chlorobium limicola* entspricht, umfassen.

**15.** Bakterium gemäß einem der Ansprüche 9 bis 14, wobei das Bakterium ein Bakterium ist, das zur Gattung *Escherichia* gehört, wobei es sich vorzugsweise bei dem Bakterium um *Escherichia coli* handelt.

**Revendications**

**1.** Procédé de production d'ergothionéine, ou d'une ergothionéine oxydée ou alkylée, ou d'une sélénonéine, ou d'un mélange de celles-ci, comprenant les étapes consistant à mettre en culture une bactérie appartenant à la famille des *Enterobacteriaceae* et ayant la capacité de produire de l'ergothionéine dans un milieu de culture, puis à recueillir l'ergothionéine, ou l'ergothionéine oxydée ou alkylée, ou la sélénonéine, ou le mélange de celles-ci, à partir du milieu de culture ou des cellules obtenues par culture, où ladite bactérie est une bactérie qui est modifiée de façon à présenter une activité réduite d'une protéine comprenant une région de séquence centrale comprenant comme partie 5 résidus d'acides aminés : Ser-Arg-Gly-Arg-Thr (SEQ ID N° : 7).

**2.** Procédé de la revendication 1, dans lequel la région de séquence centrale présente la séquence : Phe-X15aa-Ala-Arg-X16aa-Ser-Arg-Gly-Arg-Thr-X17aa-Arg
dans laquelle X15aa, X16aa et X17aa sont des résidus d'acides aminés arbitraires (SEQ ID N° : 8).

**3.** Procédé de la revendication 1, dans lequel la région de séquence centrale est la séquence ayant les résidus d'acides aminés sélectionnés parmi les résidus (1) à (3) suivants :

(1) X15aa est Leu ou Ile ;
(2) X16aa est Ile ou Val ;
(3) X17aa est Ile ou Val.

**4.** Procédé de la revendication 1, dans lequel la protéine comprenant une région de séquence centrale est soit une protéine BetT soit une protéine CaiT, ou les deux, où la protéine BetT est la protéine de (a), (b) ou (c) ci-après, et où la protéine CaiT est la protéine de (d), (e) ou (f) ci-après :

(a) une protéine comprenant la séquence d'acides aminés montrée dans le SEQ ID N° : 10 ;
(b) une protéine comprenant la séquence d'acides aminés montrée dans le SEQ ID N° : 10 dans laquelle un à deux, trois, quatre, cinq, six, sept, huit, neuf ou dix résidus d'acides aminés est/sont substitué(s), supprimé(s), inséré(s) ou ajouté(s) ;
(c) une protéine comprenant une séquence d'acides aminés ayant 90 % ou plus d'identité avec la séquence d'acides aminés présentée dans le SEQ ID N° : 10 ;

(d) une protéine comprenant la séquence d'acides aminés montrée dans le SEQ ID N° : 11 ;
(e) une protéine comprenant la séquence d'acides aminés montrée dans le SEQ ID N° : 11 dans laquelle un à deux, trois, quatre, cinq, six, sept, huit, neuf ou dix résidus d'acides aminés est/sont substitué(s), supprimé(s), inséré(s) ou ajouté(s) ;
(f) une protéine comprenant une séquence d'acides aminés ayant 90 % ou plus d'identité avec la séquence d'acides aminés présentée dans le SEQ ID N° : 11.

**5.** Procédé de l'une quelconque des revendications 1 à 4, dans lequel la bactérie appartenant à la famille des *Enterobacteriaceae* et ayant la capacité de produire de l'ergothionéine est une bactérie modifiée de façon à améliorer l'expression de gènes de biosynthèse d'ergothionéine.

**6.** Procédé de la revendication 5, dans lequel les gènes de biosynthèse d'ergothionéine comprennent l'un quelconque ou plusieurs d'un gène correspondant à egtA, egtB, egtC, egtD ou egtE de *Mycobacterium smegmatis,* d'un gène correspondant à Egt1 ou Egt2 de *Shizosaccaromyces pombe,* d'un gène correspondant à Egt1 ou Egt2 de *Nerospora crassa*, d'un gène correspondant à eanA ou eanB de *Chlorobium limicola.*

**7.** Procédé de l'une quelconque des revendications 1 à 6, dans lequel la bactérie appartenant à la famille des *Enterobacteriaceae* et ayant la capacité de produire de l'ergothionéine est une bactérie appartenant au genre *Escherichia.*

**8.** Procédé de la revendication 7, dans lequel la bactérie appartenant à la famille des *Enterobacteriaceae* et ayant la capacité de produire de l'ergothionéine est *Escherichia coli.*

**9.** Bactérie appartenant à la famille des *Enterobacteriaceae* ayant la capacité de produire de l'ergothionéine modifiée de façon à présenter une activité réduite d'une protéine comprenant une région de séquence centrale comprenant comme partie 5 résidus d'acides aminés : Ser-Arg-Gly-Arg-Thr (SEQ ID N° : 7).

**10.** Bactérie de la revendication 9, dans laquelle la région de séquence centrale a la séquence : Phe-X15aa-Ala-Arg-X16aa-Ser-Arg-Gly-Arg-Thr-X17aa-Arg
où X15aa, X16aa et X17aa sont des résidus d'acides aminés arbitraires (SEQ ID N° : 8).

**11.** Bactérie de la revendication 10, dans laquelle la région de séquence centrale est la séquence ayant les résidus d'acides aminés sélectionnés parmi les résidus (1) à (3) suivants :

(1) X15aa est Leu ou Ile ;
(2) X16aa est Ile ou Val ;
(3) X17aa est Ile ou Val.

**12.** Bactérie de la revendication 9, dans laquelle la protéine comprenant une région de séquence centrale est soit une protéine BetT soit une protéine CaiT, ou les deux, où la protéine BetT est la protéine de (a), (b) ou (c) ci-après, et où la protéine CaiT est la protéine de (d), (e) ou (f) ci-après :

(a) une protéine comprenant la séquence d'acides aminés montrée dans le SEQ ID N° : 10 ;
(b) une protéine comprenant la séquence d'acides aminés montrée dans le SEQ ID N° : 10 dans laquelle un à deux, trois, quatre, cinq, six, sept, huit, neuf ou dix résidus d'acides aminés est/sont substitué(s), supprimé(s), inséré(s) ou ajouté(s) ;
(c) une protéine comprenant une séquence d'acides aminés ayant 90 % ou plus d'identité avec la séquence d'acides aminés présentée dans le SEQ ID N° : 10 ;
(d) une protéine comprenant la séquence d'acides aminés montrée dans le SEQ ID N° : 11 ;
(e) une protéine comprenant la séquence d'acides aminés montrée dans le SEQ ID N° : 11 dans laquelle un à deux, trois, quatre, cinq, six, sept, huit, neuf ou dix résidus d'acides aminés est/sont substitué(s), supprimé(s), inséré(s) ou ajouté(s) ;
(f) une protéine comprenant une séquence d'acides aminés ayant 90 % ou plus d'identité avec la séquence d'acides aminés présentée dans le SEQ ID N° : 11.

**13.** Bactérie de l'une quelconque des revendications 9 à 12, dans laquelle la bactérie est une bactérie modifiée de façon à améliorer l'expression des gènes de biosynthèse d'ergothionéine.

**14.** Bactérie de la revendication 13, dans laquelle les gènes de biosynthèse d'ergothionéine comprennent l'un quelcon-

que ou plusieurs d'un gène correspondant à egtA, egtB, egtC, egtD ou egtE de *Mycobacterium smegmatis,* d'un gène correspondant à Egt1 ou Egt2 de *Shizosaccaromyces pombe,* d'un gène correspondant à Egt1 ou Egt2 de *Nerospora crassa*, d'un gène correspondant à eanA ou eanB de *Chlorobium limicola*.

**15.** Bactérie de l'une quelconque des revendications 9 à 14, dans laquelle la bactérie est une bactérie appartenant à la famille du genre *Escherichia,* et la bactérie est de préférence *Escherichia coli.*

Fig. 1

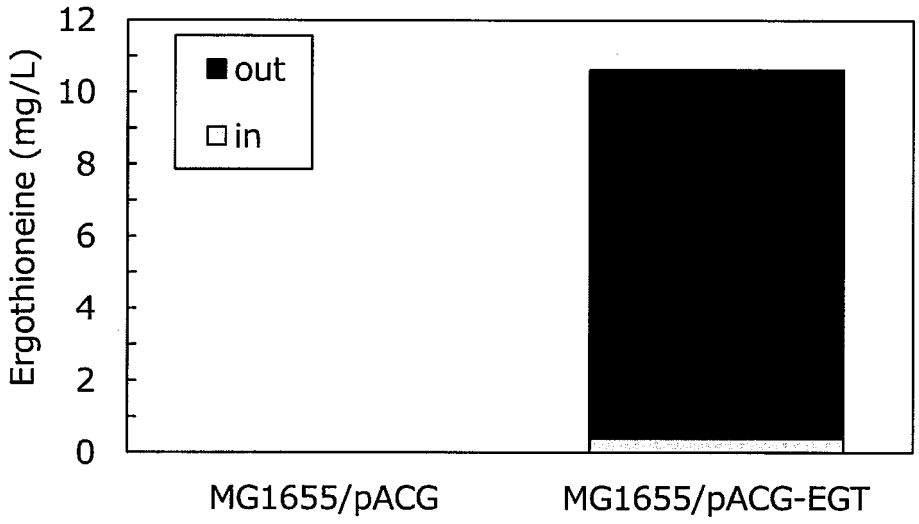

Ergothioneine (mg/L)
12
10
8
6
4
2
0
out
in
MG1655/pACG
MG1655/pACG-EGT

Fig. 2

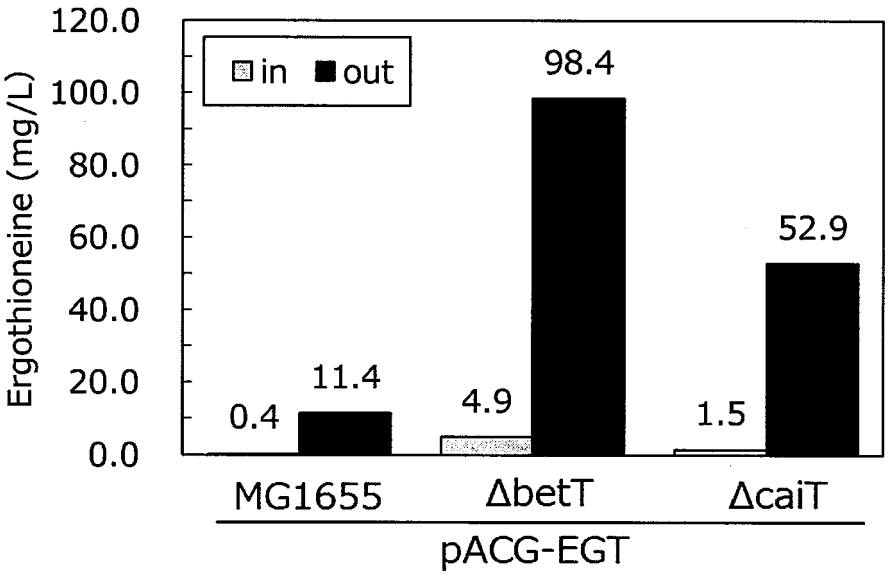

Ergothioneine (mg/L)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
in
out
0.4
11.4
4.9
98.4
1.5
52.9
MG1655
ΔbetT
ΔcaiT
pACG-EGT

Fig. 3

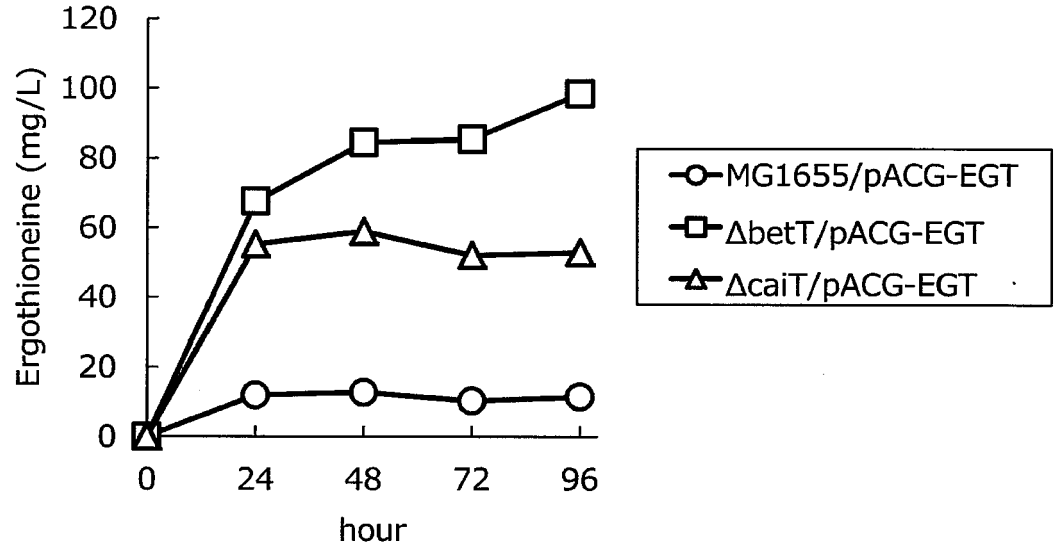
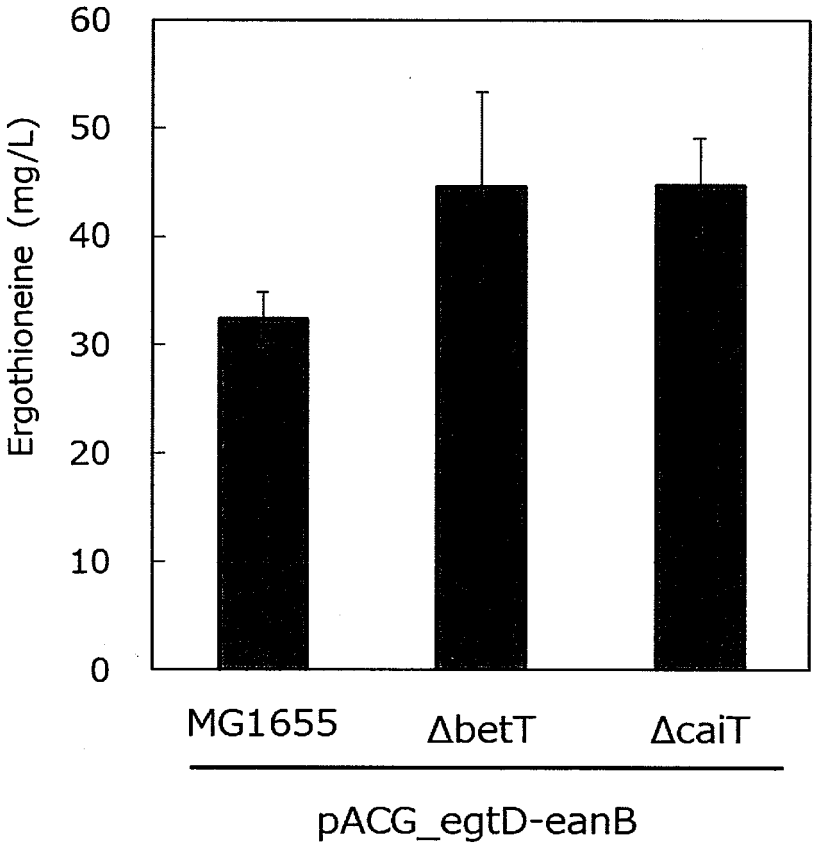

120
100
80
60
40
20
0
Ergothioneine (mg/L)
0
24
48
72
96
hour
MG1655/pACG-EGT
ΔbetT/pACG-EGT
ΔcaiT/pACG-EGT

Fig. 4


60
50
40
30
20
10
0
Ergothioneine (mg/L)
MG1655
ΔbetT
ΔcaiT
pACG_egtD-eanB

## REFERENCES CITED IN THE DESCRIPTION

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