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WO-A1-2005/103278
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MICHAEL BOTT ET AL: "Two-component signal transduction inand other corynebacteria: on the way towards stimuli and targets", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, BERLIN, DE, vol. 94, no. 5, 28 April 2012 (2012-04-28) , pages 1131-1150, XP035056736, ISSN: 1432-0614, DOI: 10.1007/S00253-012-4060-X

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DESCRIPTION

Technical Field

[0001] The present invention relates to a method for secretory production of a heterologous protein as defined by the claims.

Background Art

[0002] As secretory production of heterologous proteins by microorganisms, there have been reported secretory productions of heterologous proteins by a *Bacillus* bacterium (Non-patent document 1), methanol-assimilating yeast, *Pichia pastoris* (Non-patent document 2), filamentous fungi of the genus *Aspergillus* (Non-patent documents 3 and 4), and so forth.

[0003] There are also attempted secretory productions of heterologous proteins by coryneform bacteria. As for secretory productions of heterologous proteins by coryneform bacteria, there have been reported secretion of a nuclease and a lipase by *Corynebacterium glutamicum* (henceforth also abbreviated as C. *glutamicum)* (Patent document 1, Non-patent document 5), secretion of a protease such as subtilisin (Non-patent document 6), secretion of a protein using signal peptides of cell surface layer proteins PS1 and PS2 (also referred to as CspB) of coryneform bacteria (Patent document 2), secretion of a fibronectin-binding protein using the signal peptide of PS2 (CspB) (Non-patent document 7), secretion of protransglutaminase using signal peptides of PS2 (CspB) and SlpA (also referred to as CspA) (Patent document 3), secretion of a protein using a variant type secretion system (Patent document 4), secretion of a protransglutaminase by a variant strain (Patent document 5), and so forth. In addition, as techniques for improving secretory production amounts of heterologous proteins by coryneform bacteria, there are known reducing the activity of a cell surface layer protein (Patent documents 6 and 7), reducing the activity of a penicillin-binding protein (Patent document 6), enhancing the expression of a gene encoding a metallopeptidase (Patent document 7), introducing a mutation into a ribosomal protein S1 gene (Patent document 8), expressing a heterologous protein with an amino acid sequence comprising Gln-Glu-Thr inserted between a signal peptide and the heterologous protein (Patent document 9), and so forth.

[0004] A general protein secretion pathway is a pathway called "Sec system", which is widely present from prokaryotes to eukaryotes, however, a protein secretion pathway completely different from the Sec system has recently been found in thylakoid membranes of chloroplasts of plant cells (Non-patent document 8). This novel secretory pathway has been named "Tat system" (Twin-Arginine Translocation system) because an arginine-arginine sequence is commonly present in the signal sequence of a protein secreted thereby (Non-patent document 8). It is known that proteins are secreted by the Sec system in a state before forming a higher-

order structure, while proteins are secreted by the Tat system through a cell membrane after forming a higher-order structure in the cell (Non-patent document 9). Also for coryneform bacteria, secretory production of proteins utilizing a Tat-dependent signal peptide has been reported (Patent documents 8 and 10) .

[0005] As a system by which bacteria respond to various environmental changes inside and outside the cell, a signaling pathway called "two-component regulatory system" is known. The two-component regulatory system is a regulatory system consisting of two components: a sensor kinase that is responsible for sensing a stimulus of an environmental change, and a response regulator that is responsible for receiving a signal from the sensor kinase and regulating the expression of downstream genes. When the sensor kinase senses a stimulus, a specific histidine residue thereof is autophosphorylated, a signal is transduced via transfer of the phosphate group to a specific aspartic acid residue of the response regulator, and thereby the phosphorylated response regulator is activated to regulate the expression of downstream genes as a transcription factor.

[0006] Knowledge concerning the two-component regulatory system of C. *glutamicum* is detailed in Non-patent document 10 etc. For C. *glutamicum,* at least 13 types of systems have been known as the two-component regulatory system. One of them is the PhoRS system, which consists of a sensor kinase PhoS protein and a response regulator PhoR protein. Analysis of a PhoRS-deficient strain revealed that the PhoRS system is a regulatory system that senses phosphate depletion in the environment and performs signal transduction (Non-patent document 11).

[0007] The PhoS protein is a membrane protein having two transmembrane domains. The PhoS protein consists of a sensor domain that senses a stimulus, a linker domain called HAMP domain, a HisKA domain having a histidine residue that is autophosphorylated, and a HATPase domain having an ATP binding ability and a function of catalyzing autophosphorylation of the histidine residue. The PhoR protein is an intracellular protein. The PhoR protein consists of a receiver domain on the N-terminal side that receives a signal and an effector domain on the C-terminal side that regulates the expression of downstream genes (Non-patent document 10).

[0008] WO 2006/069610 A2 discloses a process for the production of fine chemicals. EP 1748077 A1 discloses a method of producing protein. WO 2004/035792 A1 discloses E. coli host cells with modified PhoS/PstS periplasmic phosphate-binding proteins and a method of manufacturing recombinant Fabs. Bott et al. (Applied Microbiology and Biotechnology, vo. 94, no. 5, 2012, pages 1131-1150) discloses two-component signal transduction in Corynebacterium glutamicum and other corynebacteria.

[0009] However, there is not known the relationship between the PhoRS system and secretory production of heterologous proteins. Also, it is not known that mutation of the PhoS protein is effective for secretory production of heterologous proteins in coryneform bacteria. Furthermore, it is not known that specific mutations of the PhoS protein are effective for secretory production of heterologous proteins.

Prior art references

Patent documents

[0010]

Patent document 1: U.S. Patent No. 4,965,197

Patent document 2: Japanese Patent Laid-open (Kohyo) No. 6-502548

Patent document 3: Japanese Patent No. 4320769

Patent document 4: Japanese Patent Laid-open (Kokai) No. 11-169182

Patent document 5: Japanese Patent No. 4362651

Patent document 6: WO2013/065869

Patent document 7: WO2013/065772

Patent document 8: WO2013/118544

Patent document 9: WO2013/062029

Patent document 10: Japanese Patent No. 4730302

Non-patent documents

[0011]

Non-patent document 1: Microbiol. Rev., 57, 109-137 (1993)

Non-patent document 2: Biotechnol., 11, 905-910 (1993)

Non-patent document 3: Biotechnol., 6, 1419-1422 (1988)

Non-patent document 4: Biotechnol., 9, 976-981 (1991)

Non-patent document 5: J. Bacteriol., 174, 1854-1861 (1992)

Non-patent document 6: Appl. Environ. Microbiol., 61, 1610-1613 (1995)

Non-patent document 7: Appl. Environ. Microbiol., 63, 4392-4400 (1997)

Non-patent document 8: EMBO J., 14, 2715-2722(1995)

Non-patent document 9: J. Biol. Chem., 25;273(52), 34868-74(1998)

Non-patent document 10: Appl. Microbiol. Biotechnol., 94, 1131-1150(2012)

Non-patent document 11: J. Bacteriol., 188, 724-732(2006)

Summary of the Invention

Object to be Achieved by the Invention

[0012] An object of the present invention is to develop a novel technique for improving secretory production of a heterologous protein by a coryneform bacterium, and thereby to provide a method for secretory production of a heterologous protein using a coryneform bacterium as defined by the claims.

Means for Achieving the Object

[0013] The inventors of the present invention conducted various researches in order to achieve the aforementioned object. As a result, they found that an ability of a coryneform bacterium to produce a heterologous protein by secretory production can be improved by modifying the coryneform bacterium so as to harbor a *phoS* gene having a specific mutation, and accomplished the present invention.

[0014] The present invention can be thus embodied as follows.

[0015] A method for producing a heterologous protein comprising:

culturing a coryneform bacterium having a genetic construct for secretory expression of the heterologous protein; and

collecting the heterologous protein produced by secretory production,

wherein the coryneform bacterium has been modified so as to harbor a phoS gene encoding a mutant PhoS protein,

wherein the mutant PhoS protein is a PhoS protein having a mutation of replacing an amino acid residue corresponding to the tryptophan residue at position 302 in SEQ ID NO: 4 with a lysine residue, alanine residue, valine residue, serine residue, cysteine residue, methionine residue, aspartic acid residue, or asparagine residue in a wild-type PhoS protein,

wherein the genetic construct comprises, in the direction from 5' to 3', a promoter sequence that functions in the coryneform bacterium, a nucleic acid sequence encoding a signal peptide that functions in the coryneform bacterium, and a nucleic acid sequence encoding the heterologous protein, and

wherein the heterologous protein is expressed as a fusion protein with the signal peptide.

[0016] Further embodiments of the invention are defined in the claims.

Brief Description of the Drawings

[0017]

Fig. 1 is a photograph showing the results of SDS-PAGE observed upon expressing CspB50TEV-Teri (Teriparatide fused with CspB signal sequence and mature CspB N-terminal sequence) in the C. *glutamicum* YDK010 strain, and PhoS(W302C)-mutant and PhoS-deletion strains thereof.

Fig. 2 is a diagram showing an alignment of the amino acid sequences of HisKA domains of PhoS homologues of *Corynebacterium* bacteria.

Fig. 3 is a photograph showing the results of SDS-PAGE observed upon expressing CspB6Xa-LFABP (LFABP fused with CspB signal sequence and mature CspB N-terminal sequence) in the C. *glutamicum* YDK010 strain and PhoS(W302C)-mutant strain thereof.

Fig. 4 is a photograph showing the results of SDS-PAGE observed upon expressing CspB6TEV-ExCP (Exenatide fused with CspB signal sequence and mature CspB N-terminal sequence) in the C. *glutamicum* YDK010 strain and PhoS(W302C)-mutant strain thereof.

Fig. 5 is a diagram showing a construction scheme of pPK6 vector.

Fig. 6 is a photograph showing results of SDS-PAGE observed upon expressing protransglutaminase fused with E. *coli* TorA signal sequence in the C. *glutamicum* YDK010 strain and PhoS(W302C)-mutant strain thereof.

Fig. 7 is a photograph showing results of SDS-PAGE observed upon expressing protein glutaminase comprising a pro-structure moiety and fused with E. *coli* TorA signal sequence in the C. *glutamicum* YDK010 strain and PhoS(W302C)-mutant strain thereof.

Fig. 8 is a photograph showing results of SDS-PAGE observed upon expressing *Arthrobacter globiformis* isomaltodextranase including a signal sequence in the C. *glutamicum* YDK010 strain and PhoS(W302C)-mutant strain thereof.

Fig. 9 is a photograph showing results of SDS-PAGE observed upon expressing CspB50TEV-

Teri in PhoS-deletion strain of the C. *glutamicum* YDK010 strain, and PhoS-complemented strains thereof.

Fig. 10 is a photograph showing results of SDS-PAGE observed upon expressing CspB50TEV-Teri in PhoS-deletion strains of C. *glutamicum* YDK010 introduced with various mutant *phoS(W302X)* genes.

Fig. 11 is a photograph showing results of SDS-PAGE observed upon expressing CspB6TEV-ExCP in the C. *glutamicum* ATCC13869 strain and PhoS(W302C)-mutant strain thereof.

Fig. 12 is a photograph showing results of SDS-PAGE observed upon expressing CspB6Xa-LFABP in the C. *glutamicum* ATCC13869ΔcspB strain and PhoS(W302C)-mutant strain thereof.

Modes for Carrying out the Invention

[0018] Hereinafter, the present invention will be explained in detail.

<1> Method for producing heterologous protein of the present invention

[0019] The present invention as defined by the claims provides a method for producing a heterologous protein, the method comprising culturing a coryneform bacterium having a genetic construct for secretory expression of the heterologous protein, and collecting the heterologous protein produced by secretory production, wherein the coryneform bacterium has been modified so as to harbor a *phoS* gene encoding a mutant PhoS protein as further defined in the claims (henceforth also referred to as "method of the present invention" or "method for producing a heterologous protein of the present invention").

<1-1> Coryneform bacterium used for the method of the present invention as defined by the claims

[0020] The coryneform bacterium used for the method of the present invention as further defined in the claims a coryneform bacterium having a genetic construct for secretory expression of a heterologous protein, which has been modified so as to harbor a mutant *phoS* gene. The coryneform bacterium used for the method of the present invention as further defined in the claims is also referred to as "bacterium of the present invention" or "coryneform bacterium of the present invention". Furthermore, the genetic construct for secretory expression of a heterologous protein harbored by the bacterium of the present invention as further defined in the claims is also referred to as "genetic construct used for the present

invention".

<1-1-1> Coryneform bacterium having ability of secretory production of heterologous protein as further defined in the claims

[0021] The coryneform bacterium of the present invention as further defined in the claims has the genetic construct for secretory expression of a heterologous protein (genetic construct used for the method of the present invention as further defined in the claims), and therefore has an ability of secretory production of the heterologous protein.

[0022] In the present invention, the expression that a protein is "secreted" means that the protein is transported out of a bacterial cell (extracellularly transported). Examples of a position outside of a bacterial cell (outside of a cell) include a medium and a cell surface layer. That is, the expression that a protein is "secreted" is not limited to cases where all the molecules of the protein eventually exist in the medium in completely free forms, and also include cases where all the molecules of the protein exist in the cell surface layer, and cases where a part of the molecules of the protein exists in the medium and the remaining part of the molecules of the protein exists in the cell surface layer.

[0023] That is, in the present disclosure, the term "ability to produce a heterologous protein by secretory production" refers to an ability of the bacterium of the present invention to secrete the heterologous protein into a medium or a cell surface layer, and accumulate it there to such an extent that the heterologous protein can be collected from the medium or the cell surface layer, when the bacterium is cultured in the medium. The accumulation amount may be, for example, in terms of the accumulation amount in the medium, preferably 10 μg/L or more, more preferably 1 mg/L or more, particularly preferably 100 mg/L or more, still more preferably 1 g/L or more. Also, the accumulation amount may be, for example, in terms of the accumulation amount in the cell surface layer, such an amount that if the heterologous protein in the cell surface layer is collected and suspended in a liquid of the same volume as the medium, the concentration of the heterologous protein in the suspension is preferably 10 μg/L or more, more preferably 1 mg/L or more, particularly preferably 100 mg/L or more. In addition, in the present invention, the term "protein" to be produced by secretory production refers to a concept also including those called peptide, such as oligopeptides and polypeptides.

[0024] In the present invention, the term "heterologous protein" refers to an exogenous protein relative to a coryneform bacterium that expresses and secretes that protein. The heterologous protein may be, for example, a protein derived from a microorganism, a protein derived from a plant, a protein derived from an animal, a protein derived from a virus, or even a protein of which the amino acid sequence is artificially designed. The heterologous protein may be a monomeric protein or a multimeric protein. The term "multimeric protein" refers to a protein that may exist as a multimer consisting of two or more subunits. In the multimer, the subunits may be linked by covalent bonds such as disulfide bonds, linked by non-covalent bonds such as hydrogen bonds and hydrophobic interaction, or linked by a combination thereof. The

multimer preferably comprises one or more intermolecular disulfide bonds. The multimer may be a homo-multimer consisting of a single kind of subunit, or may be a hetero-multimer consisting of two or more kinds of subunits. In the case where the multimeric protein is a hetero-multimer, it is sufficient that at least one subunit selected from the subunits constituting the hetero-multimer is a heterologous protein. That is, all the subunits may be heterologous, or only a part of subunits may be heterologous. Although the heterologous protein may be a secretory protein in nature, or may be a non-secretory protein in nature, it is preferably a secretory protein in nature. Furthermore, the heterologous protein may be a Tat-dependent secretory protein in nature, or may be a Sec-dependent secretory protein in nature. Specific examples of the "heterologous protein" will be mentioned later.

[0025] The heterologous protein to be produced may consist of a single kind of protein, or two or more kinds of proteins. Moreover, when the heterologous protein is a hetero-multimer, only one kind of subunit may be produced, or two or more kinds of subunits may be produced. That is, the term "secretory production of a heterologous protein" includes secretory production of all the subunits constituting an objective heterologous protein, as well as secretory production of only a part of the subunits constituting an objective heterologous protein.

[0026] Coryneform bacteria are aerobic gram-positive bacilli. Examples of the coryneform bacteria include *Corynebacterium* bacteria, *Brevibacterium* bacteria, *Microbacterium* bacteria, and so forth. Advantages of use of the coryneform bacteria include that they inherently secrete an extremely small amount of proteins out of cells compared with fungi, yeasts, *Bacillus* bacteria, etc., which are conventionally used for secretory production of proteins, and therefore the purification process of a heterologous protein produced by secretory production is expected to be simplified or eliminated, that they can grow well in a simple medium containing a saccharide, ammonia, mineral salts, etc., and therefore they are excellent in view of cost of medium, culture method, and culture productivity, and so forth.

[0027] Specific examples of coryneform bacteria include the following species:

Corynebacterium acetoacidophilum

Corynebacterium acetoglutamicum

Corynebacterium alkanolyticum

Corynebacterium callunae

Corynebacterium crenatum

Corynebacterium glutamicum

Corynebacterium lilium

Corynebacterium melassecola

Corynebacterium thermoaminogenes (Corynebacterium efficiens)

Corynebacterium herculis

Brevibacterium divaricatum (Corynebacterium glutamicum)

Brevibacterium flavum (Corynebacterium glutamicum)

Brevibacterium immariophilum

Brevibacterium lactofermentum (Corynebacterium glutamicum)

Brevibacterium roseum

Brevibacterium saccharolyticum

Brevibacterium thiogenitalis

Corynebacterium ammoniagenes (Corynebacterium stationis)

Brevibacterium album

Brevibacterium cerinum

Microbacterium ammoniaphilum

[0028] Specific examples of coryneform bacteria include the following strains:

Corynebacterium acetoacidophilum ATCC 13870

Corynebacterium acetoglutamicum ATCC 15806

Corynebacterium alkanolyticum ATCC 21511

Corynebacterium callunae ATCC 15991

Corynebacterium crenatum AS1.542

Corynebacterium glutamicum ATCC 13020, ATCC 13032, ATCC 13060, ATCC 13869, FERM BP-734

Corynebacterium lilium ATCC 15990

Corynebacterium melassecola ATCC 17965

Corynebacterium thermoaminogenes (Corynebacterium efficiens) AJ12340 (FERM BP-1539)

Corynebacterium herculis ATCC 13868

Brevibacterium divaricatum (Corynebacterium glutamicum) ATCC 14020

Brevibacterium flavum (Corynebacterium glutamicum) ATCC 13826, ATCC 14067, AJ12418

(FERM BP-2205)

Brevibacterium immariophilum ATCC 14068

Brevibacterium lactofermentum (Corynebacterium glutamicum) ATCC 13869

Brevibacterium roseum ATCC 13825

Brevibacterium saccharolyticum ATCC 14066

Brevibacterium thiogenitalis ATCC 19240

Corynebacterium ammoniagenes (Corynebacterium stationis) ATCC 6871, ATCC 6872

Brevibacterium album ATCC 15111

Brevibacterium cerinum ATCC 15112

Microbacterium ammoniaphilum ATCC 15354

[0029] The *Corynebacterium* bacteria include bacteria that had previously been classified into the genus *Brevibacterium,* but are presently united into the genus *Corynebacterium* (Int. J. Syst. Bacteriol., 41, 255 (1991)). Moreover, *Corynebacterium stationis* includes bacteria that had previously been classified as *Corynebacterium ammoniagenes,* but are presently re-classified into *Corynebacterium stationis* on the basis of nucleotide sequence analysis of 16S rRNA etc. (Int. J. Syst. Evol. Microbiol., 60, 874-879 (2010)).

[0030] These strains are available from, for example, the American Type Culture Collection (Address: 12301 Parklawn Drive, Rockville, Maryland 20852, P.O. Box 1549, Manassas, VA 20108, United States of America). That is, registration numbers are assigned to the respective strains, and the strains can be ordered by using these registration numbers (refer to http://www.atcc.org/). The registration numbers of the strains are listed in the catalogue of the American Type Culture Collection. These strains can also be obtained from, for example, the depositories at which the strains were deposited.

[0031] In particular, the *Corynebacterium glutamicum (C. glutamicum)* AJ12036 strain (FERM BP-734), which was isolated as a streptomycin (Sm) resistant mutant strain from a wild-type strain C. *glutamicum* ATCC 13869 is predicted to have a mutation in a gene responsible for a function involved in secretion of proteins, and shows an extremely high secretory production ability for proteins as high as about 2 to 3 times in terms of accumulation amount of proteins under optimum culture conditions, compared with the parent strain (wild-type strain), and therefore it is preferred as a host bacterium. The AJ12036 strain was originally deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology (currently, independent administrative agency, National Institute of Technology and Evaluation, International Patent Organism Depositary, #120, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-

ken, 292-0818, Japan) on March 26, 1984 as an international deposit, and assigned an accession number of FERM BP-734.

[0032] *Corynebacterium thermoaminogenes* AJ12340 (FERM BP-1539) was originally deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology (currently, independent administrative agency, National Institute of Technology and Evaluation, International Patent Organism Depositary, #120, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken, 292-0818, Japan) on March 13, 1987 as an international deposit, and assigned an accession number of FERM BP-1539. *Brevibacterium flavum* AJ12418 (FERM BP-2205) was originally deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology (currently, independent administrative agency, National Institute of Technology and Evaluation, International Patent Organism Depositary, #120, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken, 292-0818, Japan) on December 24, 1988 as an international deposit, and assigned an accession number of FERM BP-2205.

[0033] Moreover, a strain having an enhanced ability to produce a protein by secretory production may be selected from such a coryneform bacterium as mentioned above as a parent strain by using a mutagenesis method or a genetic recombination method, and used as a host. For example, after a parent strain is treated with ultraviolet irradiation or a chemical mutation agent such as N-methyl-N'-nitrosoguanidine, a strain having an enhanced ability to produce a protein by secretory production can be selected.

[0034] Furthermore, if a strain obtained by modifying such a strain as mentioned above so that it does not produce a cell surface layer protein is used as a host, purification of the heterologous protein secreted in the medium or on the cell surface layer becomes easy, Such modification can be carried out by introducing a mutation into the coding region of the cell surface layer protein or an expression control region thereof, on the chromosome by mutagenesis or genetic recombination. Examples of coryneform bacterium modified so that it does not produce a cell surface layer protein include the C. *glutamicum* YDK010 strain (WO2004/029254), which is a cell surface layer protein PS2 deficient strain of the C. *glutamicum* AJ12036 strain (FERM BP-734).

[0035] A coryneform bacterium having an ability of secretory production of a heterologous protein can be obtained by introducing the genetic construct used for the method of the present invention as further defined in the claims into such a coryneform bacterium as described above so as to make the bacterium harbor the genetic construct. The genetic construct used for the method of the present invention as further defined in the claims and methods for introduction of the same will be described later.

<1-1-2> Introduction of mutant *phoS* gene

[0036] The bacterium of the present invention as defined by the claims has been modified so as to harbor a mutant *phoS* gene as defined by the claims. The expression "to harbor a mutant

phoS gene" is also referred to as "to have a mutant *phoS* gene" or "to have a mutation in a *phoS* gene". In addition, the expression "to harbor a mutant *phoS* gene" is also referred to as "to have a mutant PhoS protein" or "to have a mutation in a PhoS protein". The bacterium of the present invention can be obtained by modifying a coryneform bacterium having an ability of secretory production of a heterologous protein so that it harbors a mutant *phoS* gene. The bacterium of the present invention as defined by the claims can also be obtained by modifying a coryneform bacterium so as to harbor a mutant *phoS* gene, and then imparting an ability of secretory production of a heterologous protein thereto. In the present invention, modifications for constructing the bacterium of the present invention as defined by the claims can be performed in an arbitrary order. A strain to be used for constructing the bacterium of the present invention as defined by the claims and before being modified so as to harbor a mutant *phoS* gene may or may not be able to produce a heterologous protein, on the assumption that the strain has the genetic construct for secretory expression of the heterologous protein. That is, the bacterium of the present invention as defined by the claims may also be, for example, a bacterium that has acquired an ability of secretory production of a heterologous protein due to being modified so as to harbor a mutant *phoS* gene. Specifically, for example, the bacterium of the present invention as defined by the claims may also be a bacterium obtained from a strain that is not able to produce a heterologous protein by secretory production even when it has the genetic construct for secretory expression of the heterologous protein before it is modified so as to harbor a mutant *phoS* gene, which came to be able to produce the heterologous protein by secretory production due to being modified so as to harbor a mutant *phoS* gene.

[0037] Hereinafter, the *phoS* gene and the PhoS protein will be explained. The *phoS* gene is a gene encoding a PhoS protein, which is a sensor kinase of the PhoRS system. The PhoRS system is one of two-component regulatory systems, and induces a response against phosphate depletion. The PhoRS system consists of a sensor kinase PhoS encoded by a *phoS* gene and a response regulator PhoR encoded by a *phoR* gene.

[0038] A PhoS protein as used in the present invention having the "specific mutation" is also referred to as "mutant PhoS protein", and a gene encoding it is also referred to as "mutant *phoS* gene". The mutant *phoS* gene is, in other words, a *phoS* gene having the "specific mutation". Furthermore, a PhoS protein not having the "specific mutation" is also referred to as "wild-type PhoS protein", and a gene encoding it is also referred to as "wild-type *phoS* gene". The wild-type *phoS* gene is, in other words, a *phoS* gene not having the "specific mutation". The term "wild-type" referred to herein is used for convenience to distinguish "wild-type" ones from "mutant" ones, and "wild-type" ones are not limited to those obtained as natural substances, so long as those do not have the "specific mutation". The "specific mutation" will be described later.

[0039] Examples of the wild-type *phoS* gene include, for example, *phoS* genes of coryneform bacteria. Specific examples of the *phoS* genes of coryneform bacteria include, for example, the *phoS* genes of C. *glutamicum* YDK010, C. *glutamicum* ATCC 13032, *C. glutamicum* ATCC 14067, C. *callunae, C. crenatum,* and C. *efficiens.* The nucleotide sequence of the *phoS* gene of C. *glutamicum* YDK010 is shown as SEQ ID NO: 3. The amino acid sequences of the wild-

type PhoS proteins encoded by these *phoS* genes are shown as SEQ ID NOS: 4, 54, 55, 56, 57, and 58, respectively. That is, the wild-type *phoS* gene may be, for example, a gene having the nucleotide sequence shown as SEQ ID NO: 3. Also, the wild-type PhoS protein may be, for example, a protein having the amino acid sequence shown as SEQ ID NO: 4, 54, 55, 56, 57, or 58. The expression "a gene or protein has a nucleotide or amino acid sequence" encompasses cases where a gene or protein comprises the nucleotide or amino acid sequence, and cases where a gene or protein consists of the nucleotide or amino acid sequence.

[0040] The wild-type *phoS* gene may be a variant of any of the wild-type *phoS* genes exemplified above, so long as it does not have the "specific mutation" and the original function thereof is maintained. Similarly, the wild-type PhoS protein may be a variant of any of the proteins encoded by the wild-type *phoS* genes exemplified above, so long as it does not have the "specific mutation" and the original function thereof is maintained. Such a variant is also referred to as "conservative variant". In the present invention, the term "wild-type *phoS* gene" includes not only the wild-type *phoS* genes exemplified above, but also includes conservative variants thereof that do not have the "specific mutation". Similarly, the term "wild-type PhoS protein" includes not only the proteins encoded by the wild-type *phoS* genes exemplified above, but also includes conservative variants thereof that do not have the "specific mutation". Examples of the conservative variants include, for example, homologues and artificially modified versions of the wild-type *phoS* genes and wild-type PhoS proteins exemplified above.

[0041] The expression "the original function is maintained" means that a variant of a gene or protein has a function (such as activity or property) corresponding to the function (such as activity or property) of the original gene or protein. That is, the expression "the original function is maintained" used for the wild-type *phoS* gene may mean that a variant of the gene encodes a protein that maintains the original function. Furthermore, the expression "the original function is maintained" used for the wild-type PhoS protein may mean that a variant of the protein has a function as a sensor kinase of the PhoRS system. The term "function as a sensor kinase of the PhoRS system" may specifically refer to a function of inducing a response against phosphate depletion in the environment in combination with a response regulator PhoR protein. The term "function as a sensor kinase of the PhoRS system" may more specifically refer to a function of sensing phosphate depletion in the environment to be autophosphorylated, and activating the PhoR protein via transfer of phosphate group.

[0042] Whether or not a variant of the PhoS protein has a function as a sensor kinase of the PhoRS system can be confirmed by, for example, introducing a gene encoding the variant into a phoS-gene-deletion strain of a coryneform bacterium, and confirming whether or not responsiveness against phosphate depletion is complemented. Complementation of responsiveness against phosphate depletion can be detected, for example, as improvement of growth under phosphate depletion conditions, or as induction of the expression of genes of which the expression is known to be induced under phosphate depletion conditions (J. Bacteriol., 188, 724-732(2006)). As the phoS-gene-deletion strain of a coryneform bacterium, for example, a phoS-gene-deletion strain of C. *glutamicum* YDK010 or a phoS-gene-deletion strain of C. *glutamicum* ATCC13032 can be used.

[0043] Hereinafter, examples of the conservative variants will be explained.

[0044] Homologues of the wild-type *phoS* genes can be easily obtained from public databases by, for example, BLAST search or FASTA search using any of the nucleotide sequences of the wild-type *phoS* genes exemplified above as a query sequence. Furthermore, homologues of the wild-type *phoS* genes can be obtained by, for example, PCR using a chromosome of coryneform bacteria as the template, and oligonucleotides prepared on the basis of any of the nucleotide sequences of these known wild-type *phoS* genes as primers.

[0045] The wild-type PhoS protein may be a protein having any of the amino acid sequences of the wild-type PhoS proteins exemplified above (SEQ ID NO: 4, 54, 55, 56, 57, or 58), but which includes substitution, deletion, insertion, or addition of one or several amino acid residues at one or several positions, so long as it does not have the "specific mutation" and the original function thereof is maintained. Although the number meant by the term "one or several" mentioned above may differ depending on the positions of amino acid residues in the three-dimensional structure of the protein or the types of amino acid residues, specifically, it is preferably 1 to 20, more preferably 1 to 10, still more preferably 1 to 5, particularly preferably 1 to 3.

[0046] The aforementioned substitution, deletion, insertion, or addition of one or several amino acid residues is a conservative mutation that maintains the normal function of the protein. Typical examples of the conservative mutation are conservative substitutions. The conservative substitution is a mutation wherein substitution takes place mutually among Phe, Trp, and Tyr, if the substitution site is an aromatic amino acid; among Leu, Ile, and Val, if it is a hydrophobic amino acid; between Gln and Asn, if it is a polar amino acid; among Lys, Arg, and His, if it is a basic amino acid; between Asp and Glu, if it is an acidic amino acid; and between Ser and Thr, if it is an amino acid having a hydroxyl group. Examples of substitutions considered as conservative substitutions include, specifically, substitution of Ser or Thr for Ala, substitution of Gln, His, or Lys for Arg, substitution of Glu, Gln, Lys, His, or Asp for Asn, substitution of Asn, Glu, or Gln for Asp, substitution of Ser or Ala for Cys, substitution of Asn, Glu, Lys, His, Asp, or Arg for Gln, substitution of Gly, Asn, Gln, Lys, or Asp for Glu, substitution of Pro for Gly, substitution of Asn, Lys, Gln, Arg, or Tyr for His, substitution of Leu, Met, Val, or Phe for Ile, substitution of Ile, Met, Val, or Phe for Leu, substitution of Asn, Glu, Gln, His, or Arg for Lys, substitution of Ile, Leu, Val, or Phe for Met, substitution of Trp, Tyr, Met, Ile, or Leu for Phe, substitution of Thr or Ala for Ser, substitution of Ser or Ala for Thr, substitution of Phe or Tyr for Trp, substitution of His, Phe, or Trp for Tyr, and substitution of Met, Ile, or Leu for Val. Furthermore, such substitution, deletion, insertion, or addition of amino acid residues as mentioned above includes a naturally occurring mutation due to an individual difference, or a difference of species of the bacterium from which the gene is derived (mutant or variant).

[0047] The wild-type PhoS protein may also be a protein having an amino acid sequence showing a homology of, for example, 80% or more, preferably 90% or more, more preferably 95% or more, still more preferably 97% or more, particularly preferably 99% or more, to the

total amino acid sequence of any of the amino acid sequences of the wild-type PhoS proteins exemplified above (SEQ ID NO: 4, 54, 55, 56, 57, or 58), so long as it does not have the "specific mutation" and the original function thereof is maintained. In this description, "homology" can mean "identity".

[0048] It is preferred that a histidine residue that is autophosphorylated is conserved. That is, it is preferred that a conservative mutation occurs at an amino acid residue other than the histidine residue that is autophosphorylated. The term "histidine residue that is autophosphorylated" refers to a histidine residue at position 276 of the wild-type PhoS protein. Furthermore, it is preferred that, for example, the wild-type PhoS protein has a conservative sequence of the wild-type PhoS proteins exemplified above. That is, it is preferred that a conservative mutation occurs at, for example, an amino acid residue not conserved in the wild-type PhoS proteins exemplified above.

[0049] The wild-type *phoS* gene may also be DNA that is able to hybridize under stringent conditions with a complementary sequence of any of the nucleotide sequences of the wild-type *phoS* genes exemplified above (e.g. SEQ ID NO: 3), or with a probe that can be prepared from the complementary sequence, so long as it does not have the "specific mutation" and the original function thereof is maintained. The term "stringent conditions" refers to conditions under which a so-called specific hybrid is formed, and a non-specific hybrid is not formed. Examples of the stringent conditions include those under which highly homologous DNAs hybridize to each other, for example, DNAs not less than 80% homologous, preferably not less than 90% homologous, more preferably not less than 95% homologous, still more preferably not less than 97% homologous, particularly preferably not less than 99% homologous, hybridize to each other, and DNAs less homologous than the above do not hybridize to each other, or conditions of washing of typical Southern hybridization, i.e., conditions of washing once, preferably 2 or 3 times, at a salt concentration and temperature corresponding to 1 x SSC, 0.1% SDS at 60°C, preferably 0.1 x SSC, 0.1% SDS at 60°C, more preferably 0.1 x SSC, 0.1% SDS at 68°C.

[0050] The probe may be, for example, a part of a sequence that is complementary to the gene as described above. Such a probe can be prepared by PCR using oligonucleotides prepared on the basis of the nucleotide sequences of known genes as primers and a DNA fragment containing any of these nucleotide sequences as a template. As the probe, for example, a DNA fragment having a length of about 300 bp can be used. In such a case, the washing conditions of the hybridization may be, for example, 50°C, 2 x SSC and 0.1% SDS.

[0051] Furthermore, the wild-type *phoS* gene may be a gene having a nucleotide sequence corresponding to any of the nucleotide sequences of the wild-type *phoS* genes exemplified above or conservative variants thereof in which arbitrary codon(s) is/are replaced with respective equivalent codon(s). For example, the wild-type *phoS* gene may be a gene modified so that it has optimal codons according to codon frequencies in a host to be used.

[0052] The percentage of the sequence identity between two sequences can be determined

by, for example, using a mathematical algorithm. Non-limiting examples of such a mathematical algorithm include the algorithm of Myers and Miller (1988) CABIOS 4:11-17, the local homology algorithm of Smith et al (1981) Adv. Appl. Math. 2:482, the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48:443-453, the method for searching homology of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. 85:2444-2448, and an modified version of the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87:2264, such as that described in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5877.

[0053] By using a program based on such a mathematical algorithm, sequence comparison (i.e. alignment) for determining the sequence identity can be performed. The program can be appropriately executed by a computer. Examples of such a program include, but not limited to, CLUSTAL of PC/Gene program (available from Intelligenetics, Mountain View, Calif.), ALIGN program (Version 2.0), and GAP, BESTFIT, BLAST, FASTA, and TFASTA of Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wis., USA). Alignment using these programs can be performed by using, for example, initial parameters. The CLUSTAL program is well described in Higgins et al. (1988) Gene 73:237-244 (1988), Higgins et al. (1989) CABIOS 5:151-153, Corpet et al. (1988) Nucleic Acids Res. 16:10881-90, Huang et al. (1992) CABIOS 8:155-65, and Pearson et al. (1994) Meth. Mol. Biol. 24:307-331.

[0054] In order to obtain a nucleotide sequence homologous to a target nucleotide sequence, in particular, for example, BLAST nucleotide search can be performed by using BLASTN program with score of 100 and word length of 12. In order to obtain an amino acid sequence homologous to a target protein, in particular, for example, BLAST protein search can be performed by using BLASTX program with score of 50 and word length of 3. See http://www.ncbi.nlm.nih.gov for BLAST nucleotide search and BLAST protein search. In addition, Gapped BLAST (BLAST 2.0) can be used in order to obtain an alignment including gap(s) for the purpose of comparison. In addition, PSI-BLAST can be used in order to perform repetitive search for detecting distant relationships between sequences. See Altschul et al. (1997) Nucleic Acids Res. 25:3389 for Gapped BLAST and PSI-BLAST. When using BLAST, Gapped BLAST, or PSI-BLAST, initial parameters of each program (e.g. BLASTN for nucleotide sequences, and BLASTX for amino acid sequences) can be used. Alignment can also be manually performed.

[0055] The sequence identity between two sequences is calculated as the ratio of residues matching in the two sequences when aligning the two sequences so as to fit maximally with each other.

[0056] The above descriptions concerning variants of the genes and proteins can also be applied *mutatis mutandis* to arbitrary proteins such as PhoR protein, cell surface layer protein, Tat secretion system, and heterologous proteins to be produced by secretory production in the present disclosure, and genes encoding them.

[0057] The mutant PhoS protein has the "specific mutation" in the amino acid sequence of such a wild-type PhoS protein as described above.

[0058] That is, in other words, the mutant PhoS protein may be identical to any of the wild-type PhoS proteins exemplified above or conservative variants thereof except that the mutant PhoS protein has the "specific mutation". Specifically, the mutant PhoS protein may be, for example, a protein having the amino acid sequence shown in SEQ ID NO: 4, 54, 55, 56, 57, or 58 except that the mutant PhoS protein has the "specific mutation". Specifically, the mutant PhoS protein may also be, for example, a protein having the amino acid sequence shown in SEQ ID NO: 4, 54, 55, 56, 57, or 58 but including substitution, deletion, insertion, or addition of one or several amino acid residues, except that the mutant PhoS protein has the "specific mutation". Specifically, the mutant PhoS protein may also be, for example, a protein showing a homology of 80% or more, preferably 90% or more, more preferably 95% or more, still more preferably 97% or more, particularly preferably 99% or more, to the amino acid sequence shown in SEQ ID NO: 4, 54, 55, 56, 57, or 58 except that the mutant PhoS protein has the "specific mutation".

[0059] Furthermore, in other words, the mutant PhoS protein may be a variant of any of the wild-type PhoS proteins exemplified above having the "specific mutation", and further including a conservative mutation at a site other than that of the "specific mutation". Specifically, the mutant PhoS protein may be, for example, a protein having the amino acid sequence shown in SEQ ID NO: 4, 54, 55, 56, 57, or 58 but having the "specific mutation", and further including substitution, deletion, insertion, or addition of one or several amino acid residues at a site other than that of the "specific mutation".

[0060] The mutant *phoS* gene is not particularly limited so long as it encodes such a mutant PhoS protein as described above.

[0061] Hereinafter, the "specific mutation" of the mutant PhoS protein will be explained.

[0062] The "specific mutation" is not particularly limited, so long as it is a mutation that changes the amino acid sequence of such a wild-type PhoS protein described above, and that is effective for secretory production a heterologous protein.

[0063] It is preferred that the "specific mutation" is a mutation that improves the secretory production amount of a heterologous protein. The expression "to improve the secretory production amount of a heterologous protein" means that a coryneform bacterium modified so as to have a mutant *phoS* gene (modified strain) is able to produce the heterologous protein by secretory production in an amount larger than that obtainable with a non-modified strain. The "non-modified strain" refers to a control strain not having the "specific mutation" in the *phoS* gene, i.e. a control strain not having any mutant *phoS* gene, and it may be, for example, a wild-type strain or a parent strain. Although the degree of increase meant by the expression "to produce a heterologous protein by secretory production in an amount larger than that obtainable with a non-modified strain" is not particularly limited so long as the secretory production amount of the heterologous protein is increased compared with that obtainable with

a non-modified strain, the expression may mean that the heterologous protein is produced by secretory production in an amount of, for example, preferably 1.1 times or more, more preferably 1.2 times or more, still more preferably 1.3 times or more, still more preferably 2 times or more, particularly preferably 5 times or more, of that obtainable with a non-modified strain, in terms of the accumulation amount in the medium and/or on the cell surface layer. In addition, the expression "to produce a heterologous protein by secretory production in an amount larger than that obtainable with a non-modified strain" may also mean that whereas the heterologous protein cannot be detected when a non-concentrated culture supernatant of a non-modified strain is applied to SDS-PAGE and stained with CBB, the heterologous protein can be detected when a non-concentrated culture supernatant of a modified strain is applied to SDS-PAGE and stained with CBB. Incidentally, the expression "to improve the secretory production amount of a heterologous protein" does not necessarily mean that the secretory production amount of every heterologous protein is improved, and it is sufficient that the secretory production amount of a heterologous protein chosen as the target of secretory production is improved. The expression "to improve the secretory production amount of a heterologous protein" may specifically mean, for example, that the secretory production amount of a heterologous protein described in the Example section, such as CspB50TEV-Teri, CspB6Xa-LFABP, CspB6TEV-ExCP, protransglutaminase fused with E. *coli* TorA signal sequence, protein glutaminase comprising a pro-structure moiety and fused with E. *coli* TorA signal sequence, or isomaltodextranase comprising a signal sequence, is improved.

[0064] Whether a certain mutation is a mutation that improves the secretory production amount of a heterologous protein can be confirmed by, for example, preparing a strain modified so as to have a gene encoding the PhoS protein having the certain mutation from a strain belonging to a coryneform bacterium, quantifying the amount of the heterologous protein produced by secretory production when the strain is cultured in a medium, and comparing it with the amount of the heterologous protein produced by secretory production when the strain before the modification (non-modified strain) is cultured in the medium.

[0065] Preferred examples of the change of the amino acid sequence include substitution of an amino acid residue. That is, it is preferred that the "specific mutation" is a mutation of replacing an amino acid residue with another amino acid residue. The amino acid residue substituted by the "specific mutation" may be one residue, or may be a combination of two or more residues. The amino acid residue substituted by the "specific mutation" may preferably be an amino acid residue other than the histidine residue that is autophosphorylated. The amino acid residue substituted by the "specific mutation" may more preferably be an amino acid residue in the HisKA domain other than the histidine residue that is autophosphorylated. The term "histidine residue that is autophosphorylated" refers to a histidine residue at position 276 of the wild-type PhoS protein. The term "HisKA domain" refers to a region consisting of amino acid residues at positions 266-330 of the wild-type PhoS protein. The amino acid residue substituted by the "specific mutation" corresponds to the tryptophan residue at position 302 of SEQ. ID. No. 4.

[0066] In the aforementioned mutation, the amino acid residue after substitution is K(Lys),

A(Ala), V(Val), S(Ser), C(Cys), M(Met), D(Asp) or, N(Asn), provided that the amino acid residue after substitution is other than the original one. As the amino acid residue after substitution, for example, one resulting in improvement in the secretory production amount of a heterologous protein can be chosen.

[0067] When substitution occurs at W302, examples of the amino acid residue after substitution include amino acid residues other than aromatic amino acid and histidine residues, specifically the "amino acid residues other than aromatic amino acid and histidine residues" include K(Lys), A(Ala), V(Val), S(Ser), C(Cys), M(Met), D(Asp), or N(Asn).

[0068] Incidentally, the term "specific mutation" used for the *phoS* gene refers to a mutation on the nucleotide sequence thereof that results in such a "specific mutation" as described above into the encoded PhoS protein.

[0069] An An "amino acid residue at position X of the wild-type PhoS protein" refers to an amino acid residue corresponding to the amino acid residue at position X in SEQ ID NO: 4. For example, "W302" refers to an amino acid residue corresponding to the tryptophan residue at position 302 in SEQ ID NO: 4. The aforementioned positions of amino acid residues indicate relative positions, and the absolute positions thereof may shift due to deletion, insertion, addition, of an amino acid residue or residues. For example, if one amino acid residue is deleted or inserted at a position on the N-terminal side of position X in the amino acid sequence shown as SEQ ID NO: 4, the amino acid residue originally at position X is relocated at position X-1 or X+1 counted from the N-terminus, however, it is still regarded as the "amino acid residue at position X of the wild-type PhoS protein". Specifically, for example, "W302" refers to the tryptophan residue at positions 302, 302, 302, 321, 275, and 286, respectively, in the amino acid sequences of wild-type PhoS proteins shown in SEQ ID NOS: 4, 54, 55, 56, 57, and 58. Furthermore, the "histidine residue at position 276 of the wild-type PhoS protein (histidine residue that is autophosphorylated)" refers to the histidine residue at positions 276, 276, 276, 295, 249, and 260, respectively, in the amino acid sequences of wild-type PhoS proteins shown in SEQ ID NOS: 4, 54, 55, 56, 57, and 58. Furthermore, the "region consisting of amino acid residues at positions 266-330 of the wild-type PhoS protein (HisKA domain)" refers to the region consisting of amino acid residues at positions 266-330, 266-330, 266-330, 285-349, 239-303, and 250-314, respectively, in the amino acid sequences of wild-type PhoS proteins shown in SEQ ID NOS: 4, 54, 55, 56, 57, and 58.

[0070] Incidentally, while "W302" referred to herein is typically a tryptophan residue, it may also be other than a tryptophan residue. That is, when the wild-type PhoS protein has an amino acid sequence other than the amino acid sequences shown in SEQ ID NOS: 4, 54, 55, 56, 57, and 58, "W302" can be other than a tryptophan residue. Hence, for example, the "mutation replacing W302 with a cysteine residue" includes not only a mutation, when "W302" is a tryptophan residue, for replacing this tryptophan residue with a cysteine residue, but also includes a mutation, when "W302" is K(Lys), R(Arg), H(His), A(Ala), V(Val), L(Leu), I(Ile), G(Gly), S(Ser), T(Thr), P(Pro), F(Phe), Y(Tyr), M(Met), D(Asp), E(Glu), N(Asn), or Q(Gln), for replacing this residue with a cysteine residue. The same can be applied *mutatis mutandis* to

the other mutations.

[0071] Which amino acid residue is the "amino acid residue corresponding to the amino acid residue at position X in SEQ ID NO: 4" in the amino acid sequence of an arbitrary PhoS protein can be determined by alignment between the amino acid sequence of the arbitrary PhoS protein and the amino acid sequence of SEQ ID NO: 4. The alignment can be performed by, for example, using known gene analysis software. Specific examples of such software include DNASIS produced by Hitachi Solutions, GENETYX produced by Genetyx, and so forth (Elizabeth C. Tyler et al., Computers and Biomedical Research, 24 (1) 72-96, 1991; Barton GJ et al., Journal of Molecular Biology, 198 (2), 327-37, 1987).

[0072] The mutant *phoS* gene can be obtained by, for example, modifying a wild-type *phoS* gene so that the encoded PhoS protein has the aforementioned "specific mutation". The wild-type *phoS* gene to be modified can be obtained by, for example, cloning from an organism having the wild-type *phoS* gene, or chemical synthesis. Furthermore, the mutant *phoS* gene can also be obtained without using a wild-type *phoS* gene. For example, the mutant *phoS* gene may be directly obtained by chemical synthesis. The obtained mutant *phoS* gene may be further modified before use.

[0073] Genes can be modified by known methods. For example, an objective mutation can be introduced into a target site of DNA by the site-specific mutagenesis method. Examples of the site-specific mutagenesis method include a method of using PCR (Higuchi, R., 61, in PCR Technology, Erlich, H.A. Eds., Stockton Press (1989); Carter P., Meth. In Enzymol., 154, 382 (1987)), and a method of using a phage (Kramer, W. and Frits, H.J., Meth. in Enzymol., 154, 350 (1987); Kunkel, T.A. et al., Meth. in Enzymol., 154, 367 (1987)).

[0074] Hereinafter, methods for modifying a coryneform bacterium so as to have a mutant *phoS* gene will be explained.

[0075] A coryneform bacterium can be modified so as to have a mutant *phoS* gene by introducing the mutant *phoS* gene into the coryneform bacterium. A coryneform bacterium can be modified so as to have a mutant *phoS* gene also by introducing a mutation into the *phoS* gene on the chromosome of the coryneform bacterium. A mutation can be introduced into a gene on a chromosome by natural mutation, mutagenesis treatment, or genetic engineering means.

[0076] Methods for introducing a mutant *phoS* gene into a coryneform bacterium are not particularly limited. It is sufficient that the mutant *phoS* gene is harbored by the bacterium of the present invention so that it can be expressed under control of a promoter that functions in a coryneform bacterium. The promoter may be a promoter derived from the host, or a heterogenous promoter. The promoter may be the native promoter of the *phoS* gene, or a promoter of another gene. In the bacterium of the present invention, the mutant *phoS* gene may be present on a vector that autonomously replicates out of the chromosome, such as plasmid, or may be incorporated into the chromosome. The bacterium of the present invention

may have only one copy of the mutant *phoS* gene, or two or more copies of the mutant *phoS* gene. The bacterium of the present invention may have only one kind of mutant *phoS* gene, or two or more kinds of mutant *phoS* genes. The mutant *phoS* gene can be introduced, for example, in the same manner as that for introduction of a gene in methods for increasing the expression of a gene described below, or for introduction of the genetic construct used for the present invention described below.

[0077] The bacterium of the present invention as defined by the claims may or may not have the wild-type *phoS* gene. It is preferred that the bacterium of the present invention does not have the wild-type *phoS* gene.

[0078] A coryneform bacterium not having the wild-type *phoS* gene can be obtained by disrupting the wild-type *phoS* gene on the chromosome. The wild-type *phoS* gene can be disrupted by known methods. Specifically, the wild-type *phoS* gene can be disrupted by, for example, deleting a part or the whole of the promoter region and/or the coding region of the wild-type *phoS* gene.

[0079] Furthermore, by replacing the wild-type *phoS* gene on the chromosome with a mutant *phoS* gene, a coryneform bacterium modified so that it does not have the wild-type *phoS* gene and has the mutant *phoS* gene can be obtained. Examples of methods for performing such gene substitution include, for example, a method of using a linear DNA such as a method called "Red driven integration" (Datsenko, K.A, and Wanner, B.L., Proc. Natl. Acad. Sci. USA, 97:6640-6645 (2000)), a method of utilizing the Red driven integration in combination with an excision system derived from λ phage (Cho, E.H., Gumport, R.I., Gardner, J.F., J. Bacteriol., 184:5200-5203 (2002)) (refer to WO2005/010175), a method of using a plasmid including a temperature sensitive replication origin, a method of using a plasmid capable of conjugative transfer, a method of utilizing a suicide vector not including a replication origin that functions in a host (U.S. Patent No. 6,303,383, Japanese Patent Laid-open (Kokai) No. 05-007491), and so forth.

[0080] The PhoS protein functions, i.e. induces a response against phosphate depletion in the environment, in combination with a response regulator PhoR protein. Hence, the bacterium of the present invention has a *phoR* gene so that the mutant PhoS protein functions. The *phoR* gene is a gene encoding a PhoR protein, which is a response regulator of the PhoRS system. The expression "to have a *phoR* gene" is also referred to as "to have a PhoR protein". Typically, it is sufficient that the PhoR protein inherently possessed by the bacterium of the present invention functions in combination with the mutant PhoS protein. Alternatively, the bacterium of the present invention may be introduced with an appropriate *phoR* gene, in addition to or instead of the *phoR* gene inherently possessed by the bacterium of the present invention. The *phoR* gene to be introduced is not particularly limited, as long as it encodes a PhoR protein that functions in combination with the mutant PhoS protein.

[0081] Examples of the *phoR* gene include, for example, *phoR* genes of coryneform bacteria. Specific examples of the *phoR* genes of coryneform bacteria include, for example, the *phoR*

genes of C. *glutamicum* YDK010, *C. glutamicum* ATCC 13032, C. *glutamicum* ATCC 14067, *C. callunae, C. crenatum,* and C. *efficiens.* The nucleotide sequence of the *phoR* gene of *C. glutamicum* ATCC 13032 and the amino acid sequence of the PhoR protein of the same are shown as SEQ ID NO: 96 and 97, respectively.

[0082] The *phoR* gene may be a variant of any of the *phoR* genes exemplified above, so long as the original function thereof is maintained. Similarly, the PhoR protein may be a variant of any of the PhoR proteins exemplified above, so long as the original function thereof is maintained. That is, the term *"phoR* gene" includes not only the *phoR* genes exemplified above, but also includes conservative variants thereof. Similarly, the term "PhoR protein" includes not only the PhoR proteins exemplified above, but also includes conservative variants thereof. The above descriptions concerning conservative variants of the *phoS* gene and PhoS protein can be applied *mutatis mutandis* to variants of the *phoR* gene and PhoR protein. For example, the *phoR* gene may be a gene encoding a protein having the aforementioned amino acid sequence, but including substitution, deletion, insertion, or addition of one or several amino acid residues at one or several positions, so long as the gene encodes a protein of which the original function is maintained. Incidentally, the expression "the original function is maintained" used for the PhoR protein may mean that a variant of the protein has a function as a response regulator of the PhoRS system. The term "function as a response regulator of the PhoRS system" may specifically refer to a function of inducing a response against phosphate depletion in the environment in combination with a sensor kinase PhoS protein. The term "function as a response regulator of the PhoRS system" may more specifically refer to a function of being activated via transfer of phosphate group from the PhoS protein that sensed phosphate depletion in the environment to be autophosphorylated, and regulating the expression of genes that respond to phosphate depletion in the environment.

[0083] Whether or not a variant of the PhoR protein has a function as a response regulator of the PhoRS system can be confirmed by, for example, introducing a gene encoding the variant into a phoR-gene-deletion strain of a coryneform bacterium, and confirming whether or not responsiveness against phosphate depletion is complemented. Complementation of responsiveness against phosphate depletion can be detected, for example, as improvement of growth under phosphate depletion conditions, or as induction of the expression of genes of which the expression is known to be induced under phosphate depletion conditions (J. Bacteriol., 188, 724-732(2006)). As the phoR-gene-deletion strain of a coryneform bacterium, for example, a phoR-gene-deletion strain of C. *glutamicum* YDK010 or a phoR-gene-deletion strain of C. *glutamicum* ATCC13032 can be used.

<1-1-3> Reduction in activity of cell surface layer protein

[0084] The bacterium of the present invention as defined by the claims may be a bacterium of which the activity(s) of cell surface layer protein(s) is/are reduced. Hereinafter, the cell surface layer proteins and genes encoding them will be explained.

[0085] The cell surface layer protein is a protein constituting the surface layer (S layer) of bacteria or archaea. Examples of cell surface layer proteins of coryneform bacteria include PS1 and PS2 (CspB) of C. *glutamicum* (Japanese Patent Laid-open (Kohyo) No. 6-502548), and SlpA (CspA) of C. *stationis* (Japanese Patent Laid-open (Kokai) No. 10-108675). It is preferable to reduce the activity of the PS2 protein among these.

[0086] The nucleotide sequence of the *cspB* gene of C. *glutamicum* ATCC 13869 and the amino acid sequence of the PS2 protein (CspB protein) encoded by the gene are shown in SEQ ID NOS: 67 and 68, respectively.

[0087] Furthermore, for example, amino acid sequences of CspB homologues were reported for 28 strains of C *.glutamicum* (J. Biotechnol., 112, 177-193 (2004)). These 28 strains of C. *glutamicum* and the GenBank accession numbers of the *cspB* gene homologues in NCBI database are exemplified below (the GenBank accession numbers are shown in the parentheses).

C. glutamicum ATCC 13058 (AY524990)

C. *glutamicum* ATCC 13744 (AY524991)

C. glutamicum ATCC 13745 (AY524992)

C. *glutamicum* ATCC 14017 (AY524993)

C. glutamicum ATCC 14020 (AY525009)

C. glutamicum ATCC 14067 (AY524994)

C. glutamicum ATCC 14068 (AY525010)

C. *glutamicum* ATCC 14747 (AY525011)

C. *glutamicum* ATCC 14751 (AY524995)

C. glutamicum ATCC 14752 (AY524996)

C. glutamicum ATCC 14915 (AY524997)

C. glutamicum ATCC 15243 (AY524998)

C. *glutamicum* ATCC 15354 (AY524999)

C. glutamicum ATCC 17965 (AY525000)

C. glutamicum ATCC 17966 (AY525001)

C. glutamicum ATCC 19223 (AY525002)

C. *glutamicum* ATCC 19240 (AY525012)

C. glutamicum ATCC 21341 (AY525003)

C. glutamicum ATCC 21645 (AY525004)

C. glutamicum ATCC 31808 (AY525013)

C. glutamicum ATCC 31830 (AY525007)

C. *glutamicum* ATCC 31832 (AY525008)

C. *glutamicum* LP-6 (AY525014)

C. glutamicum DSM20137 (AY525015)

C. glutamicum DSM20598 (AY525016)

C. glutamicum DSM46307 (AY525017)

C. glutamicum 22220 (AY525005)

C. glutamicum 22243 (AY525006)

[0088] Since the nucleotide sequence of a gene encoding a cell surface layer protein may differ depending on species or strain to which the coryneform bacterium belongs, the gene encoding a cell surface layer protein may be a variant of any of genes encoding the cell surface layer proteins exemplified above, so long as the original function thereof is maintained. Similarly, the cell surface layer protein may be a variant of any of the cell surface layer proteins exemplified above, so long as the original function thereof is maintained. That is, the term *"cspB* gene" includes not only the *cspB* genes exemplified above, but also includes conservative variants thereof. Similarly, the term "CspB protein" includes not only the CspB proteins exemplified above, but also includes conservative variants thereof. The above descriptions concerning conservative variants of the *phoS* gene and PhoS protein can be applied *mutatis mutandis* to variants of the cell surface layer protein and the gene encoding it. For example, the gene encoding the cell surface layer protein may be a gene encoding a protein having the aforementioned amino acid sequence, but including substitution, deletion, insertion, or addition of one or several amino acid residues at one or several positions, so long as the gene encodes a protein of which the original function is maintained. Incidentally, the expression "original function is maintained" used for the cell surface layer protein may mean that the protein has a property that if the activity of the protein is reduced in a coryneform bacterium, the secretory production amount of a heterologous protein is increased compared with that obtainable with a non-modified strain.

[0089] The "property that if the activity of the protein is reduced in a coryneform bacterium, the secretory production amount of a heterologous protein is increased compared with that obtainable with a non-modified strain" refers to a property imparting an ability to produce a heterologous protein by secretory production in an amount larger than that obtainable with a

non-modified strain to a coryneform bacterium when the activity thereof is reduced in the coryneform bacterium. The "non-modified strain" refers to a control strain of which the activity(s) of cell surface layer protein(s) is/are not reduced, and it may be, for example, a wild-type strain or a parent strain. Although the degree of increase meant by the expression "to produce a heterologous protein by secretory production in an amount larger than that obtainable with a non-modified strain" is not particularly limited so long as the secretory production amount of the heterologous protein is increased compared with that obtainable with a non-modified strain, the expression may mean that the heterologous protein is produced by secretory production in an amount of, for example, preferably 1.1 times or more, more preferably 1.2 times or more, still more preferably 1.3 times or more, particularly preferably 2 times or more, of that obtainable with a non-modified strain, in terms of the accumulation amount in the medium and/or on the cell surface layer. In addition, the expression "to produce a heterologous protein by secretory production in an amount larger than that obtainable with a non-modified strain" may also mean that whereas the heterologous protein cannot be detected when a non-concentrated culture supernatant of a non-modified strain is applied to SDS-PAGE and stained with CBB, the heterologous protein can be detected when a non-concentrated culture supernatant of a modified strain is applied to SDS-PAGE and stained with CBB.

[0090] Whether a protein has a property that if the activity of the protein is reduced in a coryneform bacterium, the secretory production amount of a heterologous protein is increased compared with that obtainable with a non-modified strain can be confirmed by preparing a strain modified so that the activity of the protein is reduced from a strain belonging to the coryneform bacteria, quantifying the secretory production amount of the heterologous protein observed when the modified strain is cultured in a medium, and comparing the quantified amount with the secretory production amount of the heterologous protein observed when the strain before being modified (un-modified strain) is cultured in the medium.

[0091] In the present disclosure, the expression "activity of a cell surface layer protein is reduced" includes a case where a coryneform bacterium has been modified so that the activity of a cell surface layer protein is reduced and a case where the activity of a cell surface layer protein is inherently reduced in a coryneform bacterium. The "case where activity of a cell surface layer protein is inherently reduced in a coryneform bacterium" includes a case where a coryneform bacterium is inherently deficient in a cell surface layer protein. That is, examples of a coryneform bacterium in which the activity of a cell surface layer protein is reduced include a coryneform bacterium that is inherently deficient in a cell surface layer protein. Examples of the "case where a coryneform bacterium is inherently deficient in a cell surface layer protein" include a case where a coryneform bacterium is inherently deficient in the gene encoding a cell surface layer protein. The expression "a coryneform bacterium is inherently deficient in a cell surface layer protein" may mean that a coryneform bacterium is inherently deficient in one or more proteins selected from cell surface layer protein(s) found in other strain(s) of the species to which the coryneform bacterium belongs. For example, "C. *glutamicum* is inherently deficient in a cell surface layer protein" may mean that a C. *glutamicum* strain is inherently deficient in one or more proteins selected from cell surface layer protein(s) found in other C. *glutamicum* strain(s), i.e. for example, deficient in PS1 and/or PS2 (CspB). Examples of the

coryneform bacterium that is inherently deficient in a cell surface layer protein include C. *glutamicum* ATCC 13032, which is inherently deficient in the *cspB* gene.

[0092] Hereinafter, methods for reducing the activity of a protein such as cell surface layer proteins will be explained. The methods for reducing the activity of a protein described below can be utilized for disruption of the wild-type *phoS* gene.

[0093] The expression "the activity of a protein is reduced" means that the activity of the protein per cell is reduced as compared with that of a non-modified strain. The term "non-modified strain" used herein refers to a control strain that has not been modified so that the activity of an objective protein is reduced. Examples of the non-modified strain include a wild-type strain and parent strain. The state that "the activity of a protein is reduced" also includes a state that the activity of the protein has completely disappeared. Specifically, the expression "the activity of a protein is reduced" means that the number of molecules of the protein per cell is reduced, and/or the function of each molecule of the protein is reduced as compared with those of a non-modified strain. That is, the term "activity" in the expression "the activity of a protein is reduced" is not limited to the catalytic activity of the protein, but may also mean the transcription amount of a gene (i.e. the amount of mRNA) encoding the protein or the translation amount of the protein (i.e. the amount of the protein). The state that "the number of molecules of the protein per cell is reduced" also includes a state that the protein does not exist at all. The state that "the function of each molecule of the protein is reduced" also includes a state that the function of each protein molecule has completely disappeared. The degree of the reduction in the activity of a protein is not particularly limited, so long as the activity is reduced as compared with that of a non-modified strain. The activity of a protein may be reduced to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0% of that of a non-modified strain.

[0094] The modification for reducing the activity of a protein can be attained by, for example, reducing the expression of a gene encoding the protein. The expression "the expression of a gene is reduced" means that the expression of the gene per cell is reduced as compared with that of a non-modified strain such as a wild-type strain and parent strain. The expression "the expression of a gene is reduced" may specifically mean that the transcription amount of the gene (i.e. the amount of mRNA) is reduced, and/or the translation amount of the gene (i.e. the amount of the protein expressed from the gene) is reduced. The state that "the expression of a gene is reduced" also includes a state that the gene is not expressed at all. The state that "the expression of a gene is reduced" is also referred to as "the expression of a gene is attenuated". The expression of a gene may be reduced to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0% of that of a non-modified strain.

[0095] The reduction in gene expression may be due to, for example, a reduction in the transcription efficiency, a reduction in the translation efficiency, or a combination of them. The expression of a gene can be reduced by modifying an expression control sequence of the gene such as promoter, Shine-Dalgarno (SD) sequence (also referred to as ribosome-binding site (RBS)), and spacer region between RBS and the start codon of the gene. When an

expression control sequence is modified, preferably one or more nucleotides, more preferably two or more nucleotides, particularly preferably three or more nucleotides, of the expression control sequence are modified. Furthermore, a part or the whole of an expression control sequence may be deleted. The expression of a gene can also be reduced by, for example, manipulating a factor responsible for expression control. Examples of the factor responsible for expression control include low molecules responsible for transcription or translation control (inducers, inhibitors, etc.), proteins responsible for transcription or translation control (transcription factors etc.), nucleic acids responsible for transcription or translation control (siRNA etc.), and so forth. Furthermore, the expression of a gene can also be reduced by, for example, introducing a mutation that reduces the expression of the gene into the coding region of the gene. For example, the expression of a gene can be reduced by replacing a codon in the coding region of the gene with a synonymous codon used less frequently in a host. Furthermore, for example, the gene expression may be reduced due to disruption of a gene as described later.

[0096] The modification for reducing the activity of a protein can also be attained by, for example, disrupting a gene encoding the protein. The expression "a gene is disrupted" means that a gene is modified so that a protein that can normally function is not produced. The state that "a protein that normally functions is not produced" includes a state that the protein is not produced at all from the gene, and a state that the protein of which the function (such as activity or property) per molecule is reduced or eliminated is produced from the gene.

[0097] Disruption of a gene can be attained by, for example, deleting a part or the whole of the coding region of the gene on a chromosome. Furthermore, the whole of a gene including sequences upstream and downstream from the gene on a chromosome may be deleted. The region to be deleted may be any region such as an N-terminus region, an internal region, or a C-terminus region, so long as the activity of the protein can be reduced. Deletion of a longer region can usually more surely inactivate the gene. Furthermore, it is preferred that reading frames of the sequences upstream and downstream from the region to be deleted are not the same.

[0098] Disruption of a gene can also be attained by, for example, introducing a mutation for an amino acid substitution (missense mutation), a stop codon (nonsense mutation), a frame shift mutation which adds or deletes one or two nucleotide residues, or the like into the coding region of the gene on a chromosome (Journal of Biological Chemistry, 272:8611-8617 (1997); Proceedings of the National Academy of Sciences, USA, 95 5511-5515 (1998); Journal of Biological Chemistry, 26 116, 20833-20839 (1991)).

[0099] Disruption of a gene can also be attained by, for example, inserting another sequence into a coding region of the gene on a chromosome. Site of the insertion may be in any region of the gene, and insertion of a longer region can usually more surely inactivate the gene. It is preferred that reading frames of the sequences upstream and downstream from the insertion site are not the same. The other sequence is not particularly limited so long as a sequence that reduces or eliminates the activity of the encoded protein is chosen, and examples thereof

include, for example, a marker gene such as antibiotic resistance genes, and a gene useful for production of an objective substance.

[0100] Such modification of a gene on a chromosome as described above can be attained by, for example, preparing a deficient type gene modified so that it is unable to produce a protein that normally functions, and transforming a host with a recombinant DNA containing the deficient type gene to cause homologous recombination between the deficient type gene and the wild-type gene on a chromosome and thereby substitute the deficient type gene for the wild-type gene on the chromosome. In this procedure, if a marker gene selected according to the characteristics of the host such as auxotrophy is included in the recombinant DNA, the operation becomes easier. Examples of the deficient type gene include a gene including deletion of all or a part of the gene, gene including a missense mutation, gene including insertion of a transposon or marker gene, gene including a nonsense mutation, and gene including a frame shift mutation. The structure of the recombinant DNA to be used for homologous recombination is not particularly limited as long as it causes homologous recombination in a desired manner. For example, a host can be transformed with a linear DNA containing the deficient type gene and further containing upstream and downstream sequences of the wild-type gene on the chromosome at the respective ends, so that homologous recombination occurs at each of upstream and downstream sides of the wild-type gene, to thereby replace the wild-type gene with the deficient type gene in one step. The protein encoded by the deficient type gene has a conformation different from that of the wild-type protein, even if it is produced, and thus the function thereof is reduced or eliminated. Such gene disruption based on gene substitution utilizing homologous recombination has already been established, and there are methods of using a linear DNA such as a method called "Red driven integration" (Datsenko, K.A, and Wanner, B.L., Proc. Natl. Acad. Sci. USA, 97:6640-6645 (2000)), and a method utilizing the Red driven integration in combination with an excision system derived from λ phage (Cho, E.H., Gumport, R.I., Gardner, J.F., J. Bacteriol., 184:5200-5203 (2002)) (refer to WO2005/010175), a method of using a plasmid having a temperature sensitive replication origin, a method of using a plasmid capable of conjugative transfer, a method of utilizing a suicide vector not having a replication origin that functions in a host (U.S. Patent No. 6,303,383, Japanese Patent Laid-open (Kokai) No. 05-007491), and so forth.

[0101] Modification for reducing activity of a protein can also be attained by, for example, a mutagenesis treatment. Examples of the mutagenesis treatment include irradiation of X-ray or ultraviolet and treatment with a mutation agent such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), and methyl methanesulfonate (MMS).

[0102] A reduction in the activity of a protein can be confirmed by measuring the activity of the protein.

[0103] A reduction in the activity of a protein can also be confirmed by confirming a reduction in the expression of a gene encoding the protein. A reduction in the expression of a gene can be confirmed by confirming a reduction in the transcription amount of the gene or a reduction in the amount of the protein expressed from the gene.

[0104] A reduction in the transcription amount of a gene can be confirmed by comparing the amount of mRNA transcribed from the gene with that of a non-modified strain. Examples of the method for evaluating the amount of mRNA include Northern hybridization, RT-PCR, and so forth (Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001). The amount of mRNA is preferably reduced to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0% of that of a non-modified strain.

[0105] A reduction in the amount of a protein can be confirmed by performing SDS-PAGE and confirming the intensity of the separated protein band. A reduction in the amount of a protein can be confirmed by Western blotting using antibodies (Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA) 2001). The amount of the protein is preferably reduced to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0% of that of a non-modified strain.

[0106] Disruption of a gene can be confirmed by determining nucleotide sequence of a part or the whole of the gene, restriction enzyme map, full length, or the like of the gene depending on the means used for the disruption.

<1-1-4> Protein secretion system

[0107] The bacterium of the present invention has a protein secretion system as defined in the claims. The protein secretion system is not particularly limited, so long as it can secrete an objective heterologous protein. Examples of the protein secretion system include Sec system (Sec secretion system) and Tat system (Tat secretion system). The bacterium of the present disclosure may have been modified so that the protein secretion system is enhanced. For example, the bacterium of the present disclosure may have been modified so that the expression of one or more genes selected from genes encoding the Tat secretion system is increased. In the present disclosure, such a modification is also referred to as "enhancement of the Tat secretion system". Enhancement of the Tat secretion system is preferable particularly for cases of producing a heterologous protein by secretory production using a Tat-dependent signal peptide. Methods for increasing the expression of genes encoding the Tat secretion system are described in Japanese Patent No. 4730302.

[0108] Examples of the genes encoding the Tat secretion system include *tatA, tatB,* and *tatC* genes of C. *glutamicum.* The *tatA, tatB,* and *tatC* genes of C. *glutamicum* ATCC 13032 correspond to the complementary sequence of positions 1571065-1571382, the sequence of positions 1167110-1167580, and the complementary sequence of positions 1569929-1570873 in the genome sequence registered as GenBank accession NC_003450 (VERSION NC_003450.3 GI:58036263) in NCBI database, respectively. The TatA, TatB, and TatC proteins of C. *glutamicum* ATCC 13032 have been registered as GenBank accession NP_600707 (version NP_600707.1 GI:19552705, locus_tag="NCgl1434"), GenBank accession NP_600350 (version NP_600350.1 GI:19552348, locus_tag="NCgl1077"), and GenBank accession

NP_600706 (version NP_600706.1 GI:19552704, locus tag="NCgl1433"), respectively. The nucleotide sequences of the *tatA, tatB,* and *tatC* genes of C. *glutamicum* ATCC 13032 and the amino acid sequences of the TatA, TatB, and TatC proteins of the same are shown as SEQ ID NOS: 69-74.

[0109] Examples of the genes encoding the Tat secretion system also include *tatA, tatB, tatC,* and *tatE* genes of E. *coli.* The *tatA, tatB, tatC,* and *tatE* genes of E. *coli* K-12 MG1655 correspond to the sequence of positions 4019968-4020237, the sequence of positions 4020241-4020756, the sequence of positions 4020759-4021535, and the sequence of positions 658170-658373 in the genome sequence registered as GenBank accession NC_000913(VERSION NC_000913.2 GI:49175990) in NCBI database, respectively. The TatA, TatB, TatC, and TatE proteins of E. *coli* K-12 MG1655 have been registered as GenBank accession NP_418280 (version NP_418280.4 GI:90111653, locus_tag="b3836"), GenBank accession YP_026270 (version YP_026270.1 GI:49176428, locus_tag="b3838"), GenBank accession NP_418282 (version NP_418282.1 GI:16131687, locus_tag="b3839"), and GenBank accession NP_415160 (version NP_415160.1 GI:16128610, locus_tag="b0627") , respectively.

[0110] The gene encoding the Tat secretion system may be a variant of any of the genes encoding the Tat-secretion-system exemplified above, so long as the original function thereof is maintained. Similarly, the Tat-secretion-system may be a variant of any of the Tat-secretion-systems exemplified above, so long as the original function thereof is maintained. That is, the terms *"tatA* gene", *"tatB* gene", *"tatC* gene", and *"tatE* gene" include not only the *tatA, tatB, tatC,* and *tatE* genes exemplified above, respectively, but also includes conservative variants thereof. Similarly, the terms "TatA protein", "TatB protein", "TatC protein", and "TatE protein" include not only the TatA, TatB, TatC, and TatE proteins exemplified above, respectively, but also includes conservative variants thereof. The above descriptions concerning conservative variants of the *phoS* gene and PhoS protein can be applied *mutatis mutandis* to variants of the Tat-secretion-system and the gene encoding it. For example, the gene encoding the Tat-secretion-system may be a gene encoding a protein having any of the aforementioned amino acid sequences, but including substitution, deletion, insertion, or addition of one or several amino acid residues at one or several positions, so long as the gene encodes a protein of which the original function is maintained. Incidentally, the expression "original function is maintained" used for the Tat-secretion-system may mean that the system has a function of secreting a protein fused with a Tat-dependent signal peptide at the N-terminus out of the cell.

[0111] Hereinafter, methods for increasing the expression of a gene such as genes encoding the Tat secretion system will be explained.

[0112] The expression "the expression of a gene is increased" means that the expression of the gene is increased as compared with that of a non-modified strain. The term "non-modified strain" used herein refers to a control strain that has not been modified so that the expression of an objective gene is increased. Examples of the non-modified strain include a wild-type strain and parent strain. The expression "the expression of a gene is increased" may

specifically mean that the transcription amount of the gene (i.e. the amount of mRNA) is increased, and/or the translation amount of the gene (i.e. the amount of the protein expressed from the gene) is increased. The state that "the expression of a gene is increased" may also be referred to as "the expression of a gene is enhanced". The degree of the increase in the expression of a gene is not particularly limited, so long as the expression of the gene is increased as compared with that of a non-modified strain. The expression of a gene may be increased to preferably 1.5 times or more, more preferably 2 times or more, or still more preferably 3 times or more, of that of a non-modified strain. Furthermore, the state that "the expression of a gene is increased" includes not only a state that the expression amount of an objective gene is increased in a strain that inherently expresses the objective gene, but also a state that the gene is introduced into a strain that does not inherently express the objective gene, and expressed therein. That is, the phrase "the expression of a gene is increased" may also mean, for example, that an objective gene is introduced into a strain that does not possess the gene, and is expressed therein.

[0113] The expression of a gene can be increased by, for example, increasing the copy number of the gene.

[0114] The copy number of a gene can be increased by introducing the gene into the chromosome of a host. A gene can be introduced into a chromosome by, for example, using homologous recombination (Miller, J.H., Experiments in Molecular Genetics, 1972, Cold Spring Harbor Laboratory). Examples of the gene transfer method utilizing homologous recombination include, for example, a method using a linear DNA such as Red-driven integration (Datsenko, K.A., and Wanner, B.L., Proc. Natl. Acad. Sci. USA, 97:6640-6645 (2000)), a method of using a plasmid containing a temperature sensitive replication origin, a method of using a plasmid capable of conjugative transfer, a method of using a suicide vector not having a replication origin that functions in a host, or a transduction method using a phage. The structure of the recombinant DNA to be used for homologous recombination is not particularly limited as long as it causes homologous recombination in a desired manner. For example, a host can be transformed with a linear DNA containing an objective gene and further containing upstream and downstream sequences of the homologous recombination target region on the chromosome at the respective ends, so that homologous recombination occurs at each of upstream and downstream sides of the target region, to thereby replace the target region with the arbitrary sequence. The recombinant DNA to be used for homologous recombination may contain a marker gene for selection of transformants. Only one copy of, or two or more copies of a gene may be introduced. For example, by performing homologous recombination using a sequence which is present in multiple copies on a chromosome as a target, multiple copies of a gene can be introduced into the chromosome. Examples of such a sequence which is present in multiple copies on a chromosome include repetitive DNAs, and inverted repeats located at the both ends of a transposon. Alternatively, homologous recombination may be performed by using an appropriate sequence on a chromosome such as a gene unnecessary for the production of an objective substance as a target. Furthermore, a gene can also be randomly introduced into a chromosome by using a transposon or Mini-Mu (Japanese Patent Laid-open (Kokai) No. 2-109985, U.S. Patent No. 5,882,888, EP 805867 B1). As the

transposon, an artificial transposon may also be used (Japanese Patent Laid-open (Kokai) No. 9-70291).

[0115] Introduction of a target gene into a chromosome can be confirmed by Southern hybridization using a probe having a sequence complementary to the whole gene or a part thereof, PCR using primers prepared on the basis of the sequence of the gene, or the like.

[0116] Furthermore, the copy number of a gene can also be increased by introducing a vector containing the gene into a host. For example, the copy number of a target gene can be increased by ligating a DNA fragment containing the target gene with a vector that functions in a host to construct an expression vector of the gene, and transforming the host with the expression vector. The DNA fragment containing the target gene can be obtained by, for example, PCR using the genomic DNA of a microorganism having the target gene as the template. As the vector, a vector autonomously replicable in the cell of the host can be used. The vector is preferably a multi-copy vector. Furthermore, the vector preferably has a marker such as an antibiotic resistance gene for selection of transformant. Furthermore, the vector may have a promoter and/or terminator for expressing the introduced gene. The vector may be, for example, a vector derived from a bacterial plasmid, a vector derived from a yeast plasmid, a vector derived from a bacteriophage, cosmid, phagemid, or the like. Specific examples of vector autonomously replicable in coryneform bacteria include pHM1519 (Agric. Biol. Chem., 48, 2901-2903 (1984)); pAM330 (Agric. Biol. Chem., 48, 2901-2903 (1984)); plasmids obtained by improving these and having a drug resistance gene; plasmid pCRY30 described in Japanese Patent Laid-open (Kokai) No. 3-210184; plasmids pCRY21, pCRY2KE, pCRY2KX, pCRY31, pCRY3KE, and pCRY3KX described in Japanese Patent Laid-open (Kokai) No. 2-72876 and U.S. Patent No. 5,185,262; plasmids pCRY2 and pCRY3 described in Japanese Patent Laid-open (Kokai) No. 1-191686; pAJ655, pAJ611, and pAJ1844 described in Japanese Patent Laid-open (Kokai) No. 58-192900; pCG1 described in Japanese Patent Laid-open (Kokai) No. 57-134500; pCG2 described in Japanese Patent Laid-open (Kokai) No. 58-35197; pCG4 and pCG11 described in Japanese Patent Laid-open (Kokai) No. 57-183799; pVK7 described in Japanese Patent Laid-open (Kokai) No. 10-215883; and pVC7 described in Japanese Patent Laid-open (Kokai) No. 9-070291.

[0117] When a gene is introduced, it is sufficient that the gene is expressibly harbored by the bacterium of the present invention. Specifically, it is sufficient that the gene is introduced so that it is expressed under control by a promoter sequence that functions in the bacterium of the present invention. The promoter may be a promoter derived from the host, or a heterogenous promoter. The promoter may be the native promoter of the gene to be introduced, or a promoter of another gene. As the promoter, such a promoter as mentioned later which functions in a coryneform bacterium can be used.

[0118] A terminator for terminating the gene transcription can be provided downstream of the gene. The terminator is not particularly limited so long as it functions in the bacterium of the present invention. The terminator may be a terminator derived from the host, or may be a heterogenous terminator. The terminator may be the native terminator of the gene to be

introduced, or may be a terminator of another gene.

[0119] Vectors, promoters, and terminators available in various microorganisms are disclosed in detail in "Fundamental Microbiology Vol. 8, Genetic Engineering, KYORITSU SHUPPAN CO., LTD, 1987", and those can be used.

[0120] Furthermore, when two or more of genes are introduced, it is sufficient that the genes each are expressibly harbored by the bacterium of the present invention. For example, all the genes may be carried by a single expression vector or a chromosome. Furthermore, the genes may be separately carried by two or more expression vectors, or separately carried by a single or two or more expression vectors and a chromosome. An operon constituted by two or more genes may also be introduced.

[0121] The gene to be introduced is not particularly limited so long as it codes for a protein that functions in a host. The gene to be introduced may be a gene derived from the host, or may be a heterogenous gene. The gene to be introduced can be obtained by, for example, PCR using primers designed on the basis of the nucleotide sequence of the gene, and using the genomic DNA of an organism having the gene, a plasmid carrying the gene, or the like as the template. The gene to be introduced may also be totally synthesized, for example, on the basis of the nucleotide sequence of the gene (Gene, 60(1), 115-127 (1987)). The obtained gene can be used as it is, or after being modified as required.

[0122] Furthermore, the expression of a gene can be increased by improving the transcription efficiency of the gene. In addition, the expression of a gene can also be increased by improving the translation efficiency of the gene. The transcription efficiency of the gene and the translation efficiency of the gene can be improved by, for example, modifying an expression control sequence of the gene. The term "expression control sequence" collectively refers to sites that affect the expression of a gene. Examples of the expression control sequence include, for example, promoter, Shine-Dalgarno (SD) sequence (also referred to as ribosome binding site (RBS)), and spacer region between RBS and the start codon. Expression control sequences can be identified by using a promoter search vector or gene analysis software such as GENETYX. These expression control sequences can be modified by, for example, homologous recombination. Examples of methods for modification using homologous recombination include a method of using a temperature sensitive vector, or the Red driven integration method (WO2005/010175).

[0123] The transcription efficiency of a gene can be improved by, for example, replacing the promoter of the gene on a chromosome with a stronger promoter. The "stronger promoter" means a promoter providing an improved transcription of a gene compared with an inherently existing wild-type promoter of the gene. Examples of strong promoters usable in coryneform bacteria include the artificially modified P54-6 promoter (Appl. Microbiol. Biotechnol., 53, 674-679 (2000)), *pta, aceA, aceB, adh,* and *amyE* promoters inducible in coryneform bacteria with acetic acid, ethanol, pyruvic acid, or the like, *cspB, SOD,* and tuf (EF-Tu) promoters, which are strong promoters capable of providing a large expression amount in coryneform bacteria

(Journal of Biotechnology, 104 (2003) 311-323; Appl. Environ. Microbiol., 2005 Dec; 71 (12):8587-96), as well as *lac* promoter, *tac* promoter, and *trc* promoter. Furthermore, as the stronger promoter, a highly-active type of an existing promoter may also be obtained by using various reporter genes. For example, by making the -35 and -10 regions in a promoter region closer to the consensus sequence, the activity of the promoter can be enhanced (WO00/18935). Methods for evaluating the strength of promoters and examples of strong promoters are described in the paper of Goldstein et al. (Prokaryotic Promoters in Biotechnology, Biotechnol. Annu. Rev., 1, 105-128 (1995)), and so forth.

[0124] The translation efficiency of a gene can be improved by, for example, replacing the Shine-Dalgarno (SD) sequence (also referred to as ribosome binding site (RBS)) for the gene on a chromosome with a stronger SD sequence. The "stronger SD sequence" means a SD sequence that provides an improved translation of mRNA compared with the inherently existing wild-type SD sequence of the gene. Examples of stronger SD sequences include, for example, RBS of the gene 10 derived from phage T7 (Olins P.O. et al, Gene, 1988, 73, 227-235). Furthermore, it is known that substitution, insertion, or deletion of several nucleotides in a spacer region between RBS and the start codon, especially in a sequence immediately upstream of the start codon (5'-UTR), significantly affects the stability and translation efficiency of mRNA, and hence, the translation efficiency of a gene can also be improved by modifying them.

[0125] The translation efficiency of a gene can also be improved by, for example, modifying codons. For example, the translation efficiency of a gene can be improved by replacing a rare codon present in the gene with a synonymous codon more frequently used. That is, the gene to be introduced may be modified, for example, so as to contain optimal codons according to the frequencies of codons observed in a host to be used. Codons can be replaced by, for example, the site-specific mutation method for introducing an objective mutation into an objective site of DNA. Examples of the site-specific mutation method include the method utilizing PCR (Higuchi, R., 61, in PCR Technology, Erlich, H.A. Eds., Stockton Press (1989); Carter, P., Meth. in Enzymol., 154, 382 (1987)), and the method utilizing phage (Kramer, W. and Frits, H.J., Meth. in Enzymol., 154, 350 (1987); Kunkel, T.A. et al., Meth. in Enzymol., 154, 367 (1987)). Alternatively, a gene fragment in which objective codons are replaced may be totally synthesized. Frequencies of codons in various organisms are disclosed in the "Codon Usage Database" (http://www.kazusa.or.jp/codon; Nakamura, Y. et al, Nucl. Acids Res., 28, 292 (2000)).

[0126] Furthermore, the expression of a gene can also be increased by amplifying a regulator that increases the expression of the gene, or deleting or attenuating a regulator that reduces the expression of the gene.

[0127] Such methods for increasing the gene expression as mentioned above may be used independently or in an arbitrary combination.

[0128] The method for the transformation is not particularly limited, and conventionally known

methods can be used. There can be used, for example, a method of treating recipient cells with calcium chloride so as to increase the permeability thereof for DNA, which has been reported for the *Escherichia coli* K-12 strain (Mandel, M. and Higa, A., J. Mol. Biol., 1970, 53, 159-162), and a method of preparing competent cells from cells which are in the growth phase, followed by transformation with DNA, which has been reported for *Bacillus subtilis* (Duncan, C.H., Wilson, G.A. and Young, F.E., Gene, 1977, 1:153-167). Alternatively, there can also be used a method of making DNA-recipient cells into protoplasts or spheroplasts, which can easily take up recombinant DNA, followed by introducing a recombinant DNA into the DNA-recipient cells, which is known to be applicable to *Bacillus subtilis,* actinomycetes, and yeasts (Chang, S. and Choen, S.N., 1979, Mol. Gen. Genet., 168:111-115; Bibb, M.J., Ward, J.M. and Hopwood, O.A., 1978, Nature, 274:398-400; Hinnen, A., Hicks, J.B. and Fink, G.R., 1978, Proc. Natl. Acad. Sci. USA, 75:1929-1933). Transformation of coryneform bacteria can be carried out by, specifically, for example, the protoplast method (Gene, 39, 281-286(1985)), the electroporation method (Bio/Technology, 7, 1067-1070(1989)), the electric pulse method (Japanese Patent Laid-open (Kokai) No. 2-207791), or the like.

[0129] An increase in the expression of a gene can be confirmed by, for example, confirming an increase in the activity of the protein expressed from the gene. An increase in the activity of a protein can be confirmed by measuring the activity of the protein. For example, an increase in the activity of the Tat secretion system can be confirmed by confirming an increase in the secretory production amount of a protein fused with a Tat-dependent signal peptide at the N-terminus. In such a case, it is preferred that the secretory production amount of the protein fused with a Tat-dependent signal peptide at the N-terminus is increased to 1.5 times or more, 2 times or more, or 3 times or more, of that of a non-modified strain.

[0130] An increase in the expression of a gene can also be confirmed by, for example, confirming an increase in the transcription amount of the gene, or by confirming an increase in the amount of a protein expressed from the gene.

[0131] An increase of the transcription amount of a gene can be confirmed by comparing the amount of mRNA transcribed from the gene with that of a non-modified strain such as a wild-type strain or parent strain. Examples of the method for evaluating the amount of mRNA include Northern hybridization, RT-PCR, and so forth (Sambrook, J., et al., Molecular Cloning A Laboratory Manual/Third Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001). It is preferred that the amount of mRNA is increased to, for example, 1.5 times or more, 2 times or more, or 3 times or more, of that of a non-modified strain.

[0132] An increase in the amount of a protein can be confirmed by performing SDS-PAGE and confirming the intensity of the separated protein band. An increase in the amount of a protein can be confirmed by Western blotting using antibodies (Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001). It is preferred that the amount of the protein is increased to, for example, 1.5 times or more, 2 times or more, or 3 times or more, of that of a non-modified strain.

<1-1-5> Genetic construct for secretory expression of heterologous protein

[0133] It is known that a secretory protein is generally translated as a preprotein (also referred to as prepeptide) or a preproprotein (also referred to as prepropeptide), and then becomes a mature protein through processing. Specifically, a secretory protein is generally translated as a preprotein or preproprotein, then a signal peptide as the pre-moiety is cleaved with a protease (generally called signal peptidase), and the secretory protein is thereby converted into a mature protein or proprotein. As for the proprotein, the pro-moiety thereof is further cleaved by a protease, and the proprotein thereby becomes a mature protein. Therefore, a signal peptide is used for the secretory production of a heterologous protein in the method of the present invention as futher defined in the claims. In the present disclosure a preprotein and a preproprotein of a secretory protein may be collectively referred to as "secretory protein precursor". In the present invention, the "signal peptide" (also referred to as "signal sequence") refers to an amino acid sequence present at the N-terminus of a secretory protein precursor, and not usually present in the natural mature protein.

[0134] The genetic construct used for the present invention comprises, in the direction from 5' to 3', a promoter sequence that functions in a coryneform bacterium, a nucleic acid sequence encoding a signal peptide that functions in a coryneform bacterium, and a nucleic acid sequence encoding a heterologous protein. The nucleic acid sequence encoding the signal peptide may be ligated downstream from the promoter sequence so that the signal peptide is expressed under the control of the promoter. The nucleic acid sequence encoding the heterologous protein may be ligated downstream from the nucleic acid sequence encoding the signal peptide so that the heterologous protein is expressed as a fusion protein with the signal peptide. This fusion protein is also referred to as "fusion protein of the present invention". In the fusion protein of the present invention as further defined in the claims, the signal peptide and the heterologous protein may be or may not be adjacent to each other. That is, the expression "a heterologous protein is expressed as a fusion protein with a signal peptide" includes not only cases where a heterologous protein is expressed as a fusion protein with a signal peptide in which the signal peptide and the heterologous protein are adjacent to each other, but also include cases where a heterologous protein is expressed as a fusion protein in which the signal peptide and the heterologous protein are fused with each other via another amino acid sequence. For example, as described later, the fusion protein of the present disclosure can contain an insertion sequence, such as an amino acid sequence comprising Gln-Glu-Thr and an amino acid sequence used for enzymatic digestion, between the signal peptide and the heterologous protein. A nucleic acid sequence may also be read as "gene". For example, a nucleic acid sequence encoding a heterologous protein is also referred to as "gene encoding a heterologous protein" or "heterologous protein gene". Examples of the nucleic acid sequence include DNA. The genetic construct used for the present disclosure may also comprise a control sequence (operator, terminator, etc.) effective for expression of the fusion protein of the present invention in a coryneform bacterium at such an appropriate position that it can function.

[0135] The promoter used in the present invention is not particularly limited so long as a promoter that functions in a coryneform bacterium is chosen. The promoter may be a promoter derived from a coryneform bacterium, such as one derived from the host, or it may be a heterologous promoter. The promoter may be the native promoter of the heterologous protein, or a promoter of another gene. The "promoter that functions in a coryneform bacterium" refers to a promoter that possesses promoter activity in a coryneform bacterium.

[0136] Specific examples of the heterologous promoter include, for example, promoters derived from E. *coli* such as *tac* promoter, *lac* promoter, *trp* promoter, and *araBAD* promoter. Among these, strong promoters such as *tac* promoter and inducible promoters such as *araBAD* promoter are preferred.

[0137] Examples of the promoter derived from a coryneform bacterium include, for example, promoters of the genes of the cell surface layer proteins PS1, PS2 (also referred to as CspB), and SlpA (also referred to as CspA), and promoters of various amino acid biosynthesis system genes. Specific examples of the promoters of various amino acid biosynthesis system genes include, for example, promoters of the glutamate dehydrogenase gene of the glutamic acid biosynthesis system, the glutamine synthetase gene of the glutamine synthesis system, the aspartokinase gene of the lysine biosynthesis system, the homoserine dehydrogenase gene of the threonine biosynthesis system, the acetohydroxy acid synthetase gene of the isoleucine and valine biosynthesis system, 2-isopropylmalate synthetase gene of the leucine biosynthesis system, the glutamate kinase gene of the proline and arginine biosynthesis system, the phosphoribosyl-ATP pyrophosphorylase gene of the histidine biosynthesis system, the deoxyarabinoheptulonate phosphate (DAHP) synthetase gene of the aromatic amino acid biosynthesis systems such as those for tryptophan, tyrosine, and phenylalanine, the phosphoribosyl pyrophosphate (PRPP) amidotransferase gene of the nucleic acid biosynthesis systems such as those for inosinic acid and guanylic acid, the inosinic acid dehydrogenase gene, and the guanylic acid synthetase gene.

[0138] Examples of the promoter that functions in a coryneform bacterium include such strong promoters as described above usable in coryneform bacteria. As the promoter, a high activity type of an existing promoter may be obtained by using various reporter genes, and used. For example, by making the -35 and -10 regions in a promoter region closer to a consensus sequence, activity of the promoter can be enhanced (International Patent Publication WO00/18935). Examples of the method for evaluating strength of a promoter and strong promoters are described in the paper of Goldstein et al. (Prokaryotic promoters in biotechnology, Biotechnol. Annu. Rev., 1, 105-128 (1995)) and so forth. Furthermore, it is known that substitution, insertion, or deletion of several nucleotides in a spacer region between the ribosome-binding site (RBS) and the start codon, especially in a sequence immediately upstream of the start codon (5'-UTR), significantly affects stability and translation efficiency of mRNA, and these sequences can also be modified.

[0139] The signal peptide used in the present invention is not particularly limited so long as a signal peptide that functions in a coryneform bacterium is chosen. The signal peptide may be a

signal peptide derived from a coryneform bacterium, such as one derived from the host, or it may be a heterologous signal peptide. The signal peptide may be the native signal peptide of the heterologous protein, or a signal peptide of another gene. The "signal peptide that functions in a coryneform bacterium" refers to a peptide that when it is ligated to the N-terminus of an objective protein, allows the coryneform bacterium to secrete the protein. Whether a signal peptide functions in a coryneform bacterium can be confirmed by, for example, expressing an objective protein in a form of being fused with the signal peptide, and confirming whether the protein is secreted.

[0140] Examples of the signal peptide include Tat-dependent signal peptides and Sec-dependent signal peptides.

[0141] The term "Tat-dependent signal peptide" refers to a signal peptide recognized by the Tat system. The term "Tat-dependent signal peptide" may specifically refer to a signal peptide that, upon being linked at the N-terminus of an objective protein, results in secretion of the protein by the Tat secretion system.

[0142] Examples of the Tat-dependent signal peptide include the signal peptide of the TorA protein (trimethylamine-N-oxidoreductase) of *E. coli,* the signal peptide of SufI protein (suppressor of ftsI) of *E. coli,* the PhoD protein (phosphodiesterase) of *Bacillus subtilis,* the signal peptide of LipA protein (lipoic acid synthase) of *Bacillus subtilis,* and the signal peptide of IMD protein (isomaltodextranase) of *Arthrobacter globiformis.* The amino acid sequences of these signal peptides are as follows.

TorA signal peptide:
MNNNDLFQASRRRFLAQLGGLTVAGMLGPSLLTPRRATA (SEQ ID NO: 75)

SufI signal peptide: MSLSRRQFIQASGIALCAGAVPLKASA (SEQ ID NO: 76)

PhoD signal peptide:
MAYDSRFDEWVQKLKEESFQNNTFDRRKFIQGAGKIAGLSLGLTIAQS (SEQ ID NO: 77)

LipA signal peptide: MKFVKRRTTALVTTLMLSVTSLFALQPSAKAAEH (SEQ ID NO: 78)

IMD signal peptide: MMNLSRRTLLTTGSAATLAYALGMAGSAQA (SEQ ID NO: 79)

[0143] The Tat-dependent signal peptide has a twin-arginine motif. Examples of the twin-arginine motif include S/T-R-R-X-F-L-K (SEQ ID NO: 80) and R-R-X-#-# (#: hydrophobic residue) (SEQ ID NO: 81).

[0144] The term "Sec-dependent signal peptide" refers to a signal peptide recognized by the Sec system. The term "Sec-dependent signal peptide" may specifically refer to a signal peptide that, upon being linked at the N-terminus of an objective protein, results in secretion of the protein by the Sec secretion system.

[0145] Examples of the Sec-dependent signal peptide include a signal peptide of a cell surface layer protein of a coryneform bacterium. The cell surface layer protein of coryneform bacteria is as described above. Examples of the cell surface layer protein of coryneform bacteria include PS1 and PS2 (CspB) derived from C. *glutamicum* (Japanese Patent Laid-open (Kohyo) No. 6-502548), and SlpA (CspA) derived from C. *ammoniagenes (C. stationis)* (Japanese Patent Laid-open (Kokai) No. 10-108675). The amino acid sequence of the signal peptide of PS1 (PS1 signal peptide) of C. *glutamicum* is shown in SEQ ID NO: 82, the amino acid sequence of the signal peptide of PS2 (CspB) (PS2 signal peptide) of C. *glutamicum* is shown in SEQ ID NO: 83, and the amino acid sequence of the signal peptide of SlpA (CspA) (SlpA signal peptide)of C. stationis is shown in SEQ ID NO: 84. Moreover, U.S. Patent No. 4,965,197 describes that there are signal peptides for DNases derived from coryneform bacteria, and such signal peptides can also be used for the present invention.

[0146] The Tat-dependent signal peptide may be a variant of any of the Tat-dependent signal peptides exemplified above, so long as it contains a twin-arginine motif and the original function thereof is maintained. The Sec-dependent signal peptide may be a variant of any of the Sec-dependent signal peptides exemplified above, so long as the original function thereof is maintained. The above descriptions concerning conservative variants of the *phoS* gene and PhoS protein can be applied *mutatis mutandis* to variants of the signal peptide and the gene encoding it. For example, the signal peptide may be a peptide having any of the aforementioned amino acid sequences, but including substitution, deletion, insertion, or addition of one or several amino acid residues at one or several positions. The number meant by the term "one or several" used for a variant of the signal peptide is specifically, preferably 1 to 7, more preferably 1 to 5, still more preferably 1 to 3, particularly preferably 1 to 2. In the present invention, the terms "TorA signal peptide", "SufI signal peptide", "PhoD signal peptide", "LipA signal peptide", "IMD signal peptide", "PS1 signal peptide", "PS2 signal peptide", and "SlpA signal peptide" include not only the peptides of SEQ ID NOS: 75, 76, 77, 78, 79, 82, 83, and 84, respectively, but also includes conservative variants thereof.

[0147] The expression "original function is maintained" used for the Tat-dependent signal peptide means that the peptide is recognized by the Tat system, and specifically, may mean that the peptide has a function of, upon being linked at the N-terminus of an objective protein, resulting in secretion of the protein by the Tat secretion system. Whether a peptide function as the Tat-dependent signal peptide can be confirmed by, for example, confirming an increase in the secretory production amount of a protein linked with the peptide at the N-terminus due to enhancement of the Tat secretion system, or confirming a reduction in the secretory production amount of a protein linked with the peptide at the N-terminus due to deletion of the Tat secretion system.

[0148] The expression "original function is maintained" used for the Sec-dependent signal peptide means that the peptide is recognized by the Sec system, and specifically, may mean that the peptide has a function of, upon being linked at the N-terminus of an objective protein, resulting in secretion of the protein by the Sec secretion system. Whether a peptide function as

the Sec-dependent signal peptide can be confirmed by, for example, confirming an increase in the secretory production amount of a protein linked with the peptide at the N-terminus due to enhancement of the Sec secretion system, or confirming a reduction in the secretory production amount of a protein linked with the peptide at the N-terminus due to deletion of the Sec secretion system.

[0149] The signal sequence is generally cleaved by a signal peptidase, when the translation product is secreted out of the cell. As a gene encoding a signal peptide, although a naturally occurring gene may be used as it is, it may be modified so that it has the optimal codons according to codon frequencies in a host to be used.

[0150] In the genetic construct used for the present disclosure, a nucleic acid sequence encoding an amino acid sequence comprising Gln-Glu-Thr may be inserted between the nucleic acid sequence encoding the signal peptide and the nucleic acid sequence encoding the heterologous protein (WO2013/062029). The "amino acid sequence comprising Gln-Glu-Thr" is also referred to as "insertion sequence used for the present invention". Examples of the insertion sequence used for the present disclosure include amino acid sequences comprising Gln-Glu-Thr described in WO2013/062029. Particularly, the insertion sequence used for the present disclosure can be used preferably in combination with the Sec-dependent signal peptide.

[0151] The insertion sequence used for the present disclosure is preferably a sequence consisting of 3 or more amino acid residues from the N-terminus of the mature protein of the cell surface layer protein CspB of a coryneform bacterium (henceforth also referred to as "mature CspB" or "CspB mature protein"). The term "sequence consisting of 3 or more amino acid residues from the N-terminus" refers to an amino acid sequence starting from the amino acid residue at position 1 of the N-terminus to an amino acid residue at position 3 or a more remote position.

[0152] The cell surface layer protein CspB of coryneform bacteria is as described above. Specific examples of CspB include, for example, CspB of C. *glutamicum* ATCC 13869, CspB of 28 strains of C .*glutamicum* exemplified above, and variants thereof. In the amino acid sequence of the CspB protein of C. *glutamicum* ATCC 13869 shown in SEQ ID NO: 68, the amino acid residues at positions 1 to 30 correspond to the signal peptide, and the amino acid residues at positions 31 to 499 correspond to the CspB mature protein. The amino acid sequence of the CspB mature protein of C. *glutamicum* ATCC 13869 except for the 30 amino acid residues as the signal peptide moiety is shown in SEQ ID NO: 85. In the mature CspB of C. *glutamicum* ATCC 13869, the amino acid residues at positions 1 to 3 of the N-terminus correspond to Gln-Glu-Thr.

[0153] The insertion sequence used for the present disclosured is preferably an amino acid sequence starting from the amino acid residue at position 1 to an amino acid residue at any of the positions 3 to 50 of the mature CspB. The insertion sequence used for the present disclosure is more preferably an amino acid sequence starting from the amino acid residue at

position 1 to an amino acid residue at any of the positions 3 to 8, 17, and 50 of the mature CspB. The insertion sequence used for the present disclosure is particularly preferably an amino acid sequence starting from the amino acid residue at position 1 to an amino acid residue at any of the positions 4, 6, 17 and 50.

[0154] The insertion sequence used for the present disclosure is preferably an amino acid sequence selected from the group consisting of the following amino acid sequences (A) to (H):

(A) Gln-Glu-Thr

(B) Gln-Glu-Thr-Xaa1 (SEQ ID NO: 86)

(C) Gln-Glu-Thr-Xaa1-Xaa2 (SEQ ID NO: 87)

(D) Gln-Glu-Thr-Xaa1-Xaa2-Xaa3 (SEQ ID NO: 88)

(E) an amino acid sequence consisting of Gln-Glu-Thr fused with the amino acid residues at positions 4 to 7 of a mature CspB,

(F) an amino acid sequence consisting of Gln-Glu-Thr fused with the amino acid residues at positions 4 to 8 of a mature CspB,

(G) an amino acid sequence consisting of Gln-Glu-Thr fused with the amino acid residues at positions 4 to 17 of a mature CspB,

(H) an amino acid sequence consisting of Gln-Glu-Thr fused with the amino acid residues at positions 4 to 50 of a mature CspB.

[0155] In the amino acid sequences (A) to (H), Xaa1 is Asn, Gly, Thr, Pro, or Ala, Xaa2 is Pro, Thr, or Val, and Xaa3 is Thr or Tyr. As for the amino acid sequences (A) to (H), "Gln-Glu-Thr fused with the amino acid residues at positions 4 to X of a mature CspB" means that the amino acid residues at positions 4 to X of the N-terminus of a mature CspB is fused to Thr of Gln-Glu-Thr. The first to third amino acid residues of the N-terminus of a mature CspB are usually Gln-Glu-Thr, and in such a case, "an amino acid sequence consisting of Gln-Glu-Thr fused with the amino acid residues at positions 4 to X of a mature CspB" is synonymous with an amino acid sequence consisting of the amino acid residues at position 1 to X of the mature CspB.

[0156] Furthermore, specifically, the insertion sequence used for the present disclosure is preferably an amino acid sequence selected from the group consisting of Gln-Glu-Thr-Asn-Pro-Thr (SEQ ID NO: 89), Gln-Glu-Thr-Gly-Thr-Tyr (SEQ ID NO: 90), Gln-Glu-Thr-Thr-Val-Thr (SEQ ID NO: 91), Gln-Glu-Thr-Pro-Val-Thr (SEQ ID NO: 92), and Gln-Glu-Thr-Ala-Val-Thr (SEQ ID NO: 93).

[0157] In the present disclosure, the "amino acid residue at position X of the mature CspB" refers to an amino acid residue corresponding to the amino acid residue at position X in SEQ

ID NO: 85. Which amino acid residue is the "amino acid residue corresponding to the amino acid residue at position X in SEQ ID NO: 85" in the amino acid sequence of an arbitrary mature CspB can be determined by alignment between the amino acid sequence of the arbitrary mature CspB and the amino acid sequence of SEQ ID NO: 85.

[0158] Examples of the heterologous protein to be produced by secretory production according to the method of the present invention include, for example, physiologically active proteins, receptor proteins, antigenic proteins to be used as vaccines, and enzymes.

[0159] Examples of the enzymes include, for example, transglutaminase, protein glutaminase, isomaltodextranase, protease, endopeptidase, exopeptidase, aminopeptidase, carboxypeptidase, collagenase, chitinase, and so forth. Examples of transglutaminase include, for example, secretory-type transglutaminases of Actinomycetes such as *Streptoverticillium mobaraense* IFO 13819 (WO01/23591), *Streptoverticillium cinnamoneum* IFO 12852, *Streptoverticillium griseocarneum* IFO 12776, and *Streptomyces lydicus* (WO96/06931), and of filamentous fungi such as Oomycetes (WO96/22366). Examples of protein glutaminase include, for example, protein glutaminase of *Chryseobacterium proteolyticum* (WO2005/103278). Examples of isomaltodextranase include, for example, isomaltodextranase of *Arthrobacter globiformis* (WO2005/103278).

[0160] Examples of the physiologically active proteins include, for example, growth factors, hormones, cytokines, and antibody-related molecules.

[0161] Specific examples of the growth factors include, for example, epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), transforming growth factor (TGF), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), vesicular endothelial growth factor (VEGF), granulocyte-colony stimulating factor (G-CSF), granulocytemacrophage-colony stimulating factor (GM-CSF), plateletderived growth factor (PDGF), erythropoietin (EPO), thrombopoietin (TPO), acidic fibroblast growth factor (aFGF or FGF1), basic fibroblast growth factor (bFGF or FGF2), keratinocyte growth factor (KGF-1 or FGF7, and, KGF-2 or FGF10), and hepatocyte growth factor (HGF).

[0162] Specific examples of the hormones include, for example, insulin, glucagon, somatostatin, human growth hormone (hGH), parathyroid hormone (PTH), calcitonin, and exenatide.

[0163] Specific examples of the cytokines include, for example, interleukins, interferons, and tumor necrosis factors (TNFs).

[0164] The growth factors, hormones, and cytokines may not be strictly distinguished from one another. For example, a physiologically active protein may be a protein belonging to a single group selected from growth factors, hormones, and cytokines, or may be a protein belonging to a plurality of groups selected from those.

[0165] Furthermore, a physiologically active protein may be an intact protein, or may be a part of a protein. Examples of a part of a protein include, for example, a part having physiological activity. Specific examples of a part having physiological activity include, for example, Teriparatide, a physiologically active peptide consisting of the N-terminal 34 amino acid residues of parathyroid hormone (PTH) .

[0166] The term "antibody-related molecule" refers to a protein containing a molecular species consisting of a single domain or a combination of two or more domains selected from the domains constituting a complete antibody. Examples of the domains constituting a complete antibody include heavy chain domains VH, CH1, CH2, and CH3, and light chain domains VL and CL. The antibody-related molecule may be a monomeric protein, or may be a multimeric protein, so long as it contains the above-mentioned molecular species. When the antibody-related molecule is a multimeric protein, it may be a homo-multimer consisting of a single kind of subunit, or may be a hetero-multimer consisting of two or more kinds of subunits. Specific examples of the antibody-related molecules include, for example, complete antibody, Fab, F(ab'), $F(ab')_2$, Fc, dimer consisting of a heavy chain (H chain) and a light chain (L chain), Fc-fusion protein, heavy chain (H chain), light chain (L chain), light chain Fv (scFv), $sc(Fv)_2$, disulfidebonded Fv (sdFv), and diabody.

[0167] The receptor proteins are not particularly limited. A receptor protein may be, for example, a receptor protein for any of physiologically active proteins and other physiologically active substances. Examples of the other physiologically active substances include, for example, neurotransmitters such as dopamine. Furthermore, a receptor protein may be an orphan receptor of which the corresponding ligand is not known.

[0168] The antigen proteins to be used as vaccines are not particularly limited, so long as they are proteins that can induce an immune response. An antigen protein can be appropriately selected depending on the intended object of the immune response.

[0169] In addition, examples of other proteins include Liver-type fatty acid-binding protein (LFABP).

[0170] Genes encoding these proteins can be modified according to a host to be used and for obtaining a desired activity. For example, the genes encoding these proteins may each be modified so that the proteins include addition, deletion, substitution, or the like of one or several amino acid residues. The above descriptions concerning variants of the PhoS protein and *phoS* gene can be applied *mutatis mutandis* to the heterologous protein to be produced by secretory production by the method of the present invention as further defined in the claims and the gene encoding it. Furthermore, in the genes encoding these proteins, an arbitrary codon may be replaced with an equivalent codon thereof. For example, in the genes encoding these proteins, codons may be optimized as required according to codon frequencies observed in the host.

[0171] The genetic construct of the present disclosure may further comprise a nucleic acid

sequence encoding an amino acid sequence used for enzymatic digestion between the nucleic acid sequence encoding the amino acid sequence comprising Gln-Glu-Thr and the nucleic acid sequence encoding the heterologous protein. If the amino acid sequence used for enzymatic digestion is inserted in the fusion protein of the present disclosure, the expressed fusion protein can be enzymatically digested to obtain the objective heterologous protein.

[0172] The amino acid sequence used for enzymatic digestion is not particularly limited so long as it is a sequence that can be recognized and digested by an enzyme that hydrolyzes a peptide bond, and a usable sequence can be appropriately chosen according to the amino acid sequence of the objective heterologous protein. The nucleic acid sequence encoding the amino acid sequence used for enzymatic digestion may be designed on the basis of that amino acid sequence, and for example, optimal codons can be used according to codon frequencies observed in the host.

[0173] The amino acid sequence used for enzymatic digestion is preferably a recognition sequence of a protease showing high substrate specificity. Specific examples of such an amino acid sequence include, for example, a recognition sequence of factor Xa protease and a recognition sequence of proTEV protease. The factor Xa protease and the proTEV protease recognize the amino acid sequence of Ile-Glu-Gly-Arg (= IEGR, SEQ ID NO: 94) and the amino acid sequence of Glu-Asn-Leu-Tyr-Phe-Gln (= ENLYFQ, SEQ ID NO: 95) in a protein, respectively, to specifically digest the protein at the C-terminal side of each recognition sequence.

[0174] The N-terminal region of the heterologous protein eventually obtained by the method of the present invention as further defined in the claims may be the same as that of the natural protein, or may not be the same as that of the natural protein. For example, the N-terminal region of the eventually obtained heterologous protein may be that of the natural protein including addition or deletion of one or several amino acid residues. Although the number of the "one or several" amino acid residues may differ depending on the full length or structure of the objective heterologous protein, specifically, it is preferably 1 to 20, more preferably 1 to 10, still more preferably 1 to 5, particularly preferably 1 to 3.

[0175] Furthermore, the heterologous protein to be produced by secretory production may be a protein comprising a pro-structure moiety (proprotein). When the heterologous protein to be produced by secretory production is a proprotein, the heterologous protein to be eventually obtained may be the proprotein or may not be the proprotein. That is, the proprotein may be processed into the mature protein by cleavage of the pro-structure moiety. The cleavage can be attained with, for example, a protease. When a protease is used, generally, the proprotein is preferably cleaved at a position substantially the same as that of the natural protein, or more preferably at exactly the same position as that of the natural protein so that the same mature protein as the natural mature protein is obtained, in view of the activity of the eventually obtained protein. Therefore, generally, a specific protease that cleaves the proprotein at such a position that the same protein as the naturally occurring mature protein is generated is most preferred. However, the N-terminal region of the heterologous protein to be eventually

obtained may not be the same as that of the natural protein as described above. For example, depending on type, purpose of use etc. of the heterologous protein to be produced, a protein having an N-terminus longer or shorter by one to several amino acid residues compared with the natural protein may have more appropriate activity. Proteases usable in the present invention include, for example, commercially available proteases such as Dispase (produced by Boehringer Mannheim) as well as those obtainable from culture broth of a microorganism such as culture broth of actinomycetes. Such proteases may be used in an un-purified state, or may be used after purification to an appropriate purity as required. When the pro-structure moiety is cleaved to obtain a mature protein, the inserted amino acid sequence comprising Gln-Glu-Thr is removed together with the pro-structure moiety, and therefore the objective protein can be obtained without providing an amino acid sequence used for enzymatic digestion downstream from the amino acid sequence comprising Gln-Glu-Thr.

[0176] The method for introducing the genetic construct used for the present invention into the coryneform bacterium is not particularly limited. The term "introduction of the genetic construct used for the present invention" refers to making a host harbor the genetic construct. The term "introduction of the genetic construct used for the present invention" includes not only cases where the genetic construct that has been preliminarily constructed is collectively introduced into a host, but also includes cases where at least the heterologous protein gene is introduced into a host and the genetic construct is constructed in the host. In the bacterium of the present invention, the genetic construct used for the present invention may be present on a vector that autonomously replicates out of the chromosome such as a plasmid, or may be incorporated into the chromosome. The genetic construct used for the present invention can be introduced, for example, in the same manner as that for introduction of a gene in methods for increasing the expression of a gene described above. In addition, for constructing the bacterium of the present invention, introduction of the genetic structure used for the present invention, introduction of the mutant *phoS* gene, and other modifications can be performed in an arbitrary order.

[0177] The genetic construct used for the present invention can be introduced into a host by using, for example, a vector comprising the genetic construct. For example, the genetic construct used for the present invention can be introduced into a host by ligating the genetic construct with a vector to construct an expression vector of the genetic construct, and transforming the host with the expression vector. Also, when the vector contains a promoter that functions in a coryneform bacterium, an expression vector of the genetic construct used for the present invention can be constructed by ligating the nucleic acid sequence encoding the fusion protein of the present invention downstream from the promoter. The vector is not particularly limited so long as a vector autonomously replicable in a coryneform bacterium is chosen. The vector usable in a coryneform bacterium is as described above.

[0178] Furthermore, the genetic construct used for the present invention can be introduced into the chromosome of a host by using, for example, a transposon such as an artificial transposon. When a transposon is used, the genetic construct used for the present invention is introduced into the chromosome by homologous recombination or translocation ability of the

transposon itself. Furthermore, the genetic construct used for the present invention can also be introduced into the chromosome of a host by other introduction methods utilizing homologous recombination. Examples of the introduction methods utilizing homologous recombination include, for example, methods utilizing a linear DNA, a plasmid having a temperature sensitive replication origin, a plasmid capable of conjugative transfer, a suicide vector not having a replication origin that functions in a host, and so forth. In addition, at least the heterologous protein gene may be introduced into the chromosome so that the genetic construct used for the present invention is constituted on the chromosome. In this case, a part or all of the constituents contained in the genetic construct, other than the heterologous protein gene, may be inherently present on the chromosome of the host. Specifically, for example, by using a promoter sequence inherently present on the chromosome of the host and a nucleic acid sequence encoding a signal peptide inherently present on the chromosome of the host and ligated downstream from the promoter sequence as they are, and replacing only the gene ligated downstream from the nucleic acid sequence encoding the signal peptide with an objective heterologous protein gene, the genetic construct used for the present invention can be constituted on the chromosome, and the bacterium of the present invention can be thereby constructed. A part of the genetic construct used for the present invention, such as the heterologous protein gene, can be introduced into the chromosome in the same manner as that for introduction of the genetic construct used for the present invention into the chromosome.

[0179] The genetic construct used for the present invention or a constituent thereof, such as promoter sequence, nucleic acid sequence encoding a signal peptide, or nucleic acid sequence encoding a heterologous protein, can be obtained by, for example, cloning. Specifically, for example, the genetic construct used for the present invention can be obtained by obtaining an objective heterologous protein gene by cloning from an organism having the objective heterologous protein, and then subjecting the gene to modification such as introduction of the nucleic acid sequence encoding the signal peptide and introduction of the promoter sequence. Furthermore, the genetic construct used for the present invention or a constituent thereof can also be obtained by chemical synthesis. The obtained genetic construct used for the present invention or constituent thereof can be used as it is, or after being modified as required.

[0180] Furthermore, when two or more kinds of proteins are expressed, it is sufficient that the genetic constructs for secretory expression of the proteins are harbored by the bacterium of the present invention so that secretory expression of the objective heterologous proteins can be attained. Specifically, for example, all the genetic constructs for secretory expression of the proteins may be harbored on a single expression vector, or harbored on the chromosome. Alternatively, the genetic constructs for secretory expression of the proteins may be separately harbored on a plurality of expression vectors, or may be separately harbored on one or more expression vectors and the chromosome. The "case where two or more kinds of proteins are expressed" refers to, for example, a case where two or more kinds of heterologous proteins are produced by secretory production, or a case where a heteromultimeric protein is produced by secretory production.

[0181] The method for introducing the genetic construct used for the present invention into the coryneform bacterium is not particularly limited, and a generally used method, for example, the protoplast method (Gene, 39, 281-286 (1985)), the electroporation method (Bio/Technology, 7, 1067-1070 (1989)), the electric pulse method (Japanese Patent Laid-open (Kokai) No. 2-207791), and so forth can be used.

<1-2> Method for producing heterologous protein

[0182] By culturing the bacterium of the present invention obtained as described above to express a heterologous protein, a large amount of the heterologous protein secreted out of the cells is obtained.

[0183] The bacterium of the present invention can be cultured according to a usually used method and conditions. For example, the bacterium of the present invention can be cultured in a usual medium containing a carbon source, a nitrogen source, and inorganic ions. In order to obtain still higher proliferation, organic micronutrients such as vitamins and amino acids can also be added as required.

[0184] As the carbon source, carbohydrates such as glucose and sucrose, organic acids such as acetic acid, alcohols, and others can be used. As the nitrogen source, ammonia gas, aqueous ammonia, ammonium salts, and others can be used. As the inorganic ions, calcium ions, magnesium ions, phosphate ions, potassium ions, iron ions, and so forth are appropriately used as required. The culture is performed within appropriate ranges of pH 5.0 to 8.5 and 15 to 37°C under aerobic conditions for 1 to 7 days. Furthermore, the culture conditions for L-amino acid production by coryneform bacteria and other conditions described for the methods for producing a protein using a Sec- or Tat-dependent signal peptide can be used (refer to WO01/23591 and WO2005/103278). Furthermore, when an inducible promoter is used for expression of the heterologous protein, culture may also be performed with adding a promoterinducing agent to the medium. By culturing the bacterium of the present invention under such conditions, a large amount of the objective protein is produced in cells and efficiently secreted out of the cells. In addition, according to the method of the present invention as further defined in the claims, the produced heterologous protein is secreted out of the cells, and therefore a protein that is generally lethal if it is accumulated in a large amount in cells of microorganisms, such as transglutaminases, can also be continuously produced without lethal effect.

[0185] The protein secreted in the medium according to the method of the present invention as further defined in the claims can be separated and purified from the medium after the culture by a method well known to those skilled in the art. For example, after the cells are removed by centrifugation or the like, the protein can be separated and purified by a known appropriate method such as salting out, ethanol precipitation, ultrafiltration, gel filtration chromatography, ion exchange column chromatography, affinity chromatography, medium or

high pressure liquid chromatography, reverse phase chromatography, and hydrophobic chromatography, or a combination of these. Furthermore, in a certain case, culture or culture supernatant may be used as it is. The protein secreted in the cell surface layer according to the method of the present invention as further defined in the claims can also be separated and purified in the same manner as that for the case where the protein is secreted in the medium, after solubilizing it by a method well known to those skilled in the art such as elevation of salt concentration and use of a surfactant. Furthermore, in a certain case, the protein secreted in the cell surface layer may be used as, for example, an immobilized enzyme, without solubilizing it.

[0186] Secretory production of the objective heterologous protein can be confirmed by performing SDS-PAGE for the culture supernatant and/or a fraction containing the cell surface layer as a sample, and confirming the molecular weight of the separated protein band. Furthermore, secretory production of the objective heterologous protein can also be confirmed by performing Western blotting using antibodies for the culture supernatant and/or a fraction containing the cell surface layer as a sample (Molecular Cloning, Cold spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001). Furthermore, secretory production of the objective heterologous protein can also be confirmed by detecting an N-terminal amino acid sequence of the objective protein using a protein sequencer. Furthermore, secretory production of the objective heterologous protein can also be confirmed by determining the mass of the objective protein using a mass spectrometer. Furthermore, when the objective heterologous protein is an enzyme or a protein having a certain measurable physiological activity, secretory production of the objective heterologous protein can be confirmed by measuring enzymatic activity or the physiological activity of the objective protein in the culture supernatant and/or a fraction containing the cell surface layer as a sample.

<2> Coryneform bacterium harboring mutant *phoS* gene

[0187] The present invention as defined by the claims also provides a coryneform bacterium harboring a mutant *phoS* gene. This coryneform bacterium may or may not have an ability of secretory production of a heterologous protein. Hence, this coryneform bacterium may or may not have a genetic construct for secretory expression of a heterologous protein. The above descriptions concerning the "coryneform bacterium used for the method of the present invention" can be applied *mutatis mutandis* to this coryneform bacterium, provided that this coryneform bacterium may not have the genetic construct for secretory expression of a heterologous protein. This coryneform bacterium, for example, can be made harbor the genetic construct for secretory expression of a heterologous protein and can be used for secretory production of the heterologous protein. That is, the "coryneform bacterium used for the method of the present invention is defined by the claims".

Examples

[0188] The present invention will be further specifically explained with reference to the following examples.

Example 1: Obtainment of PhoS-mutant strains derived from C. *glutamicum* YDK010 strain

[0189] The C. *glutamicum* YDK010 strain disclosed in WO2002/081694 was transformed with pPKK50TEV-Teri disclosed in WO2014/126260, which is a secretory expression plasmid of a physiologically active peptide Teriparatide. Incidentally, pPKK50TEV-Teri is a secretory expression vector of a physiologically active peptide Teriparatide, and a plasmid having a promoter region of *cspB* gene of the C. *glutamicum* ATCC13869 strain and a nucleotide sequence expressively linked downstream from the promoter and encoding a fusion protein (hereinafter, referred to as CspB50TEV-Teri) of the CspB signal peptide of the same strain, the N-terminal 50 amino acid residues of mature CspB of the same strain, the ProTEV protease recognition sequence ENLYFQ, and Teriparatide (WO2014/126260). The C. *glutamicum* YDK010 strain is a cell-surface-layer-protein-CspB-deficient strain of the C. *glutamicum* AJ12036 strain (FERM BP-734). The obtained transformant was cultured on CMDex agar medium (5 g of glucose, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of $MnSO_4 \cdot 5H_2O$, 1 g of KH_2PO_4, 10 μg of biotin, 10 g of Difco™ Select Soytone (Becton Dickinson), 10 g of Bacto™ Yeast Extract (Becton Dickinson), 3 g of urea, 1.2 g (as total nitrogen) of soybean hydrolysate solution obtained with HCl, and 20 g of agar powder, filled up with water to 1 L, and adjusted to pH6.5) containing 25 mg/L of kanamycin at 30°C, to form colonies.

[0190] After the culture, a natural mutant strain of which the *phoS* gene was introduced with a mutation was selected, and designated as strain YDK0107. The nucleotide sequence of the mutant *phoS* gene of the YDK0107 strain and the amino acid sequence of the mutant PhoS protein of the YDK0107 strain are shown in SEQ ID NOS: 1 and 2, respectively. In the mutant *phoS* gene of the YDK0107 strain, "G" at position 906 of SEQ ID NO: 3 of the wild-type *phoS* gene of the YDK010 strain has been mutated to "T". Due to this mutation, in the mutant PhoS protein of the YDK0107 strain, the tryptophan residue at position 302 of SEQ ID NO: 4 of the wild-type PhoS protein of the YDK010 strain has been mutated to a cysteine residue. This mutation was designated as PhoS(W302C) mutation. Incidentally, genomic DNA was prepared with PurElute™ Genomic DNA Kit (EdgeBio), and nucleotide sequencing was carried out with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130 Genetic Analyzer (Applied Biosystems).

(2) Construction of phoS-gene-substitution vector encoding mutant PhoS(W302C)

[0191] PCR was carried out by using primers of SEQ ID NOS: 5 and 6, and genomic DNA of

the C. *glutamicum* YDK0107 strain prepared with PurElute™ Genomic DNA Kit (EdgeBio) as the template, to amplify a region of about 1.5 kbp containing a *phoS* gene encoding the mutant PhoS(W302C) (also referred to as mutant *phoS* gene or mutant *phoS(W302C)* gene). PCR was carried out with Pyrobest(R) DNA polymerase (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer.

[0192] Then, the amplified DNA fragment of about 1.5 kbp was subject to agarose gel electrophoresis, an objective band was cut out, and the DNA fragment was collected from the gel with Wizard(R) SV Gel and PCR Clean-Up System (Promega). The collected DNA fragment was inserted at *SmaI* site of pBS5T disclosed in WO2006/057450, and the resultant was introduced into competent cells of *E. coli* JM109 (Takara Bio). A Strain harboring a plasmid into which the DNA fragment containing the mutant *phoS* gene was cloned was obtained, the plasmid was collected from the strain, to obtain pBS5T-phoS(W302C), a plasmid into which the mutant *phoS* gene was cloned. As a result of nucleotide sequencing of the inserted fragment, it was confirmed that the expected gene was cloned. Nucleotide sequencing was carried out with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130 Genetic Analyzer (Applied Biosystems).

(3) Construction of PhoS(W302C)-mutant strain

[0193] The C. *glutamicum* YDK010 strain disclosed in WO2002/081694 was transformed with the plasmid pBS5T-phoS(W302C) constructed in Example 1(2). Strain selection from the obtained transformants was carried out according to the method disclosed in WO2006/057450, to obtain YDK010::phoS(W302C), which is a strain of which the wild-type *phoS* gene on the chromosome was replaced with the mutant *phoS* gene. Incidentally, even without using the genome DNA of the YDK0107 strain, the YDK010::phoS(W302C) strain can be reproductively constructed by using, for example, the mutant *phoS* gene obtained by genetic engineering.

(4) Construction of phoS-gene-deletion vector pBS5TΔphoS

[0194] PCR was carried out by using genomic DNA of the C. *glutamicum* ATCC13869 strain prepared with PurElute™ Genomic DNA Kit (EdgeBio) as the template, in combination with primers of SEQ ID NOS: 7 and 8 to amplify a 5'-side upstream region of the *phoS* gene of about 1 kbp, and in combination with primers of SEQ ID NOS: 9 and 10 to amplify a 3'-side downstream region of the *phoS* gene of about 1 kbp. PCR was carried out with Pyrobest(R) DNA polymerase (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer. The amplified DNA fragments of about 1 kbp each were subject to agarose gel electrophoresis, objective bands were cut out, and the DNA fragments were collected from the gel with Wizard(R) SV Gel and PCR Clean-Up System (Promega). The collected two DNA fragments were inserted at *Sma*I site of pBS5T disclosed in WO2006/057450 by infusion reaction, to obtain a *phoS*-gene-deletion vector pBS5TΔphoS.

The infusion reaction was carried out with In-Fusion(R) HD Cloning Kit (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer.

(5) Construction of PhoS-deletion strain of YDK010 strain The C. *glutamicum* YDK010 strain disclosed in

[0195] WO2002/081694 was transformed with the plasmid pBS5TΔphoS constructed in Example 1(4). Strain selection from the obtained transformants was carried out according to the method disclosed in WO2006/057450, to obtain YDK010::phoS(W302C), which is a strain deficient in the *phoS* gene.

Example 2: Secretory production of CspB50TEV-Teriparatide fusion protein using CspB signal sequence in PhoS(W302C)-mutant and PhoS-deletion strains

[0196] The C. *glutamicum* YDK010 strain disclosed in WO2002/081694, the YDK0107 strain obtained in Example 1(1), the YDK010::phoS(W302C) strain obtained in Example 1(3), and the YDK010ΔphoS strain obtained in Example 1(5) were each transformed with pPKK50TEV-Teri disclosed in WO2014/126260, which is a secretory expression plasmid of a physiologically active peptide Teriparatide, to obtain strains YDK010/pPKK50TEV-Teri, YDK0107/pPKK50TEV-Teri, YDK010::phoS(W302C)/pPKK50TEV-Teri, and YDK010ΔphoS/pPKK50TEV-Teri. The obtained transformants were each cultured on MMTG liquid medium (120 g of glucose, 3 g of $MgSO_4 \cdot 7H_2O$, 30 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 0.03 g of $FeSO_4 \cdot 7H_2O$, 0.03 g of $MnSO_4 \cdot 5H_2O$, 0.45 mg of thiamine hydrochloride, 0.45 mg of biotin, 0.15 g of DL-methionine, 0.2 g (as total nitrogen) of soybean hydrolysate solution obtained with HCl, and 50 g of $CaCO_3$, filled up with water to 1 L, and adjusted to pH7.0) containing 25 mg/L of kanamycin at 30°C for 72 hr. After completion of the culture, 5 μL of the culture supernatant obtained by centrifuging each culture broth was subjected to reduced SDS-PAGE, and then staining was carried out with SYPRO Orange (Life Technologies). As a result, the secretion amount of CspB50TEV-Teri was significantly improved in the YDK0107 and YDK010::phoS(W302C) strains, as compared with the YDK010 strain (Fig. 1). After the staining, the band intensity of CspB50TEV-Teri was digitized with image analysis software Multi Gauge (FUJIFILM), and the average value of the band intensity observed upon expressing CspB50TEV-Teri in each strain was calculated as a relative value based on the average value of the band intensity observed upon expressing CspB50TEV-Teri in the YDK010 strain which was taken as 1. As a result, it was confirmed that the secretion amount of CspB50TEV-Teri was improved to about 13.2-fold in the YDK0107 strain and about 12.5-fold in the YDK010::phoS(W302C) strain, as compared with the YDK010 strain (Table 1). By contrast, the secretion amount of CspB50TEV-Teri was reduced to about 0.2-fold in the YDK010ΔphoS strain, as compared with the YDK010 strain. From this, it was revealed that the PhoS(W302C) mutation is an effective mutation that leads to a significant improvement of the secretion amount in secretion of CspB50TEV-Teri. By contrast, deletion of the *phoS* gene showed no advantageous effect in secretion of CspB50TEV-Teri.

Table 1

Strain	Relative intensity
YDK010/pPKK50TEV-Teri	1.0
YDK0107/pPKK50TEV-Teri	13.2
YDK010::phoS(W302C)/pPKK50TEV-Teri	12.5
YDK010ΔphoS/pPKK50TEV-Teri	0.2

[0197] According to Appl. Environ. Microbiol., 94, 1131-1150(2012), the region of positions 266-330 of the PhoS protein of the C. *glutamicum* ATCC13032 strain is considered to be a HisKA domain, and the HisKA domain contains a histidine residue that is autophosphorylated. Because the tryptophan residue of position 302 is present in the HisKA domain, comparison of the amino acid sequences of HisKA domains of PhoS homologues of various *Corynebacterium* bacteria was carried out. Alignment of the amino acid sequences of HisKA domains of PhoS proteins of the C. *glutamicum* YDK0107 strain, the C. *glutamicum* YDK010 strain, and the C. *glutamicum* ATCC13869 strain predicted from the nucleotide sequences analyzed with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130 Genetic Analyzer (Applied Biosystems), and the amino acid sequences of HisKA domains of PhoS homologues of the C. *glutamicum* ATCC13032 strain (Genbank Accession No. NP_601807), the C. *glutamicum* ATCC14067 strain (Genbank Accession No. KEI24167), C. *callunae* (Genbank Accession No. WP_015652043), *C. crenatum* (Genbank Accession No. WP_031512002), and C. *efficiens* (Genbank Accession No. WP_006769148), which were obtained as homologues having a homology of 70% or more from database by BLAST search using the amino acid sequence of SEQ ID NO: 4 as the query sequence, is shown in Fig. 2, and the amino acid residue of position 302 was indicated with a frame. As a result, it was revealed that the tryptophan residue of position 302 is commonly conserved in *Corynebacterium* bacteria other than the YDK0107 strain. While the PhoS is known to be a sensor kinase of a two-component regulatory system, it has not been known the effect thereof on secretory production of a heterologous protein. Furthermore, it was difficult to predict that mutation at such a highly-conserved amino acid residue as W302 improves the secretion amount of a heterologous protein.

Example 3: Secretory production of Liver-type fatty acid-binding protein (LFABP) fused with mature CspB N-terminal amino acid residues using CspB signal sequence in PhoS(W302C)-mutant strain

(1) Construction of secretory expression plasmid of Liver-type Fatty acid-binding protein (LFABP) fused with N-terminal 6 amino acid residues of CspB mature protein

[0198] The amino acid sequence of Liver-type fatty acid-binding protein of human (hereinafter,

referred to as LFABP) has already been determined (Genbank Accession No. NP_001434). This amino acid sequence is shown as SEQ ID NO: 11. Considering the codon frequency of C. *glutamicum,* a nucleotide sequence encoding LFABP was designed. In addition, a fusion protein (hereinafter, referred to as CspB6Xa-LFABP) of the CspB signal peptide 30 amino acid residues of the C. *glutamicum* ATCC13869 strain, the N-terminal 6 amino acid residues of CspB mature protein of the same strain, the Factor Xa protease recognition sequence IEGR, and LFABP, and a nucleotide sequence encoding the fusion protein were designed. The designed nucleotide sequence encoding the fusion protein is shown as SEQ ID NO: 12, and the amino acid sequence of the fusion protein is shown as SEQ ID NO: 13.

[0199] Then, an expression cassette of CspB6Xa-LFABP, in which the promoter of *cspB* gene of the C. *glutamicum* ATCC13869 strain was linked upstream of the DNA of SEQ ID NO: 12, and *Kpn*I site was further added at both the 5'-side and 3'-side termini, was totally synthesized. The synthesized DNA fragment was treated with the restriction enzyme *Kpn*I and inserted at *Kpn*I site of pPK4 disclosed in Japanese Patent Laid-open (Kokai) No. 9-322774, to construct pPK4_CspB6Xa-LFABP, which is a secretory expression plasmid of CspB6Xa-LFABP. As a result of nucleotide sequencing of the inserted fragment, it was confirmed that the expected gene encoding CspB6Xa-LFABP was constructed. Nucleotide sequencing was carried out with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130 Genetic Analyzer (Applied Biosystems).

(2) Secretory expression Liver-type Fatty acid-binding protein (LFABP) fused with N-terminal 6 amino acid residues of CspB mature protein

[0200] The C. *glutamicum* YDK010 strain disclosed in WO2002/081694 and the YDK010::phoS(W302C) strain constructed in Example 1(3) were each transformed with pPK4_CspB6Xa-LFABP constructed in Example 3(1), which is a secretory expression plasmid of LFABP fused with the N-terminal 6 amino acid residues of mature CspB and the Factor Xa protease recognition sequence IEGR, to obtain strains YDK010/pPK4_CspB6Xa-LFABP and YDK010::phoS(W302C)/pPK4_CspB6Xa-LFABP.

[0201] The obtained transformants were each cultured on MMTG liquid medium (120 g of glucose, 3 g of $MgSO_4 \cdot 7H_2O$, 30 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 0.03 g of $FeSO_4 \cdot 7H_2O$, 0.03 g of $MnSO_4 \cdot 5H_2O$, 0.45 mg of thiamine hydrochloride, 0.45 mg of biotin, 0.15 g of DL-methionine, 0.2 g (as total nitrogen) of soybean hydrolysate solution obtained with HCl, and 50 g of $CaCO_3$, filled up with water to 1 L, and adjusted to pH7.0) containing 25 mg/L of kanamycin at 30°C for 72 hr. After completion of the culture, 6.5 μL of the culture supernatant obtained by centrifuging each culture broth was subjected to reduced SDS-PAGE, and then staining was carried out with Quick-CBB (Wako Pure Chemical Industries).

[0202] As a result, a band indicating a protein having the molecular weight of CspB6Xa-LFABP was densely detected in the YDK010::phoS(W302C) strain, as compared with the YDK010

strain, and hence, it was confirmed that the secretion amount of CspB6Xa-LFABP was significantly improved in the YDK010::phoS(W302C) strain (Fig. 3). After the staining, the band intensity of CspB6Xa-LFABP was digitized with image analysis software Multi Gauge (FUJIFILM), and the average value of the band intensity observed upon expressing CspB6Xa-LFABP in the YDK010::phoS(W302C) strain was calculated as a relative value based on the average value of the band intensity observed upon expressing CspB6Xa-LFABP in the YDK010 strain which was taken as 1. As a result, it was confirmed that the secretion amount of CspB6Xa-LFABP was improved to about 1.9-fold in the YDK010::phoS(W302C) strain, as compared with the YDK010 strain (Table 2). From this, it was revealed that the PhoS(W302C) mutation is an effective mutation that leads to a significant improvement of the secretion amount also in secretion of CspB6Xa-LFABP.

Table 2

Strain	Relative intensity
YDK010/pPK4_CspB6Xa-LFABP	1.0
YDK010::phoS(W302C)/pPK4_CspB6Xa-LFABP	1.9

Example 4: Secretory production of Exenatide precursor (ExCP) fused with mature CspB N-terminal amino acid residues using CspB signal sequence in PhoS(W302C)-mutant strain

(1) Construction of secretory expression plasmid of Exenatide precursor (ExCP) fused with N-terminal 6 amino acid residues of CspB mature protein

[0203] The amino acid sequence of a physiologically active peptide Exenatide has already been determined (Genbank Accession No. P26349). Because the activated Exenatide is a peptide of which the C-terminus was amidated, the amino acid sequence of an Exenatide precursor added with Cys-Pro at the C-terminus (hereinafter, referred to as ExCP) as a precursor of the amidated Exenatide is shown as SEQ ID NO: 14. Considering the codon frequency of C. *glutamicum,* a nucleotide sequence encoding ExCP was designed. In addition, a fusion protein (hereinafter, referred to as CspB6TEV-ExCP) of the CspB signal peptide 30 amino acid residues of the C. *glutamicum* ATCC13869 strain, the N-terminal 6 amino acid residues of CspB mature protein of the same strain, the ProTEV protease recognition sequence ENLYFQ, and ExCP, and a nucleotide sequence encoding the fusion protein were designed. The designed nucleotide sequence encoding the fusion protein is shown as SEQ ID NO: 15, and the amino acid sequence of the fusion protein is shown as SEQ ID NO: 16.

[0204] Then, an expression cassette of CspB6TEV-ExCP, in which the promoter of *cspB* gene of the C. *glutamicum* ATCC13869 strain was linked upstream of the DNA of SEQ ID NO: 15, and *Kpn*I site was further added at both the 5'-side and 3'-side termini, was totally synthesized. The synthesized DNA fragment was treated with the restriction enzyme *Kpn*I and inserted at

*Kpn*I site of pPK4 disclosed in Japanese Patent Laid-open (Kokai) No. 9-322774, to construct pPK4_CspB6TEV-ExCP, which is a secretory expression plasmid of CspB6TEV-ExCP. As a result of nucleotide sequencing of the inserted fragment, it was confirmed that the expected gene encoding CspB6TEV-ExCP was constructed. Nucleotide sequencing was carried out with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130 Genetic Analyzer (Applied Biosystems).

(2) Secretory expression Exenatide precursor (ExCP) fused with N-terminal 6 amino acid residues of CspB mature protein

[0205] The C. *glutamicum* YDK010 strain disclosed in WO2002/081694 and the YDK010::phoS(W302C) strain constructed in Example 1(3) were each transformed with pPK4_CspB6TEV-ExCP constructed in Example 4(1), which is a secretory expression plasmid of ExCP fused with the N-terminal 6 amino acid residues of mature CspB and the ProTEV protease recognition sequence, to obtain strains YDK010/pPK4_CspB6TEV-ExCP and YDK010::phoS(W302C)/pPK4_CspB6TEV-ExCP.

[0206] The obtained transformants were each cultured on MMTG liquid medium (120 g of glucose, 3 g of $MgSO_4 \cdot 7H_2O$, 30 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 0.03 g of $FeSO_4 \cdot 7H_2O$, 0.03 g of $MnSO_4 \cdot 5H_2O$, 0.45 mg of thiamine hydrochloride, 0.45 mg of biotin, 0.15 g of DL-methionine, 0.2 g (as total nitrogen) of soybean hydrolysate solution obtained with HCl, and 50 g of $CaCO_3$, filled up with water to 1 L, and adjusted to pH7.0) containing 25 mg/L of kanamycin at 30°C for 72 hr. After completion of the culture, 6.5 μL of the culture supernatant obtained by centrifuging each culture broth was subjected to reduced SDS-PAGE, and then staining was carried out with Quick-CBB (Wako Pure Chemical Industries).

[0207] As a result, a band indicating a protein having the molecular weight of CspB6TEV-ExCP was densely detected in the YDK010::phoS(W302C) strain while it was hardly detected in the YDK010 strain, and hence, it was confirmed that the secretion amount of CspB6TEV-ExCP was significantly improved in the YDK010::phoS(W302C) strain (Fig. 4). In addition, the secretion amount of CspB6TEV-ExCP observed for each of the strains was averaged and shown in Table 3 with scores ranging from "±" to "+++". From this, it was revealed that the PhoS(W302C) mutation is an effective mutation that leads to a significant improvement of the secretion amount also in secretion of CspB6TEV-ExCP.

Table 3

Strain	Secretion amount
YDK010/pPK4_CspB6TEV-ExCP	±
YDK010::phoS(W302C)/pPK4_CspB6TEV-ExCP	+++

[0208] From Examples 2-4, it was revealed that, in cases of secretory expression of a

heterologous protein using the Sec secretion system in the CspB fusion method as disclosed in WO2013/062029, which is a method for expressing a heterologous protein in a form of being fused with N-terminal amino acid residues of mature CspB, the secretory expression amount of an objective protein can be significantly improved by using the PhoS(W302C)-mutant strain regardless of the type of protein to be expressed, the number of N-terminal amino acid residues of mature CspB to be fused therewith, and the type of protease recognition sequence.

Example 5: Secretory production of protransglutaminase using TorA signal sequence in PhoS(W302C)-mutant strain

(1) Construction of co-expression plasmid of *tatABC* genes encoding Tat secretion system and gene encoding protransglutaminase added with TorA signal sequence

(a) Construction of pPK5, which is a vector corresponding to pPK4 vector of which NaeI recognition sequence was modified

[0209] In pPK4 disclosed in Japanese Patent Laid-open (Kokai) No. 9-322774, there is the recognition sequence of restriction enzyme NaeI at one site. For modifying this sequence, primers of SEQ ID NOS: 17 and 18, which contain a sequence gcaggc modified from the NaeI recognition sequence gccggc and adjacent sequence thereof in pPK4, were synthesized. Then, PCR was carried out by using primers of SEQ ID NOS: 17 and 18, and pPK4 as the template, to amplify a full length plasmid of about 5.6 kbp. PCR was carried out with Pyrobest(R) DNA polymerase (Takara Bio), and the reaction conditions consisted of 95°C for 5 min, and 12 cycles of (95°C for 30 sec. 55°C for 1 min, and 72°C for 12 min).

[0210] Then, the obtained PCR product was treated with restriction enzyme *Dpn*I, to digest the methylated template DNA. The obtained non-methylated plasmid after the *Dpn*I digestion was introduced into competent cells of *E. coli* JM109 (Takara Bio), to obtain the plasmid. As a result of nucleotide sequencing, it was confirmed that the expected plasmid in which the NaeI recognition sequence was modified. Nucleotide sequencing was carried out with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130 Genetic Analyzer (Applied Biosystems). The thus-obtained vector corresponding to the pPK4 vector of which the *Nae*I recognition sequence was modified was designated as pPK5.

(b) Construction of pPK5-tatABC, which is a vector corresponding to pPK5 vector carrying *tatABC* genes

[0211] Then, PCR was carried out by using primers of SEQ ID NOS: 19 and 20, and pVtatABC disclosed in WO2005/103278, which is an amplification plasmid of Tat secretion system, as the

template, to amplify a DNA fragment of about 3.7 kbp containing a sequence encoding *tatABC* genes. The primer of SEQ ID NO: 20 was designed to contain the recognition sequences of restriction enzymes *Kpn*I and *Apa*I. PCR was carried out with Pyrobest(R) DNA polymerase (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer. This DNA fragment was phosphorylated at the termini with BKL Kit (Takara Bio), treated with *Kpn*I, blunt-ended with BKL Kit (TakaraBio), and inserted into the pPK5 vector that was dephosphorylated at the termini with CIAP (Takara Bio), to construct pPK5-tatABC, which is a vector carrying the *tatABC* genes. Ligation reaction was carried out with DNA Ligation Kit Ver.2.1 (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer. As a result of nucleotide sequencing of the inserted fragment, it was confirmed that the expected gene was inserted. Nucleotide sequencing was carried out with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130 Genetic Analyzer (Applied Biosystems).

(c) Construction of pPK6, which is a vector corresponding to pPK5-tatABC vector of which *Kpn*I and *Xba*I recognition sequences in *tatABC* genes were modified

[0212] In the *tatABC* gene region in the pPK5-tatABC plasmid constructed in (b), there are the recognition sequences of restriction enzymes *Kpn*I and *Xba*I each at one site. For modifying these sequences, primers of SEQ ID NOS: 21 and 22, which contain a sequence ggaacc modified from the *Kpn*I recognition sequence ggtacc and adjacent sequence thereof in pPK5-tatABC, and primers of SEQ ID NOS: 23 and 24, which contain a sequence tgtaga modified from the *Xba*I recognition sequence tctaga and adjacent sequence thereof in pPK5-tatABC, were synthesized.

[0213] First, for modifying the *Kpn*I recognition sequence in the *tatABC* gene region, PCR was carried out by using primers of SEQ ID NOS: 21 and 22, and pPK5-tatABC as the template, to amplify a full length plasmid of about 9.4 kbp. PCR was carried out with Pyrobest(R) DNA polymerase (Takara Bio), and the reaction conditions consisted of 95°C for 5 min, and 12 cycles of (95°C for 30 sec. 55°C for 1 min, and 72°C for 12 min).

[0214] Then, the obtained PCR product was treated with restriction enzyme *Dpn*I, to digest the methylated template DNA. The obtained non-methylated plasmid after the *Dpn*I digestion was introduced into competent cells of *E. coli* JM109 (Takara Bio), to obtain the plasmid. Thus, pPK5-tatABCΔKpnI, which is a vector of which the *Kpn*I recognition sequences in the *tatABC* gene region was modified, was constructed.

[0215] Then, for modifying the *Xba*I recognition sequence in the *tatABC* gene region, PCR was carried out by using primers of SEQ ID NOS: 23 and 24, and pPK5-tatABCΔKpnI as the template, to amplify a full length plasmid of about 9.4 kbp. PCR was carried out with Pyrobest(R) DNA polymerase (Takara Bio), and the reaction conditions consisted of 95°C for 5 min, and 12 cycles of (95°C for 30 sec. 55°C for 1 min, and 72°C for 12 min).

[0216] Then, the obtained PCR product was treated with restriction enzyme *Dpn*I, to digest the methylated template DNA. The obtained non-methylated plasmid after the *Dpn*I digestion was introduced into competent cells of *E. coli* JM109 (Takara Bio), to obtain the plasmid. Thus, pPK5-tatABCΔKpnIΔXbaI, which is a vector of which the *Xba*I recognition sequences in the *tatABC* gene region was modified, was constructed. As a result of nucleotide sequencing, it was confirmed that the expected gene was constructed. Nucleotide sequencing was carried out with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130 Genetic Analyzer (Applied Biosystems).

[0217] The thus-obtained vector carrying the *tatABC* genes based on the pPK4 vector was designated as pPK6. The construction scheme of pPK6 from pPK4 is shown in Fig. 5. For amplifying the TatABC secretion system in secretory expression of a protein using the Tat system, two plasmids, i.e. a secretory expression plasmid of an objective protein, and pVtatABC, which is an amplification plasmid of the Tat secretion system, were necessarily used in the method of WO2005/103278. By contrast, use of the pPK6 vector enabled the expression of an objective protein and amplification of the TatABC secretion system in one plasmid.

(d) Construction of pPK6-TorAss, which is a vector corresponding to pPK6 vector carrying cspB promoter and DNA encoding TorA signal sequence

[0218] PCR was carried out by using primers of SEQ ID NOS: 25 and 26, and pPTGFP disclosed in Appl. Environ. Microbiol., 72, 7183-7192(2006) as the template, to amplify a DNA fragment of about 0.7 kbp containing a promoter region of *cspB* gene of the *C. glutamicum* ATCC13869 strain and a nucleotide sequence encoding the TorA signal sequence of *E. coli.* The primer of SEQ ID NO: 26 was designed to contain the recognition sequences of restriction enzymes *Apa*I and NaeI. PCR was carried out with Pyrobest(R) DNA polymerase (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer. This DNA fragment was treated with *Kpn*I and *Apa*I, and inserted into the *Kpn*I-*Apa*I site of the pPK6 vector constructed in (c), to construct pPK6-TorAss, which is a vector carrying the promoter region of *cspB* gene of the C. *glutamicum* ATCC13869 strain and the nucleotide sequence encoding the TorA signal sequence of *E. coli.* Ligation reaction was carried out with DNA Ligation Kit Ver.2.1 (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer. As a result of nucleotide sequencing of the inserted fragment, it was confirmed that the expected gene was inserted. Nucleotide sequencing was carried out with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130 Genetic Analyzer (Applied Biosystems).

(e) Construction of secretory expression vector of protransglutaminase using pPK6-TorAss vector

[0219] PCR was carried out by using primers of SEQ ID NOS: 27 and 28, and pPKSPTG1

disclosed in WO2001/23591, which is a secretory expression vector of protransglutaminase, as the template, to amplify a DNA fragment of about 1.1 kbp encoding protransglutaminase. The primer of SEQ ID NO: 28 was designed to contain the recognition sequence of restriction enzyme XbaI. PCR was carried out with Pyrobest(R) DNA polymerase (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer. This DNA fragment was treated with *XbaI,* and inserted into the *Nae*I-*Xba*I site of the pPK6-TorAss vector constructed in (d), to construct pPK6_T_PTG, which is a co-expression vector of the TatABC secretion system and protransglutaminase added with the TorA signal sequence. Ligation reaction was carried out with DNA Ligation Kit Ver.2.1 (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer. As a result of nucleotide sequencing of the inserted fragment, it was confirmed that the expected gene was inserted. Nucleotide sequencing was carried out with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130 Genetic Analyzer (Applied Biosystems).

(2) Secretory expression of protransglutaminase using TorA signal sequence in the YDK010 and YDK010::phoS(W302C) strains

[0220] The C. *glutamicum* YDK010 strain disclosed in WO2002/081694 and the YDK010::phoS(W302C) strain obtained in Example 1(3) were each transformed with pPK6_T_PTG obtained in Example 5(1)(e), which is a secretory expression plasmid of protransglutaminase, to obtain strains YDK010/pPK6_T_PTG and YDK010::phoS(W302C)/pPK6_T_PTG. The obtained transformants were each cultured on MMTG liquid medium (120 g of glucose, 3 g of $MgSO_4 \cdot 7H_2O$, 30 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 0.03 g of $FeSO_4 \cdot 7H_2O$, 0.03 g of $MnSO_4 \cdot 5H_2O$, 0.45 mg of thiamine hydrochloride, 0.45 mg of biotin, 0.15 g of DL-methionine, 0.2 g (as total nitrogen) of soybean hydrolysate solution obtained with HCl, and 50 g of $CaCO_3$, filled up with water to 1 L, and adjusted to pH7.0) containing 25 mg/L of kanamycin at 30°C for 72 hr. After completion of the culture, 5 μL of the culture supernatant obtained by centrifuging each culture broth was subjected to reduced SDS-PAGE, and then staining was carried out with Quick-CBB (Wako Pure Chemical Industries). As a result, the secretion amount of protransglutaminase was significantly improved in the YDK010::phoS(W302C) strain, as compared with the YDK010 strain (Fig. 6). After the staining, the band intensity of protransglutaminase was digitized with image analysis software Multi Gauge (FUJIFILM), and the average value of the band intensity observed upon expressing protransglutaminase in the YDK010::phoS(W302C) strain was calculated as a relative value based on the average value of the band intensity observed upon expressing protransglutaminase in the YDK010 strain which was taken as 1. As a result, it was confirmed that the secretion amount of protransglutaminase was improved to about 7.2-fold in the YDK010::phoS(W302C) strain, as compared with the YDK010 strain (Table 4). From this, it was revealed that the PhoS(W302C) mutation is an effective mutation that leads to a significant improvement of the secretion amount also in secretion of protransglutaminase using the TorA signal sequence.

Table 4

Strain	Relative intensity
YDK010/pPK6_T_PTG	1.0
YDK010::phoS(W302C)/pPK6_T_PTG	7.2

Example 6: Secretory production of protein glutaminase comprising pro-structure moiety using TorA signal sequence in PhoS(W302C)-mutant strain

(1) Construction of co-expression plasmid of *tatABC* genes encoding Tat secretion system and gene expressing protein glutaminase comprising pro-structure moiety added with TorA signal sequence

[0221] PCR was carried out by using primers of SEQ ID NOS: 29 and 30, and pPKT-PPG disclosed in WO2005/103278, which is an expression plasmid of protein glutaminase comprising a pro-structure moiety, as the template, to amplify a promoter region of *cspB* gene of the *C. glutamicum* ATCC13869 strain, and a nucleotide sequence expressively linked downstream from the promoter and encoding a fusion protein of the TorA signal sequence of *E. coli* and protein glutaminase comprising a pro-structure moiety of Chryseobacterium proteolyticum. The primers of SEQ ID NO: 29 and 30 were each designed to contain the recognition sequence of restriction enzyme *Xbal.* PCR was carried out with Pyrobest(R) DNA polymerase (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer. This DNA fragment was treated with *Xbal,* and inserted into the *Xba*I site of the pPK6 vector constructed in Example 5(1), to construct pPK6_T_PPG, which is a co-expression vector of the TatABC secretion system and protein glutaminase comprising a pro-structure moiety added with the TorA signal sequence. Ligation reaction was carried out with DNA Ligation Kit <Mighty Mix> (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer. As a result of nucleotide sequencing of the inserted fragment, it was confirmed that the expected gene was inserted. Nucleotide sequencing was carried out with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130 Genetic Analyzer (Applied Biosystems).

(2) Secretory expression of protein glutaminase comprising pro-structure moiety using TorA signal sequence in the YDK010 and YDK010::phoS(W302C) strains

[0222] The *C. glutamicum* YDK010 strain disclosed in WO2002/081694 and the YDK010::phoS(W302C) strain obtained in Example 1(3) were each transformed with pPK6_T_PPG obtained in Example 6(1), which is a secretory expression plasmid of protein glutaminase comprising a pro-structure moiety, to obtain strains YDK010/pPK6_T_PPG and YDK010::PhoS(W302C)/pPK6_T_PPG. The obtained transformants were each cultured on

MMTG liquid medium (120 g of glucose, 3 g of $MgSO_4 \cdot 7H_2O$, 30 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 0.03 g of $FeSO_4 \cdot 7H_2O$, 0.03 g of $MnSO_4 \cdot 5H_2O$, 0.45 mg of thiamine hydrochloride, 0.45 mg of biotin, 0.15 g of DL-methionine, 0.2 g (as total nitrogen) of soybean hydrolysate solution obtained with HCl, and 50 g of $CaCO_3$, filled up with water to 1 L, and adjusted to pH7.0) containing 25 mg/L of kanamycin at 30°C for 72 hr. After completion of the culture, 5 µL of the culture supernatant obtained by centrifuging each culture broth was subjected to reduced SDS-PAGE, and then staining was carried out with Quick-CBB (Wako Pure Chemical Industries). As a result, the secretion amount of protein glutaminase comprising a pro-structure moiety was significantly improved in the YDK010::phoS(W302C) strain, as compared with the YDK010 strain (Fig. 7). After the staining, the band intensity of protein glutaminase comprising a pro-structure moiety was digitized with image analysis software Multi Gauge (FUJIFILM), and the average value of the band intensity observed upon expressing protein glutaminase comprising a pro-structure moiety in the YDK010::phoS(W302C) strain was calculated as a relative value based on the average value of the band intensity observed upon expressing protein glutaminase comprising a pro-structure moiety in the YDK010 strain which was taken as 1. As a result, it was confirmed that the secretion amount of protein glutaminase comprising a pro-structure moiety was improved to about 8.3-fold in the YDK010::phoS(W302C) strain, as compared with the YDK010 strain (Table 5). From this, it was revealed that the PhoS(W302C) mutation is an effective mutation that leads to a significant improvement of the secretion amount also in secretion of protein glutaminase comprising a pro-structure moiety using the TorA signal sequence.

Table 5

Strain	Relative intensity
YDK010/pPK6_T_PPG	1.0
YDK010::phoS(W302C)/pPK6_T_PPG	8.3

Example 7: Secretory production of isomaltodextranase using IMD signal sequence in PhoS(W302C)-mutant strain

(1) Construction of co-expression plasmid of *tatABC* genes encoding Tat secretion system and gene encoding isomaltodextranase

[0223] PCR was carried out by using primers of SEQ ID NOS: 29 and 31, and pPKI-IMD disclosed in WO2005/103278, which is an expression plasmid of isomaltodextranase, as the template, to amplify a promoter region of *cspB* gene of the *C. glutamicum* ATCC13869 strain, and an IMD gene sequence of *Arthrobacter globiformis* (including a coding region of the IMD signal sequence) expressively linked downstream from the promoter. The primer of SEQ ID NO: 29 and 31 were each designed to contain the recognition sequence of restriction enzyme *Xbal.* PCR was carried out with PrimeSTAR(R) GXL DNA Polymerase (Takara Bio), and the

reaction conditions were according to the protocol recommended by the manufacturer. This DNA fragment was treated with *Xba*I, and inserted into the *Xba*I site of the pPK6 vector constructed in Example 5(1), to construct pPK6-I-IMD, which is a co-expression vector of the TatABC secretion system and isomaltodextranase including the IMD signal sequence. Ligation reaction was carried out with DNA Ligation Kit <Mighty Mix> (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer. As a result of nucleotide sequencing of the inserted fragment, it was confirmed that the expected gene was inserted. Nucleotide sequencing was carried out with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130 Genetic Analyzer (Applied Biosystems).

(2) Secretory expression of isomaltodextranase using IMD signal sequence in the YDK010 and YDK010::phoS(W302C) strains

[0224] The C. *glutamicum* YDK010 strain disclosed in WO2002/081694 and the YDK010::phoS(W302C) strain obtained in Example 1(3) were each transformed with pPK6_I_IMD obtained in Example 7(1), which is an expression plasmid of isomaltodextranase, to obtain strains YDK010/pPK6_I_IMD and YDK010::phoS(W302C)/pPK6_I_IMD. The obtained transformants were each cultured on MMTG liquid medium (120 g of glucose, 3 g of $MgSO_4 \cdot 7H_2O$, 30 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 0.03 g of $FeSO_4 \cdot 7H_2O$, 0.03 g of $MnSO_4 \cdot 5H_2O$, 0.45 mg of thiamine hydrochloride, 0.45 mg of biotin, 0.15 g of DL-methionine, 0.2 g (as total nitrogen) of soybean hydrolysate solution obtained with HCl, and 50 g of $CaCO_3$, filled up with water to 1 L, and adjusted to pH7.0) containing 25 mg/L of kanamycin at 30°C for 72 hr. After completion of the culture, 5 μL of the culture supernatant obtained by centrifuging each culture broth was subjected to reduced SDS-PAGE, and then staining was carried out with Quick-CBB (Wako Pure Chemical Industries). As a result, the secretion amount of isomaltodextranase was significantly improved in the YDK010::phoS(W302C) strain, as compared with the YDK010 strain (Fig. 8). After the staining, the band intensity of isomaltodextranase was digitized with image analysis software Multi Gauge (FUJIFILM), and the average value of the band intensity observed upon expressing isomaltodextranase in the YDK010::phoS(W302C) strain was calculated as a relative value based on the average value of the band intensity observed upon expressing isomaltodextranase in the YDK010 strain which was taken as 1. As a result, it was confirmed that the secretion amount of isomaltodextranase was improved to about 6.6-fold in the YDK010::phoS(W302C) strain, as compared with the YDK010 strain (Table 6). From this, it was revealed that the PhoS(W302C) mutation is an effective mutation that leads to a significant improvement of the secretion amount also in secretion of isomaltodextranase using the IMD signal sequence of *A. globiformis.*

Table 6

Strain	Relative intensity
YDK010/pPK6_I_IMD	1.0
YDK010::phoS(W302C)/pPK6_I_IMD	6.6

[0225] From Examples 5-7, it was revealed that, in cases of secretory expression of a heterologous protein using the Tat secretion system as disclosed in WO2005/103278, the secretory expression amount of an objective protein can be significantly improved by using the PhoS(W302C)-mutant strain regardless of the type of protein to be expressed and the type of signal sequence to be used.

[0226] Hence, from Examples 2-7, it was revealed that the secretion amount of an objective protein can be significantly improved by using the PhoS(W302C)-mutant strain regardless of the difference in the type of secretion pathway to be used, the type of signal sequence to be used, and the type of protein to be expressed.

Example 8: Functional complementation of PhoS-deletion strain through plasmid amplification of *phoS* gene

(1) Construction of amplification plasmids of wild-type *phoS* gene and mutant *phoS(W302C)* gene

(a) Construction of amplification plasmid of wild-type *phoS* gene

[0227] PCR was carried out by using primers of SEQ ID NOS: 32 and 33, and genomic DNA of the *C. glutamicum* YDK010 strain prepared with PurElute™ Genomic DNA Kit (EdgeBio) as the template, to amplify a region of about 1.5 kbp containing the wild-type *phoS* gene. PCR was carried out with Pyrobest(R) DNA polymerase (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer.

[0228] Then, the amplified DNA fragment of about 1.5 kbp was subject to agarose gel electrophoresis, an objective band was cut out, and the DNA fragment was collected from the gel with Wizard(R) SV Gel and PCR Clean-Up System (Promega). The collected DNA fragment was inserted at *Sma*I site of the pVC7 vector disclosed in Japanese Patent Laid-open (Kokai) No. 9-070291 by infusion reaction, which is a shuttle vector having a chloramphenicol resistance gene and capable of replicating in both *E. coli* and coryneform bacteria, to obtain pVphoS(WT), which is an amplification plasmid of the wild-type *phoS* gene. The infusion reaction was carried out with In-Fusion(R) HD Cloning Kit (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer. As a result of nucleotide sequencing of the inserted fragment, it was confirmed that the expected gene was inserted. Nucleotide sequencing was carried out with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130 Genetic Analyzer (Applied Biosystems).

(b) Construction of amplification plasmid of mutant *phoS(W302C)* gene

[0229] Similarly, PCR was carried out by using primers of SEQ ID NOS: 32 and 33, and genomic DNA of the *C. glutamicum* YDK010::phoS(W302C) strain prepared with PurElute™ Genomic DNA Kit (EdgeBio) as the template, to amplify a region of about 1.5 kbp containing the mutant *phoS(W302C)* gene. PCR was carried out with Pyrobest(R) DNA polymerase (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer.

[0230] Then, the amplified DNA fragment of about 1.5 kbp was subject to agarose gel electrophoresis, an objective band was cut out, and the DNA fragment was collected from the gel with Wizard(R) SV Gel and PCR Clean-Up System (Promega). The collected DNA fragment was inserted at *Sma*I site of the pVC7 vector disclosed in Japanese Patent Laid-open (Kokai) No. 9-070291 by infusion reaction, to obtain pVphoS(W302C), which is an amplification plasmid of the mutant *phoS(W302C)* gene. The infusion reaction was carried out with InFusion(R) HD Cloning Kit (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer. As a result of nucleotide sequencing of the inserted fragment, it was confirmed that the expected gene was inserted. Nucleotide sequencing was carried out with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130 Genetic Analyzer (Applied Biosystems).

(2) Effect of amplification of wild-type *phoS* gene or mutant *phoS(W302C)* gene on secretory expression of CspB50TEV-Teri using PhoS-deletion strain

[0231] The YDK010ΔphoS strain constructed in Example 1(5) was transformed with pPKK50TEV-Teri disclosed in WO2014/126260 in combination with pVphoS(WT) or pVphoS(W302C) constructed in Example 8(1). In addition, as a control, the YDK010ΔphoS strain constructed in Example 1(5) was transformed with the pPK4 vector disclosed in Japanese Patent Laid-open (Kokai) No. 9-322774 in combination with pVphoS(WT) or pVphoS(W302C) constructed in Example 8(1). The obtained transformants were each cultured on MMTG liquid medium (120 g of glucose, 3 g of $MgSO_4 \cdot 7H_2O$, 30 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 0.03 g of $FeSO_4 \cdot 7H_2O$, 0.03 g of $MnSO_4 \cdot 5H_2O$, 0.45 mg of thiamine hydrochloride, 0.45 mg of biotin, 0.15 g of DL-methionine, 0.2 g (as total nitrogen) of soybean hydrolysate solution obtained with HCl, and 50 g of $CaCO_3$, filled up with water to 1 L, and adjusted to pH7.0) containing 6 mg/L of chloramphenicol and 25 mg/L of kanamycin at 30°C for 72 hr. After completion of the culture, 5 μL of the culture supernatant obtained by centrifuging each culture broth was subjected to reduced SDS-PAGE, and then staining was carried out with SYPRO Orange (Life Technologies), to compare the secretion amount of CspB50TEV-Teri (Fig. 9). In addition, the secretion amount of CspB50TEV-Teri observed for a part of the strains was shown in Table 7 with scores ranging from "+" to "+++". As a result, when introducing pVphoS(WT) into the YDK010ΔphoS strain, a secretion amount of CspB50TEV-Teri equivalent

to that observed for the YDK010 strain was obtained, and when introducing pVphoS(W302C) into the YDK010ΔphoS strain, a secretion amount of CspB50TEV-Teri equivalent to that observed for the YDK010::phoS(W302C) strain was obtained. From this, it was revealed that deletion of the *phoS* gene on the chromosome can be functionally complemented by amplifying any of wild-type and mutant *phoS* genes on a plasmid. Therefore, it was revealed that an effect equivalent to that obtained by mutating the *phoS* gene on the chromosome can be obtained by utilizing an expression plasmid of a mutant *phoS* gene.

Table 7

Strain	Secretion amount
YDK010/pPKK50TEV-Teri	+
YDK010::phoS(W302C)/pPKK50TEV-Teri	++ +
YDK010ΔphoS/pVphoS(WT)/pPKK50TEV-Teri	+
YDK010ΔphoS/pVphoS(W302C)/pPKK50TEV-Teri	+++

Example 9: Secretory expression of heterologous protein using stains expressing mutant PhoS(W302X) in which tryptophan residue at position 302 of wild-type PhoS protein was modified to an arbitrary amino acid residue

(1) Construction of expression plasmids of mutant *phoS* genes encoding various mutant PhoS(W302X) proteins

[0232] Plasmids pVphoS(W302X) each for expression of mutant PhoS(W302X) in which the tryptophan residue at position 302 of the wild-type PhoS protein (W302) was modified to another amino acid residue were constructed. The "X" represents an arbitrary amino acid residue.

[0233] For constructing pVphoS(W302S), which is an expression plasmid of the mutant PhoS(W302S) protein, PCR was carried out by using the pVphoS(WT) plasmid constructed in Example 8(1) as the template, in combination with primers of SEQ ID NOS: 32 and 34 to amplify a region of about 0.9 kbp containing an N-terminal side of the *phoS* gene, and in combination with primers of SEQ ID NOS: 33 and 35 to amplify a region of about 0.6 kbp containing a C-terminal side of the *phoS* gene. The primers of SEQ ID NOS: 34 and 35 were designed so as to replace the codon (tgg) encoding the tryptophan residue at position 302 of the wild-type PhoS protein with a codon (tcc) encoding a serine residue. PCR was carried out with Pyrobest(R) DNA polymerase (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer. The amplified DNA fragments were subject to agarose gel electrophoresis, objective bands were cut out, and the DNA fragments were collected from the gel with Wizard(R) SV Gel and PCR Clean-Up System (Promega). The collected two DNA fragments were inserted at *Sma*I site of the pVC7 vector disclosed in

Japanese Patent Laid-open (Kokai) No. 9-070291 by infusion reaction, to obtain pVphoS(W302C), which is an amplification plasmid of the mutant *phoS(W302S)* gene. The infusion reaction was carried out with In-Fusion(R) HD Cloning Kit (Takara Bio), and the reaction conditions were according to the protocol recommended by the manufacturer. As a result of nucleotide sequencing of the inserted fragment, it was confirmed that the expected gene was constructed. Nucleotide sequencing was carried out with BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3130 Genetic Analyzer (Applied Biosystems).

[0234] In the same manner, a plasmid pVphoS(W302A) for expression of the mutant PhoS(W302A) protein, a plasmid pVphoS(W302V) for expression of the mutant PhoS(W302V) protein, a plasmid pVphoS(W302M) for expression of the mutant PhoS(W302M) protein, a plasmid pVphoS(W302F) for expression of the mutant PhoS(W302F) protein, a plasmid pVphoS(W302Y) for expression of the mutant PhoS(W302Y) protein, a plasmid pVphoS(W302D) for expression of the mutant PhoS(W302D) protein, a plasmid pVphoS(W302N) for expression of the mutant PhoS(W302N) protein, a plasmid pVphoS(W302H) for expression of the mutant PhoS(W302H) protein, and a plasmid pVphoS(W302K) for expression of the mutant PhoS(W302K) protein, to construct total 10 plasmids for expression of mutant PhoS(W302X) proteins. The primer sets for amplifying a region containing an N-terminal side of the *phoS* gene and the primer sets for amplifying a region containing a C-terminal side of the *phoS* gene used in PCR for constructing the respective plasmids are shown in Table 8.

Table 8

Plasmid	N-terminal region		C-terminal region	
pVphoS (W302S)	SEQ ID NO: 32	SEQ I D NO: 34	SEQ ID NO: 33	SEQ ID NO: 35
pVphoS (W302A)	SEQ ID NO: 32	SEQ I D NO: 36	SEQ ID NO: 33	SEQ ID NO: 37
pVphoS (W302V)	SEQ ID NO: 32	SEQ ID NO: 38	SEQ ID NO: 33	SEO ID NO: 39
pVphoS (W302M)	SEQ ID NO: 32	SEQ ID NO: 40	SEQ ID NO: 33	SEQ ID NO: 41
pVphoS (W302F)	SEQ ID NO: 32	SEQ ID NO: 42	SEQ ID NO: 33	SEQ ID NO: 43
pVphoS (W302Y)	SEQ ID NO: 32	SEQ ID NO: 44	SEQ I D NO: 33	SEQ ID NO: 45
pVphoS (W302D)	SEQ ID NO: 32	SEQ I D NO: 46	SEO ID NO: 33	SEQ ID NO: 47
pVphoS(W302N)	SEQ ID NO: 32	SEQ ID NO: 48	SEQ ID NO: 33	SEQ ID NO: 49
pVphoS (W302H)	SEQ ID NO: 32	SEQ ID NO: 50	SEQ ID NO: 33	SEQ ID NO: 51
pVphoS (W302K)	SEQ ID NO: 32	SEQ ID NO: 52	SEQ ID NO: 33	SEQ ID NO: 53

(2) Effect of amplification of various PhoS(W302X) proteins on secretory expression of CspB50TEV-Teri using PhoS-deletion strain

[0235] The YDK010ΔphoS strain constructed in Example 1(5) was transformed with pPKK50TEV-Teri disclosed in WO2014/126260 in combination with each of the various pVphoS(W302X) plasmids constructed in Example 9(1). The obtained transformants were each cultured on MMTG liquid medium (120 g of glucose, 3 g of $MgSO_4 \cdot 7H_2O$, 30 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 0.03 g of $FeSO_4 \cdot 7H_2O$, 0.03 g of $MnSO_4 \cdot 5H_2O$, 0.45 mg of thiamine hydrochloride, 0.45 mg of biotin, 0.15 g of DL-methionine, 0.2 g (as total nitrogen) of soybean hydrolysate solution obtained with HCl, and 50 g of $CaCO_3$, filled up with water to 1 L, and adjusted to pH7.0) containing 6 mg/L of chloramphenicol and 25 mg/L of kanamycin at 30°C for 72 hr. After completion of the culture, 5 μL of the culture supernatant obtained by centrifuging each culture broth was subjected to reduced SDS-PAGE, and then staining was carried out with SYPRO Orange (Life Technologies), to compare the secretion amount of CspB50TEV-Teri (Fig. 10). In addition, the secretion amount of CspB50TEV-Teri observed for each of the strains was shown in Table 9 with scores ranging from "+" to "+++". As a result, it was revealed that the secretion amount of CspB50TEV-Teri is significantly improved in cases of replacing the tryptophan residue at position 302 of the wild-type PhoS protein with another amino acid residue other than aromatic amino acid and histidine residues as with a case of replacing the same with a cysteine residue, as compared with a case of the wild-type PhoS protein. Incidentally, regarding the pVphoS(W302Y)-introduced strain, no transformant was obtained, and hence, the secretion amount of CspB50TEV-Teri could not be evaluated.

[0236] From this, it was revealed that mutation of the tryptophan residue at position 302 of the wild-type PhoS protein leads to a significant improvement of the secretion amount of a heterologous protein, when the residue is mutated to an arbitrary amino acid residue other than aromatic amino acid and histidine residues, as well as when the residue is mutated to a cysteine residue.

Table 9

Plasmid	Secretion amount of CspB50TEV-Teri
pVphoS(WT)	+
pVphoS(W302C)	+++
pVphoS(W302S)	+++
pVphoS(W302A)	+++
pVphoS(W302V)	+++
pVphoS(W302M)	++
pVphoS(W302F)	+
pVphoS(W302Y)	n.t.

Plasmid	Secretion amount of CspB50TEV-Teri
pVphoS(W302D)	+++
pVphoS(W302N)	++
pVphoS(W302H)	+
pVphoS(W302K)	+++
n.t; not tested	

Example 10: Construction of PhoS(W302C)-mutant strain from *Corynebacterium glutamicum* ATCC13869 strain and secretory expression of heterologous protein

(1) Construction of C. *glutamicum* ATCC13869::phoS(W302C) strain

[0237] The C. *glutamicum* ATCC13869 strain was transformed with pBS5T-phoS(W302C) constructed in Example 1(2), which is a vector for substitution of the mutant *phoS* gene. Strain selection from the obtained transformants was carried out according to the method disclosed in WO2006/057450, to obtain ATCC13869::phoS(W302C), which is a strain of which the wild-type *phoS* gene on the chromosome was replaced with the mutant *phoS* gene.

(2) Secretory expression Exenatide precursor (ExCP) fused with N-terminal 6 amino acid residues of CspB mature protein in C. *glutamicum* ATCC13869::phoS(W302C) strain

[0238] The C. *glutamicum* ATCC13869 strain and the ATCC13869::phoS(W302C) strain constructed in Example 10(1) were each transformed with pPK4_CspB6TEV-ExCP constructed in Example 4(1), which is a secretory expression plasmid of ExCP fused with the N-terminal 6 amino acid residues of mature CspB and the ProTEV protease recognition sequence, to obtain strains ATCC13869/pPK4_CspB6TEV-ExCP and ATCC13869::phoS(W302C)/pPK4_CspB6TEV-ExCP.

[0239] The obtained transformants were each cultured on MMTG liquid medium (120 g of glucose, 3 g of $MgSO_4 \cdot 7H_2O$, 30 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 0.03 g of $FeSO_4 \cdot 7H_2O$, 0.03 g of $MnSO_4 \cdot 5H_2O$, 0.45 mg of thiamine hydrochloride, 0.45 mg of biotin, 0.15 g of DL-methionine, 0.2 g (as total nitrogen) of soybean hydrolysate solution obtained with HCl, and 50 g of $CaCO_3$, filled up with water to 1 L, and adjusted to pH7.0) containing 25 mg/L of kanamycin at 30°C for 72 hr. After completion of the culture, 6.5 μL of the culture supernatant obtained by centrifuging each culture broth was subjected to reduced SDS-PAGE, and then staining was carried out with Quick-CBB (Wako Pure Chemical Industries).

[0240] As a result, as with the case of using the YDK010 strain as the genetic background, a band indicating a protein having the molecular weight of CspB6TEV-ExCP was densely detected in the ATCC13869::phoS(W302C) strain while it was hardly detected in the ATCC13869 strain, and hence, it was confirmed that the secretion amount of CspB6TEV-ExCP was significantly improved in the ATCC13869::phoS(W302C) strain (Fig. 11). From this, it was revealed that the PhoS(W302C) mutation is an effective mutation that leads to a significant improvement of the secretion amount of CspB6TEV-ExCP also when using the ATCC13869 strain as the genetic background.

Example 11: Construction of CspB-deletion strain from *Corynebacterium glutamicum* ATCC13869::phoS(W302C) strain and secretory expression of heterologous protein

(1) Construction of C. *glutamicum* ATCC13869::phoS(W302C)ΔcspB strain

[0241] The ATCC13869::phoS(W302C) strain was transformed with pBS5T-ΔcspB disclosed in WO2013/065869, which is a vector for deletion of the *cspB* gene. Strain selection from the obtained transformants was carried out according to the method disclosed in WO2006/057450, to obtain ATCC13869::phoS(W302C)ΔcspB, which is a strain deficient in the *cspB* gene.

(2) Secretory expression Liver-type Fatty acid-binding protein (LFABP) fused with N-terminal 6 amino acid residues of CspB mature protein in C. *glutamicum* ATCC13869::phoS(W302C)ΔcspB strain

[0242] The C. *glutamicum* ATCC13869ΔcspB strain disclosed in WO2013/065869 and the ATCC13869::phoS(W302C)ΔcspB strain constructed in Example 11(1) were each transformed with pPK4_CspB6Xa-LFABP constructed in Example 3(1), which is a secretory expression plasmid of LFABP fused with the N-terminal 6 amino acid residues of mature CspB and the Factor Xa protease recognition sequence IEGR, to obtain strains ATCC13869ΔcspB/pPK4_CspB6Xa-LFABP and ATCC13869::phoS(W302C)ΔcspB/pPK4_CspB6Xa-LFABP.

[0243] The obtained transformants were each cultured on MMTG liquid medium (120 g of glucose, 3 g of $MgSO_4 \cdot 7H_2O$, 30 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 0.03 g of $FeSO_4 \cdot 7H_2O$, 0.03 g of $MnSO_4 \cdot 5H_2O$, 0.45 mg of thiamine hydrochloride, 0.45 mg of biotin, 0.15 g of DL-methionine, 0.2 g (as total nitrogen) of soybean hydrolysate solution obtained with HCl, and 50 g of $CaCO_3$, filled up with water to 1 L, and adjusted to pH7.0) containing 25 mg/L of kanamycin at 30°C for 72 hr. After completion of the culture, 6.5 μL of the culture supernatant obtained by centrifuging each culture broth was subjected to reduced SDS-PAGE, and then staining was carried out with Quick-CBB (Wako Pure Chemical Industries).

[0244] As a result, as with the case of using the YDK010 strain as the genetic background, a band indicating a protein having the molecular weight of CspB6Xa-LFABP was stably and densely detected in the ATCC13869::phoS(W302C)ΔcspB strain, as compared with the ATCC13869ΔcspB strain, and hence, it was confirmed that the secretion amount of CspB6Xa-LFABP was significantly improved in the ATCC13869::phoS(W302C)ΔcspB strain (Fig. 12). From this, it was revealed that the PhoS(W302C) mutation is an effective mutation that leads to a significant improvement of the secretion amount of CspB6Xa-LFABP also when using the ATCC13869ΔcspB strain as the genetic background.

[0245] From Examples 10-11, it was revealed that the secretory expression amount of an objective protein can be significantly improved by using the PhoS(W302C)-mutant strain also when using the ATCC13869 or ATCC13869ΔcspB strain as the genetic background.

[0246] Hence, From Examples 2-11, it was revealed that the secretion amount of an objective protein can be significantly improved by using the PhoS(W302C)-mutant strain regardless of the difference in the genetic background of the host strain.

Industrial Applicability

[0247] According to the present invention, heterologous proteins can be efficiently produced by secretory production.

<Explanation of Sequence Listing>

[0248] SEQ ID NOS:

1: Nucleotide sequence of mutant *phoS* gene of C. *glutamicum* YDK0107

2: Amino acid sequence of mutant PhoS protein of *C. glutamicum* YDK0107

3: Nucleotide sequence of wild-type *phoS* gene of *C. glutamicum* YDK010

4: Amino acid sequence of wild-type PhoS protein of *C. glutamicum* YDK010

5 to 10: Primers

11: Amino acid sequence of LFABP

12: Nucleotide sequence encoding CspB6Xa-LFABP

13: Amino acid sequence of CspB6Xa-LFABP

14: Amino acid sequence of Exenatide precursor

15: Nucleotide sequence encoding CspB6TEV-ExCP

16: Amino acid sequence of CspB6TEV-ExCP

17 to 53: Primers

54: Amino acid sequence of PhoS protein of *C. glutamicum* ATCC 13032

55: Amino acid sequence of PhoS protein of *C. glutamicum* ATCC 14067

56: Amino acid sequence of PhoS protein of *C. callunae*

57: Amino acid sequence of PhoS protein of *C. crenatum*

58: Amino acid sequence of PhoS protein of C. *efficiens*

59: Amino acid sequence of HisAK domain of PhoS protein of *C. glutamicum* YDK0107

60: Amino acid sequence of HisAK domain of PhoS protein of *C. glutamicum* YDK010

61: Amino acid sequence of HisAK domain of PhoS protein of *C. glutamicum* ATCC 13869

62: Amino acid sequence of HisAK domain of PhoS protein of *C. glutamicum* ATCC 13032

63: Amino acid sequence of HisAK domain of PhoS protein of *C. glutamicum* ATCC 14067

64: Amino acid sequence of HisAK domain of PhoS protein of *C. callunae*

65: Amino acid sequence of HisAK domain of PhoS protein of *C. crenatum*

66: Amino acid sequence of HisAK domain of PhoS protein of *C. efficiens*

67: Nucleotide sequence of *cspB* gene of *C. glutamicum* ATCC 13869

68: Amino acid sequence of CspB protein of *C. glutamicum* ATCC 13869

69: Nucleotide sequence of *tatA* gene of *C. glutamicum* ATCC 13032

70: Amino acid sequence of TatA protein of *C. glutamicum* ATCC 13032

71: Nucleotide sequence of *tatB* gene of *C. glutamicum* ATCC 13032

72: Amino acid sequence of TatB protein of *C. glutamicum* ATCC 13032

73: Nucleotide sequence of *tatC* gene of *C. glutamicum* ATCC 13032

74: Amino acid sequence of TatC protein of *C. glutamicum* ATCC 13032

75: Amino acid sequence of TorA signal peptide

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77: Amino acid sequence of PhoD signal peptide

78: Amino acid sequence of LipA signal peptide

79: Amino acid sequence of IMD signal peptide

80 and 81: Amino acid sequence of twin-arginine motif

82: Amino acid sequence of PS1 signal peptide

83: Amino acid sequence of PS2 signal peptide

84: Amino acid sequence of SlpA signal peptide

85: Amino acid sequence of CspB mature protein of *C. glutamicum* ATCC 13869

86 to 93: Amino acid sequences of insertion sequence used in the present disclosure in one embodiment

94: Recognition sequence of factor Xa protease

95: Recognition sequence of ProTEV protease

96: Nucleotide sequence of *phoR* gene of *C. glutamicum* ATCC 13032

97: Amino acid sequence of PhoR protein of *C. glutamicum* ATCC 13032

SEQUENCE LISTING

[0249]

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Ala Trp Pro Asp Arg Thr Val Asn Val Ser Asn Lys Ala Glu Ser Ile
        355                 360                 365
Pro Val Val Glu Gly Asp Pro Thr Arg Leu His Gln Val Leu Thr Asn
    370                 375                 380
Leu Val Ala Asn Gly Leu Asn His Gly Gly Pro Asp Ala Glu Val Ser
385                 390                 395                 400
Ile Glu Ile Asn Thr Asp Gly Gln Asn Val Arg Ile Leu Val Ala Asp
                405                 410                 415
Asn Gly Val Gly Met Ser Glu Glu Asp Ala Gln His Ile Phe Glu Arg
            420                 425                 430
Phe Tyr Arg Ala Asp Ser Ser Arg Ser Arg Ala Ser Gly Gly Ser Gly
        435                 440                 445
Leu Gly Leu Ala Ile Thr Lys Ser Leu Val Glu Gly His Gly Gly Thr
    450                 455                 460
Val Thr Val Asp Ser Val Gln Gly Glu Gly Thr Val Phe Thr Ile Thr
465                 470                 475                 480
Leu Pro Ala Val Ser
                485
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<210> 3

<211> 1458

<212> **DNA**

<213> Corynebacterium glutamicum

<400> 3

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atggaaaacc cttatgtcgc tgcgctcgat gacgataaaa aagaagtcgg cgcaataaaa     60
gaagcagaaa aagaacctga aataggtccc atcagagctg ccggacgagc cataccgctg    120

cgcacccgca tcattttgat cgtggtgggt atcgccgggc ttggtttgct ggtcaacgcg    180
attgctgttt ccagcctcat gcgtgaagtt tcctataccc gcatggatca agagctagag    240
acctcgatgg ggacgtgggc gcataacgtt gagctgttta atttcgatgg cgtccgccaa    300
gggccaccca gcgattatta tgtggccaag gtttttcctg atggatccag cattattttc    360
aacgatgcac aatcggcacc caatctagct gaaaccacca tcggtactgg tccacacact    420
gtggatgctg ctagcggttc tgcctccaac actccgtggc gtgtgatggc ggaaaagaac    480
ggtgacatta tcaccgtggt gggtaaaagc atggggcgtg aaacaaacct gctgtaccga    540
ttggtgatgg tgcagatgat catcggcgcg ctgattctgg ttgctatttt gattacttca    600
ctcttcctag tcagacgctc gttgcggccg ttgagagaag ttgaagagac cgccaccagg    660
attgcgggcg gtgatttgga tcgacgtgtc ccgcagtggc caatgaccac agaagtcgga    720
cagctgtcga atgccctcaa tatcatgttg gagcagctcc aagcctcaat tctgaccgcc    780
cagcaaaaag aagctcagat gcgccgattc gttggcgacg cctcccacga gctccgcaca    840
ccactgacct ctgtgaaggg cttcaccgag ctgtattcat caggtgcaac agatgatgcc    900
aactgggtca tgtccaagat cggtggcgaa gcccaacgca tgagtgtgct tgtggaagac    960
ctcctgtcac tgacgcgtgc cgaaggccag caaatggaga agcaccgcgt tgacgtgctg   1020
gaactcgcat tggcagtacg cggatccatg cgagcagcct ggccagatcg caccgtcaac   1080
gtgtccaata aagccgagtc cattccagtt gttgaaggcg acccaacccg cctccaccaa   1140
gttctcacca acctggttgc caacggactc aaccacggcg gaccggacgc ggaagtcagc   1200
attgagatca acaccgatgg gcaaaacgtg aggattctcg tggcagacaa cggtgtcgga   1260
atgtctgaag aagatgccca gcatatcttc gagcgtttct accgcgccga ttcctcccgc   1320
tcacgcgcat ccggcggatc gggcctcggc cttgcgatca cgaaatccct ggtcgaaggc   1380
cacggcggca cagtcaccgt cgacagcgtg caaggcgaag gcacggtgtt cacgatcacc   1440
ttgccggcgg tttcttaa                                                 1458
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<210> 4

<211> 485

<212> **PRT**

<213> Corynebacterium glutamicum

<400> 4

```
Met Glu Asn Pro Tyr Val Ala Ala Leu Asp Asp Asp Lys Lys Glu Val
1               5                  10                  15
Gly Ala Ile Lys Glu Ala Glu Lys Glu Pro Glu Ile Gly Pro Ile Arg
            20                  25                  30
Ala Ala Gly Arg Ala Ile Pro Leu Arg Thr Arg Ile Ile Leu Ile Val
        35                  40                  45
Val Gly Ile Ala Gly Leu Gly Leu Leu Val Asn Ala Ile Ala Val Ser
    50                  55                  60
Ser Leu Met Arg Glu Val Ser Tyr Thr Arg Met Asp Gln Glu Leu Glu
65                  70                  75                  80
Thr Ser Met Gly Thr Trp Ala His Asn Val Glu Leu Phe Asn Phe Asp
                85                  90                  95
Gly Val Arg Gln Gly Pro Pro Ser Asp Tyr Tyr Val Ala Lys Val Phe
            100                 105                 110
Pro Asp Gly Ser Ser Ile Ile Phe Asn Asp Ala Gln Ser Ala Pro Asn
        115                 120                 125
Leu Ala Glu Thr Thr Ile Gly Thr Gly Pro His Thr Val Asp Ala Ala
    130                 135                 140
Ser Gly Ser Ala Ser Asn Thr Pro Trp Arg Val Met Ala Glu Lys Asn
145                 150                 155                 160
Gly Asp Ile Ile Thr Val Val Gly Lys Ser Met Gly Arg Glu Thr Asn
                165                 170                 175
Leu Leu Tyr Arg Leu Val Met Val Gln Met Ile Ile Gly Ala Leu Ile
            180                 185                 190
Leu Val Ala Ile Leu Ile Thr Ser Leu Phe Leu Val Arg Arg Ser Leu
        195                 200                 205
Arg Pro Leu Arg Glu Val Glu Glu Thr Ala Thr Arg Ile Ala Gly Gly
    210                 215                 220
Asp Leu Asp Arg Arg Val Pro Gln Trp Pro Met Thr Thr Glu Val Gly
225                 230                 235                 240
Gln Leu Ser Asn Ala Leu Asn Ile Met Leu Glu Gln Leu Gln Ala Ser
                245                 250                 255

Ile Leu Thr Ala Gln Gln Lys Glu Ala Gln Met Arg Arg Phe Val Gly
            260                 265                 270
Asp Ala Ser His Glu Leu Arg Thr Pro Leu Thr Ser Val Lys Gly Phe
        275                 280                 285
Thr Glu Leu Tyr Ser Ser Gly Ala Thr Asp Asp Ala Asn Trp Val Met
    290                 295                 300
Ser Lys Ile Gly Gly Glu Ala Gln Arg Met Ser Val Leu Val Glu Asp
305                 310                 315                 320
Leu Leu Ser Leu Thr Arg Ala Glu Gly Gln Gln Met Glu Lys His Arg
                325                 330                 335
Val Asp Val Leu Glu Leu Ala Leu Ala Val Arg Gly Ser Met Arg Ala
            340                 345                 350
Ala Trp Pro Asp Arg Thr Val Asn Val Ser Asn Lys Ala Glu Ser Ile
        355                 360                 365
Pro Val Val Glu Gly Asp Pro Thr Arg Leu His Gln Val Leu Thr Asn
    370                 375                 380
Leu Val Ala Asn Gly Leu Asn His Gly Gly Pro Asp Ala Glu Val Ser
385                 390                 395                 400
Ile Glu Ile Asn Thr Asp Gly Gln Asn Val Arg Ile Leu Val Ala Asp
                405                 410                 415
Asn Gly Val Gly Met Ser Glu Glu Asp Ala Gln His Ile Phe Glu Arg
            420                 425                 430
Phe Tyr Arg Ala Asp Ser Ser Arg Ser Arg Ala Ser Gly Gly Ser Gly
        435                 440                 445
Leu Gly Leu Ala Ile Thr Lys Ser Leu Val Glu Gly His Gly Gly Thr
    450                 455                 460
Val Thr Val Asp Ser Val Gln Gly Glu Gly Thr Val Phe Thr Ile Thr
465                 470                 475                 480
Leu Pro Ala Val Ser
                485
```

<210> 5

<211> 25

<212> DNA

<213> Artificial Sequence

<220>
<223> primer

<400> 5
aggcagcaaa acaccgagga ctcaa 25

<210> 6
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 6
cgggcttggt ttgctggtca acgcg 25

<210> 7
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 7
tcgagctcgg tacccggcta atcctctggc ctg 33

<210> 8
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 8
taactaattt ctcctaggca tcaagggccg gaa 33

<210> 9
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 9

aggagaaatt agttacgtgg 20

<210> 10
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 10
ctctagagga tcccccggat gtacgtggaa gac 33

<210> 11
<211> 127
<212> PRT
<213> Homo sapiens

<400> 11

```
Met Ser Phe Ser Gly Lys Tyr Gln Leu Gln Ser Gln Glu Asn Phe Glu
1               5                  10                  15
Ala Phe Met Lys Ala Ile Gly Leu Pro Glu Glu Leu Ile Gln Lys Gly
            20                  25                  30
Lys Asp Ile Lys Gly Val Ser Glu Ile Val Gln Asn Gly Lys His Phe
        35                  40                  45
Lys Phe Thr Ile Thr Ala Gly Ser Lys Val Ile Gln Asn Glu Phe Thr
    50                  55                  60
Val Gly Glu Glu Cys Glu Leu Glu Thr Met Thr Gly Glu Lys Val Lys
65                  70                  75                  80
Thr Val Val Gln Leu Glu Gly Asp Asn Lys Leu Val Thr Thr Phe Lys
                85                  90                  95
Asn Ile Lys Ser Val Thr Glu Leu Asn Gly Asp Ile Ile Thr Asn Thr
            100                 105                 110
Met Thr Leu Gly Asp Ile Val Phe Lys Arg Ile Ser Lys Arg Ile
        115                 120                 125
```

<210> 12
<211> 504
<212> **DNA**
<213> Homo sapiens

<400> 12

```
atgtttaaca accgtatccg cactgcagct ctcgctggtg caatcgcaat ctccaccgca     60
gcttccggcg tagctatccc agcattcgct caggagacca acccaaccat cgagggccgc    120
atgtccttct ccggcaagta ccagctgcag tcccaggaaa acttcgaggc attcatgaag    180
gctatcggtc tgccagaaga gctcatccag aagggcaagg atatcaaggg tgtttccgaa    240
atcgtgcaga acggcaagca cttcaagttc accatcaccg caggttccaa ggtcatccag    300
aacgagttca ccgttggcga agagtgcgaa ctcgagacca tgaccggtga aaaggttaag    360
accgtggtcc agctggaggg cgacaacaag ctcgtgacca ccttcaagaa catcaagtcc    420
gtcaccgaac tgaacggcga tatcatcacc aacaccatga ccctcggtga catcgtgttc    480
aagcgcatct ccaagcgtat ctaa                                           504
```

<210> 13
<211> 167
<212> PRT
<213> Homo sapiens

<400> 13

```
Met Phe Asn Asn Arg Ile Arg Thr Ala Ala Leu Ala Gly Ala Ile Ala
1               5                  10                  15
Ile Ser Thr Ala Ala Ser Gly Val Ala Ile Pro Ala Phe Ala Gln Glu
            20                  25                  30
Thr Asn Pro Thr Ile Glu Gly Arg Met Ser Phe Ser Gly Lys Tyr Gln
        35                  40                  45
Leu Gln Ser Gln Glu Asn Phe Glu Ala Phe Met Lys Ala Ile Gly Leu
    50                  55                  60
Pro Glu Glu Leu Ile Gln Lys Gly Lys Asp Ile Lys Gly Val Ser Glu
65                  70                  75                  80
Ile Val Gln Asn Gly Lys His Phe Lys Phe Thr Ile Thr Ala Gly Ser
                85                  90                  95
Lys Val Ile Gln Asn Glu Phe Thr Val Gly Glu Glu Cys Glu Leu Glu
            100                 105                 110
Thr Met Thr Gly Glu Lys Val Lys Thr Val Val Gln Leu Glu Gly Asp
        115                 120                 125
Asn Lys Leu Val Thr Thr Phe Lys Asn Ile Lys Ser Val Thr Glu Leu
    130                 135                 140
Asn Gly Asp Ile Ile Thr Asn Thr Met Thr Leu Gly Asp Ile Val Phe
145                 150                 155                 160
Lys Arg Ile Ser Lys Arg Ile
                165
```

<210> 14

<211> 41

<212> **PRT**

<213> Heloderma suspectum

<400> **14**

```
His Gly Glu Gly Thr Phe Thr Ser Asp Leu Ser Lys Gln Met Glu Glu
1               5                  10                  15
Glu Ala Val Arg Leu Phe Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser
            20                  25                  30
Ser Gly Ala Pro Pro Pro Ser Cys Pro
        35                  40
```

<210> 15

<211> 252

<212> DNA

<213> Heloderma suspectum

<400> 15

```
atgtttaaca accgtatccg cactgcagct ctcgctggtg caatcgcaat ctccaccgca     60
gcttccggcg tagctatccc agcattcgct caggagacca acccaaccga aaacctgtac    120
ttccagcacg gcgagggaac cttcacgtct gatctgtcta agcagatgga ggaagaggca    180
gttcgcctgt tcattgagtg gctgaaaaat ggcggtcctt ctagcggtgc acctcccccc    240
tcctgcccat ga                                                       252
```

<210> 16

<211> 83

<212> **PRT**

<213> Heloderma suspectum

<400> 16

```
Met Phe Asn Asn Arg Ile Arg Thr Ala Ala Leu Ala Gly Ala Ile Ala
1               5                  10                  15
Ile Ser Thr Ala Ala Ser Gly Val Ala Ile Pro Ala Phe Ala Gln Glu
            20                  25                  30
Thr Asn Pro Thr Glu Asn Leu Tyr Phe Gln His Gly Glu Gly Thr Phe
        35                  40                  45
Thr Ser Asp Leu Ser Lys Gln Met Glu Glu Glu Ala Val Arg Leu Phe
    50                  55                  60
```

```
Ile Glu Trp Leu Lys Asn Gly Gly Pro Ser Ser Gly Ala Pro Pro Pro
65                  70                  75                  80
Ser Cys Pro
```

<210> 17
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 17
cgagccacca ggcaggcggg aaaatcg 27

<210> 18
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 18
cgattttccc gcctgcctgg tggctcg 27

<210> 19
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 19
cccgcttgat cattccttta agg 23

<210> 20
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 20
aatgggccct ttggtacccc taaataatat cggtcc 36

<210> 21

<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 21
cgtgctctag gggaaccgtg cgttccc 27

<210> 22
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 22
gggaacgcac ggttccccta gagcacg 27

<210> 23
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 23
cgacgctgaa gttgtagaga tcatccg 27

<210> 24
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 24
cggatgatct ctacaacttc agcgtcg 27

<210> 25
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 25
ggcggtaccc aaattcctgt gaagtagc 28

<210> 26
<211> 44
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 26
ggcgggcccg ccggcagtcg cacgtcgcgg cgttaacaat gacg 44

<210> 27
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 27
gacaatggcg cgggggaaga gacg 24

<210> 28
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 28
caggtcgact ctagaggatc cc 22

<210> 29
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 29

atattattta ggtctagaca aattcctgtg 30

<210> 30
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 30
ctgcaggtcg actctagaat taattaaaat ccaca 35

<210> 31
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 31
ctgcaggtcg actctagatc acatgtccaa ctctatcc 38

<210> 32
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 32
ctctagagga tccccatgga aaacccttat gtcgc 35

<210> 33
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 33
tcgagctcgg taccttaag aaaccgccgg caag 34

<210> 34
<211> 30

<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 34
catgacggag ttggcatcat ctgttgcacc 30

<210> 35
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 35
gccaactccg tcatgtccaa gatcggtgg 29

<210> 36
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 36
catgactgcg ttggcatcat ctgttgcacc 30

<210> 37
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 37
gccaacgcag tcatgtccaa gatcggtgg 29

<210> 38
<211> 30
<212> DNA
<213> Artificial Sequence

<220>

<223> primer

<400> 38
catgaccacg ttggcatcat ctgttgcacc 30

<210> 39
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 39
gccaacgtgg tcatgtccaa gatcggtgg 29

<210> 40
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 40
catgaccatg ttggcatcat ctgttgcacc 30

<210> 41
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 41
gccaacatgg tcatgtccaa gatcggtgg 29

<210> 42
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 42
catgacgaag ttggcatcat ctgttgcacc 30

<210> 43
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 43
gccaacttcg tcatgtccaa gatcggtgg 29

<210> 44
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 44
catgacgtag ttggcatcat ctgttgcacc 30

<210> 45
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 45
gccaactacg tcatgtccaa gatcggtgg 29

<210> 46
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 46
catgacatcg ttggcatcat ctgttgcacc 30

<210> 47
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 47
gccaacgatg tcatgtccaa gatcggtgg 29

<210> 48
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 48
catgacgttg ttggcatcat ctgttgcacc 30

<210> 49
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 49
gccaacaacg tcatgtccaa gatcggtgg 29

<210> 50
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 50
catgacgtgg ttggcatcat ctgttgcacc 30

<210> 51
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 51

gccaaccacg tcatgtccaa gatcggtgg 29

<210> 52
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 52
catgaccttg ttggcatcat ctgttgcacc 30

<210> 53
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 53
gccaacaagg tcatgtccaa gatcggtgg 29

<210> 54
<211> 485
<212> PRT
<213> Corynebacterium glutamicum

<400> 54

```
Met Glu Asn Pro Tyr Val Ala Ala Leu Asp Asp Glu Asn Gln Glu Val
1               5                  10                  15
Gly Val Lys Lys Glu Ala Glu Lys Glu Pro Glu Ile Gly Pro Ile Arg
            20                  25                  30
Ala Ala Gly Arg Ala Ile Pro Leu Arg Thr Arg Ile Ile Leu Ile Val
        35                  40                  45
Val Gly Ile Ala Gly Leu Gly Leu Leu Val Asn Ala Ile Ala Val Ser
    50                  55                  60
Ser Leu Met Arg Glu Val Ser Tyr Thr Arg Met Asp Gln Glu Leu Glu
65                  70                  75                  80
Thr Ser Met Gly Thr Trp Ala His Asn Val Glu Leu Phe Asn Phe Asp
                85                  90                  95
Gly Val Arg Gln Gly Pro Pro Ser Asp Tyr Tyr Val Ala Lys Val Phe
            100                 105                 110
Pro Asp Gly Ser Ser Ile Ile Phe Asn Asp Ala Gln Ser Ala Pro Asp
        115                 120                 125
Leu Ala Glu Thr Thr Ile Gly Thr Gly Pro His Thr Val Asp Ala Ala
    130                 135                 140
Ser Gly Ser Ala Ser Asn Thr Pro Trp Arg Val Met Ala Glu Lys Asn
145                 150                 155                 160
Gly Asp Ile Ile Thr Val Val Gly Lys Ser Met Gly Arg Glu Thr Asn

                165                 170                 175
Leu Leu Tyr Arg Leu Val Met Val Gln Met Ile Ile Gly Ala Leu Ile
            180                 185                 190
Leu Val Ala Ile Leu Ile Thr Ser Leu Phe Leu Val Arg Arg Ser Leu
        195                 200                 205
Arg Pro Leu Arg Glu Val Glu Glu Thr Ala Thr Arg Ile Ala Gly Gly
```

```
Arg Pro Leu Arg Glu Val Glu Glu Thr Ala Thr Arg Ile Ala Gly Gly
    210                 215                 220
Asp Leu Asp Arg Arg Val Pro Gln Trp Pro Met Thr Thr Glu Val Gly
225                 230                 235                 240
Gln Leu Ser Asn Ala Leu Asn Ile Met Leu Glu Gln Leu Gln Ala Ser
                245                 250                 255
Ile Leu Thr Ala Gln Gln Lys Glu Ala Gln Met Arg Arg Phe Val Gly
            260                 265                 270
Asp Ala Ser His Glu Leu Arg Thr Pro Leu Thr Ser Val Lys Gly Phe
        275                 280                 285
Thr Glu Leu Tyr Ser Ser Gly Ala Thr Asp Asp Ala Asn Trp Val Met
    290                 295                 300
Ser Lys Ile Gly Gly Glu Ala Gln Arg Met Ser Val Leu Val Glu Asp
305                 310                 315                 320
Leu Leu Ser Leu Thr Arg Ala Glu Gly Gln Gln Met Glu Lys His Arg
                325                 330                 335
Val Asp Val Leu Glu Leu Ala Leu Ala Val Arg Gly Ser Met Arg Ala
            340                 345                 350
Ala Trp Pro Asp Arg Thr Val Asn Val Ser Asn Lys Ala Glu Ser Ile
        355                 360                 365
Pro Val Val Lys Gly Asp Pro Thr Arg Leu His Gln Val Leu Thr Asn
    370                 375                 380
Leu Val Ala Asn Gly Leu Asn His Gly Gly Pro Asp Ala Glu Val Ser
385                 390                 395                 400
Ile Glu Ile Asn Thr Asp Gly Gln Asn Val Arg Ile Leu Val Ala Asp
                405                 410                 415
Asn Gly Val Gly Met Ser Glu Glu Asp Ala Gln His Ile Phe Glu Arg
            420                 425                 430
Phe Tyr Arg Ala Asp Ser Ser Arg Ser Arg Ala Ser Gly Gly Ser Gly
        435                 440                 445
Leu Gly Leu Ala Ile Thr Lys Ser Leu Val Glu Gly His Gly Gly Thr
    450                 455                 460
Val Thr Val Asp Ser Val Gln Gly Glu Gly Thr Val Phe Thr Ile Thr
465                 470                 475                 480
Leu Pro Ala Val Ser
                485
```

<210> 55

<211> 485

<212> **PRT**

<213> Corynebacterium glutamicum

<400> 55

```
Met Glu Asn Pro Tyr Val Ala Ala Leu Asp Asp Glu Asn Gln Glu Val
1               5                  10                  15
Gly Val Lys Lys Glu Ala Glu Lys Glu Pro Glu Ile Gly Pro Ile Arg
            20                  25                  30
Ala Ala Gly Arg Ala Ile Pro Leu Arg Thr Arg Ile Ile Leu Ile Val
        35                  40                  45
Val Gly Ile Ala Gly Leu Gly Leu Leu Val Asn Ala Ile Ala Val Ser
    50                  55                  60
Ser Leu Met Arg Glu Val Ser Tyr Thr Arg Met Asp Gln Glu Leu Glu
65                  70                  75                  80
Thr Ser Met Gly Thr Trp Ala His Asn Val Glu Leu Phe Asn Phe Asp
                85                  90                  95
Gly Val Arg Gln Gly Pro Pro Ser Asp Tyr Tyr Val Ala Lys Val Phe
            100                 105                 110

Pro Asp Gly Ser Ser Ile Ile Phe Asn Asp Ala Gln Ser Ala Pro Asp
        115                 120                 125
Leu Ala Glu Thr Thr Ile Gly Thr Gly Pro His Thr Val Asp Ala Ala
    130                 135                 140
Ser Gly Ser Ala Ser Asn Thr Pro Trp Arg Val Met Ala Glu Lys Asn
145                 150                 155                 160
Gly Asp Ile Ile Thr Val Val Gly Lys Ser Met Gly Arg Glu Thr Asn
                165                 170                 175
Leu Leu Tyr Arg Leu Val Val Val Gln Met Ile Ile Gly Ala Leu Ile
            180                 185                 190
Leu Val Ala Ile Leu Ile Thr Ser Leu Phe Leu Val Arg Arg Ser Leu
        195                 200                 205
```

```
Arg Pro Leu Arg Glu Val Glu Glu Thr Ala Thr Arg Ile Ala Gly Gly
    210                 215                 220
Asp Leu Asp Arg Arg Val Pro Gln Trp Pro Met Thr Thr Glu Val Gly
225                 230                 235                 240
Gln Leu Ser Asn Ala Leu Asn Ile Met Leu Glu Gln Leu Gln Ala Ser
                245                 250                 255
Ile Leu Thr Ala Gln Gln Lys Glu Ala Gln Met Arg Arg Phe Val Gly
            260                 265                 270
Asp Ala Ser His Glu Leu Arg Thr Pro Leu Thr Ser Val Lys Gly Phe
        275                 280                 285
Thr Glu Leu Tyr Ser Ser Gly Ala Thr Asp Asp Ala Asn Trp Val Met
    290                 295                 300
Ser Lys Ile Gly Gly Glu Ala Gln Arg Met Ser Val Leu Val Glu Asp
305                 310                 315                 320
Leu Leu Ser Leu Thr Arg Ala Glu Gly Gln Gln Met Glu Lys His Arg
                325                 330                 335
Val Asp Val Leu Glu Leu Ala Leu Ala Val Arg Gly Ser Met Arg Ala
            340                 345                 350
Ala Trp Pro Asp Arg Thr Val Asn Val Ser Asn Lys Ala Glu Ser Ile
        355                 360                 365
Pro Val Val Glu Gly Asp Pro Thr Arg Leu His Gln Val Leu Thr Asn
    370                 375                 380
Leu Val Ala Asn Gly Leu Asn His Gly Gly Pro Asp Ala Glu Val Ser
385                 390                 395                 400
Ile Glu Ile Asn Thr Asp Gly Gln Asn Val Arg Ile Leu Val Ala Asp
                405                 410                 415
Asn Gly Val Gly Met Ser Glu Glu Asp Ala Gln His Ile Phe Glu Arg
            420                 425                 430
Phe Tyr Arg Ala Asp Ser Ser Arg Ser Arg Ala Ser Gly Gly Ser Gly
        435                 440                 445
Leu Gly Leu Ala Ile Thr Lys Ser Leu Val Glu Gly His Gly Gly Thr
    450                 455                 460
Val Thr Val Asp Ser Val Gln Gly Glu Gly Thr Val Phe Thr Ile Thr
465                 470                 475                 480
Leu Pro Ala Val Ser
                485
```

<210> 56

<211> 504

<212> **PRT**

<213> Corynebacterium callunae

<400> 56

```
Met Glu Asn Pro Tyr Val Ala Ala Leu Asp Lys Asn Ser Asn Phe Gly
1                   5                  10                  15
Ala Lys Asp Thr Asp Ser Ala Val Ser Asp Ser Thr Glu Val Ser Gln
            20                  25                  30
Asn Asn Asp Gly Ile Gly Thr Pro Ala Thr Ala Glu Pro Lys Val Gly
        35                  40                  45
Pro Ile Arg Thr Ala Gly Arg Ala Met Pro Leu Arg Thr Arg Ile Ile

    50                  55                  60
Leu Leu Val Val Gly Ile Ala Gly Leu Gly Leu Leu Val Asn Ala Val
65                  70                  75                  80
Ala Val Ser Ser Leu Met Arg Glu Val Ser Tyr Thr Arg Met Asp Gln
                85                  90                  95
Asp Leu Glu Ser Ala Met Gly Thr Trp Val Arg Asn Val Glu Leu Phe
            100                 105                 110
Asn Phe Asp Gly Val Arg Gln Gly Pro Pro Ser Asp Tyr Tyr Val Ala
        115                 120                 125
Lys Val Phe Pro Asp Gly Ser Ser Ile Ile Phe Asn Asp Ala Glu Ser
    130                 135                 140
Ala Pro Asp Leu Gly Gln Thr Thr Ile Gly Thr Gly Pro His Thr Val
145                 150                 155                 160
Glu Ala Ala Glu Gly Ser Ala Ser Ser Thr His Trp Arg Val Met Ala
                165                 170                 175
Ala Lys Asn Gly Asp Val Ile Thr Val Val Gly Lys Ser Met Gly Arg
            180                 185                 190
Glu Ser Thr Leu Leu Tyr Arg Leu Val Val Val Gln Met Val Ile Gly
        195                 200                 205
```

```
        195                 200                 205
Val Leu Ile Leu Ile Ala Ile Leu Ile Gly Ser Phe Phe Leu Val Arg
    210                 215                 220
Arg Ser Leu Lys Pro Leu Arg Glu Val Glu Glu Thr Ala Ser Arg Ile
225                 230                 235                 240
Ala Gly Gly Glu Leu Asp Arg Arg Val Pro Gln Trp Pro Met Thr Thr
                245                 250                 255
Glu Val Gly Gln Leu Ala Asn Ala Leu Asn Ile Met Leu Glu Gln Leu
            260                 265                 270
Gln Thr Ser Ile Met Asn Ala Gln Gln Lys Glu Ala Gln Met Arg Arg
        275                 280                 285
Phe Val Gly Asp Ala Ser His Glu Leu Arg Thr Pro Leu Thr Ser Val
    290                 295                 300
Lys Gly Phe Thr Glu Leu Tyr Ser Ser Gly Ala Thr Gln Asp Ala Asp
305                 310                 315                 320
Trp Val Leu Ser Lys Ile Gly Gly Glu Ala Gln Arg Met Ser Val Leu
                325                 330                 335
Val Glu Asp Leu Leu Ser Leu Thr Arg Ala Glu Gly Gln Gln Met Glu
            340                 345                 350
Lys His Arg Val Asp Met Leu Glu Leu Ala Leu Ala Val Arg Gly Ser
        355                 360                 365
Leu Lys Ala Ala Trp Pro Asp Arg Thr Val Asn Val Ala Asn Arg Ser
    370                 375                 380
Glu Asn Ile Pro Val Val Glu Gly Asp Pro Thr Arg Leu His Gln Val
385                 390                 395                 400
Leu Thr Asn Leu Val Ala Asn Gly Leu Asn His Gly Gly Pro Glu Ala
                405                 410                 415
Glu Val Asn Ile Gln Val Glu Thr Ala Asp Asp Lys Val Lys Ile Leu
            420                 425                 430
Val Ile Asp Asn Gly Val Gly Met Ser Lys Glu Asp Ala Glu His Ile
        435                 440                 445
Phe Glu Arg Phe Tyr Arg Ala Asp Thr Ser Arg Ser Arg Ala Ser Gly
    450                 455                 460
Gly Ser Gly Leu Gly Leu Ala Ile Thr Lys Ser Leu Val Glu Gly His
465                 470                 475                 480
Gly Gly Thr Ile Thr Val Asp Ser Glu Leu Gly Lys Gly Thr Val Phe
                485                 490                 495
Ser Ile Ile Leu Pro Ala Ala Glu
            500
```

<210> 57

<211> 458

<212> PRT

<213> Corynebacterium crenatum

<400> 57

```
Ile Gly Pro Ile Arg Ala Ala Gly Arg Ala Ile Pro Leu Arg Thr Arg
1               5                   10                  15
Ile Ile Leu Ile Val Val Gly Ile Ala Gly Leu Gly Leu Leu Val Asn
            20                  25                  30
Ala Ile Ala Val Ser Ser Leu Met Arg Glu Val Ser Tyr Thr Arg Met
        35                  40                  45
Asp Gln Glu Leu Glu Thr Ser Met Gly Thr Trp Ala His Asn Val Glu
    50                  55                  60
Leu Phe Asn Phe Asp Gly Val Arg Gln Gly Pro Pro Ser Asp Tyr Tyr
65                  70                  75                  80
Val Ala Lys Val Phe Pro Asp Gly Ser Ser Ile Ile Phe Asn Asp Ala
                85                  90                  95
Gln Ser Ala Pro Asp Leu Ala Glu Thr Thr Ile Gly Thr Gly Pro His
            100                 105                 110
Thr Val Asp Ala Ala Ser Gly Ser Ala Ser Asn Thr Pro Trp Arg Val
        115                 120                 125
Met Ala Glu Lys Asn Gly Asp Ile Ile Thr Val Val Gly Lys Ser Met
    130                 135                 140
Gly Arg Glu Thr Asn Leu Leu Tyr Arg Leu Val Met Val Gln Met Ile
145                 150                 155                 160
Ile Gly Ala Leu Ile Leu Val Ala Ile Leu Ile Thr Ser Leu Phe Leu
                165                 170                 175
Val Arg Arg Ser Leu Arg Pro Leu Arg Glu Val Glu Glu Thr Ala Thr
            180                 185                 190
Arg Ile Ala Gly Gly Asp Leu Asp Arg Arg Val Pro Gln Trp Pro Met
```

```
                195                 200                 205
Thr Thr Glu Val Gly Gln Leu Ser Asn Ala Leu Asn Ile Met Leu Glu
    210                 215                 220
Gln Leu Gln Ala Ser Ile Leu Ser Ala Gln Gln Lys Glu Ala Gln Met
225                 230                 235                 240
Arg Arg Phe Val Gly Asp Ala Ser His Glu Leu Arg Thr Pro Leu Thr
                245                 250                 255
Ser Val Lys Gly Phe Thr Glu Leu Tyr Ser Ser Gly Ala Thr Asp Asp
            260                 265                 270
Ala Asn Trp Val Met Ser Lys Ile Gly Gly Glu Ala Gln Arg Met Ser
        275                 280                 285
Val Leu Val Glu Asp Leu Leu Ser Leu Thr Arg Ala Glu Gly Gln Gln
    290                 295                 300
Met Glu Lys His Arg Val Asp Val Leu Glu Leu Ala Leu Ala Val Arg
305                 310                 315                 320
Gly Ser Met Arg Ala Ala Trp Pro Asp Arg Thr Val Asn Val Ser Asn
                325                 330                 335
Lys Ala Ala Ser Ile Pro Val Val Glu Gly Asp Pro Thr Arg Leu His
            340                 345                 350
Gln Val Leu Thr Asn Leu Val Ala Asn Gly Leu Asn His Gly Gly Pro
        355                 360                 365
Asp Ala Glu Val Ser Ile Glu Ile Asn Thr Asp Gly Gln Asn Val Arg
    370                 375                 380
Ile Leu Val Ala Asp Asn Gly Val Gly Met Ser Glu Glu Asp Ala Gln
385                 390                 395                 400
His Ile Phe Glu Arg Phe Tyr Arg Ala Asp Ser Ser Arg Ser Arg Ala
                405                 410                 415
Ser Gly Gly Ser Gly Leu Gly Leu Ala Ile Thr Lys Ser Leu Val Glu
            420                 425                 430
Gly His Gly Gly Thr Val Thr Val Asp Ser Val Gln Gly Glu Gly Thr
        435                 440                 445
Val Phe Thr Ile Thr Leu Pro Ala Val Ser
    450                 455
```

<210> 58

<211> 471

<212> PRT

<213> Corynebacterium efficiens

<400> 58

```
Met Thr Ala Pro Glu Asn Pro His Ala Gln Val Thr Pro Val Gly Arg
1               5                   10                  15
Phe Arg Gln Ala Ala Arg Gly Val Pro Leu Arg Thr Arg Ile Ile Leu
            20                  25                  30
Leu Val Val Gly Ile Ala Gly Leu Gly Leu Leu Val Asn Ala Ile Ala
        35                  40                  45
Val Ser Ser Leu Met Arg Glu Val Ser Tyr Ser Arg Met Asp Gln Glu
    50                  55                  60
Leu Glu Ser Ala Met Asn Ser Trp Ala Gln Thr Ala Glu Leu Phe Gly
65                  70                  75                  80
Ser Ile Thr Leu Gly Pro Pro Ser Asp Tyr Tyr Val Val Arg Ile Phe
                85                  90                  95
Pro Asp Gly Ser His Met Val Phe Asn Gln Ser Asp Ser Ala Pro Asp
            100                 105                 110
Leu Gly Glu Thr Thr Ile Gly Ile Gly Pro His Thr Ala Ser Ala Ala
        115                 120                 125
Pro Gly Ser Ser Ser Ser Val Pro Trp Arg Val Ile Ala Ile Ser Asp
    130                 135                 140
Asn Gly Thr Ile Thr Val Val Gly Lys Ser Leu Ala Pro Glu Ser Met
145                 150                 155                 160
Leu Leu Tyr Arg Leu Val Ile Val Gln Leu Val Ile Gly Met Leu Ile
                165                 170                 175
Val Val Ala Ile Leu Leu Ser Ser Leu Tyr Leu Val Asn Arg Ser Leu
            180                 185                 190
Arg Pro Leu Arg Glu Val Glu Lys Thr Ala Lys Ser Ile Ala Gly Gly
        195                 200                 205
Asp Leu Asp Arg Arg Val Pro Gln Trp Pro Met Thr Thr Glu Val Gly
    210                 215                 220
Gln Leu Ala Asn Ala Leu Asn Ile Met Leu Glu Gln Leu Gln Ala Ser
225                 230                 235                 240
```

```
Ile Leu Ser Ala Gln Glu Lys Glu Ser Gln Met Arg Arg Phe Val Gly
                245                 250                 255
Asp Ala Ser His Glu Leu Arg Thr Pro Leu Thr Ser Val Lys Gly Tyr
            260                 265                 270
Ser Glu Leu Tyr His Ser Gly Ala Thr Arg Asp Ala Asp Trp Val Leu
        275                 280                 285
Ser Lys Ile Ser Gly Glu Ala Gln Arg Met Ser Val Leu Val Glu Asp
    290                 295                 300
Leu Leu Ser Leu Thr Arg Ala Glu Gly Gln Gln Met Glu Lys Arg Pro
305                 310                 315                 320
Val Asp Val Leu Glu Leu Ser Leu Ser Val Ala Ser Ser Met Arg Ala
                325                 330                 335
Ala Trp Pro Glu Arg Ser Ile Thr Val Val Asn Lys Thr Gly Ser Leu
            340                 345                 350
Pro Val Val Glu Gly Asp Ala Thr Arg Leu His Gln Val Leu Thr Asn
        355                 360                 365
Leu Val Asn Asn Gly Leu Asn His Gly Gly Pro Asp Ala Ser Val Glu
    370                 375                 380
Ile Glu Ile Ser Ala Glu Gly Gly Ser Val Leu Val Arg Val Val Asp
385                 390                 395                 400
Asp Gly Val Gly Met Thr Ala Glu Asp Ala Gln His Ile Phe Glu Arg
                405                 410                 415
Phe Tyr Arg Thr Asp Thr Ser Arg Ser Arg Ala Ser Gly Gly Ser Gly
            420                 425                 430
Leu Gly Leu Ala Ile Thr Lys Ser Leu Val Glu Gly His Arg Gly Thr
        435                 440                 445
Ile Thr Val Asp Ser Glu Val Gly Glu Gly Thr Val Phe Thr Ile Thr
    450                 455                 460

Leu Pro Ser Arg Met Glu Asp
465                 470
```

<210> 59

<211> 65

<212> PRT

<213> Corynebacterium glutamicum

<400> 59

```
Gln Met Arg Arg Phe Val Gly Asp Ala Ser His Glu Leu Arg Thr Pro
1               5                  10                  15
Leu Thr Ser Val Lys Gly Phe Thr Glu Leu Tyr Ser Ser Gly Ala Thr
            20                  25                  30
Asp Asp Ala Asn Cys Val Met Ser Lys Ile Gly Gly Glu Ala Gln Arg
        35                  40                  45
Met Ser Val Leu Val Glu Asp Leu Leu Ser Leu Thr Arg Ala Glu Gly
    50                  55                  60
Gln
65
```

<210> 60

<211> 65

<212> PRT

<213> Corynebacterium glutamicum

<400> 60

```
Gln Met Arg Arg Phe Val Gly Asp Ala Ser His Glu Leu Arg Thr Pro
1               5                  10                  15
Leu Thr Ser Val Lys Gly Phe Thr Glu Leu Tyr Ser Ser Gly Ala Thr
            20                  25                  30
Asp Asp Ala Asn Trp Val Met Ser Lys Ile Gly Gly Glu Ala Gln Arg
        35                  40                  45
Met Ser Val Leu Val Glu Asp Leu Leu Ser Leu Thr Arg Ala Glu Gly
    50                  55                  60
Gln
65
```

<210> 61

<211> 65

<212> PRT

<213> Corynebacterium glutamicum

<400> 61

```
Gln Met Arg Arg Phe Val Gly Asp Ala Ser His Glu Leu Arg Thr Pro
1               5                  10                  15
Leu Thr Ser Val Lys Gly Phe Thr Glu Leu Tyr Ser Ser Gly Ala Thr
            20                  25                  30
Asp Asp Ala Asn Trp Val Met Ser Lys Ile Gly Gly Glu Ala Gln Arg
        35                  40                  45
Met Ser Val Leu Val Glu Asp Leu Leu Ser Leu Thr Arg Ala Glu Gly
    50                  55                  60
Gln
65
```

<210> 62

<211> 65

<212> PRT

<213> Corynebacterium glutamicum

<400> 62

```
Gln Met Arg Arg Phe Val Gly Asp Ala Ser His Glu Leu Arg Thr Pro
1               5                  10                  15
Leu Thr Ser Val Lys Gly Phe Thr Glu Leu Tyr Ser Ser Gly Ala Thr
            20                  25                  30
Asp Asp Ala Asn Trp Val Met Ser Lys Ile Gly Gly Glu Ala Gln Arg
        35                  40                  45
Met Ser Val Leu Val Glu Asp Leu Leu Ser Leu Thr Arg Ala Glu Gly
    50                  55                  60
Gln
65
```

<210> 63

<211> 65

<212> PRT

<213> Corynebacterium glutamicum

<400> 63

```
Gln Met Arg Arg Phe Val Gly Asp Ala Ser His Glu Leu Arg Thr Pro
1               5                  10                  15
Leu Thr Ser Val Lys Gly Phe Thr Glu Leu Tyr Ser Ser Gly Ala Thr
            20                  25                  30
Asp Asp Ala Asn Trp Val Met Ser Lys Ile Gly Gly Glu Ala Gln Arg
        35                  40                  45
Met Ser Val Leu Val Glu Asp Leu Leu Ser Leu Thr Arg Ala Glu Gly
    50                  55                  60
Gln
65
```

<210> 64

<211> 65

<212> **PRT**

<213> Corynebacterium callunae

<400> **64**

```
Gln Met Arg Arg Phe Val Gly Asp Ala Ser His Glu Leu Arg Thr Pro
1               5                  10                  15
```

```
Leu Thr Ser Val Lys Gly Phe Thr Glu Leu Tyr Ser Ser Gly Ala Thr
            20                  25                  30
Gln Asp Ala Asp Trp Val Leu Ser Lys Ile Gly Gly Glu Ala Gln Arg
        35                  40                  45
Met Ser Val Leu Val Glu Asp Leu Leu Ser Leu Thr Arg Ala Glu Gly
    50                  55                  60
Gln
65
```

<210> 65

<211> 65

<212> **PRT**

<213> Corynebacterium crenatum

<400> 65

```
Gln Met Arg Arg Phe Val Gly Asp Ala Ser His Glu Leu Arg Thr Pro
1               5                   10                  15
Leu Thr Ser Val Lys Gly Phe Thr Glu Leu Tyr Ser Ser Gly Ala Thr
            20                  25                  30
Asp Asp Ala Asn Trp Val Met Ser Lys Ile Gly Gly Glu Ala Gln Arg
        35                  40                  45
Met Ser Val Leu Val Glu Asp Leu Leu Ser Leu Thr Arg Ala Glu Gly
    50                  55                  60

Gln
65
```

<210> 66

<211> 65

<212> PRT

<213> Corynebacterium efficiens

<400> 66

```
Gln Met Arg Arg Phe Val Gly Asp Ala Ser His Glu Leu Arg Thr Pro
1               5                   10                  15
Leu Thr Ser Val Lys Gly Tyr Ser Glu Leu Tyr His Ser Gly Ala Thr
            20                  25                  30
Arg Asp Ala Asp Trp Val Leu Ser Lys Ile Ser Gly Glu Ala Gln Arg
        35                  40                  45
Met Ser Val Leu Val Glu Asp Leu Leu Ser Leu Thr Arg Ala Glu Gly
    50                  55                  60
Gln
65
```

<210> 67

<211> 1500

<212> **DNA**

<213> Corynebacterium glutamicum

<400> 67

```
atgtttaaca accgtatccg cactgcagct ctcgctggtg caatcgcaat ctccaccgca     60
gcttccggcg tagctatccc agcattcgct caggagacca acccaacctt caacatcaac    120
aacggcttca acgatgctga tggatccacc atccagccag ttgagccagt taaccacacc    180
gaggaaaccc tccgcgacct gactgactcc accggcgctt acctggaaga gttccagtac    240
ggcaacgttg aggaaatcgt tgaagcatac ctgcaggttc aggcttccgc agacggattc    300
gatccttctg agcaggctgc ttacgaggct ttcgaggctg ctcgcgttcg tgcatcccag    360
gagctcgcgg cttccgctga gaccatcact aagacccgcg agtccgttgc ttacgcactc    420
aaggctgacc gcgaagctac cgcagctttc gaggcttacc tcagcgctct tcgtcaggtt    480
tcagtcatca acgatctgat cgctgatgct aacgccaaga acaagactga ctttgcagag    540
atcgagctct acgatgttct ttacaccgac gccgacatct ctggcgatgc tccacttctt    600
gctcctgcat acaaggagct gaaggacctt caggctgagg ttgacgcaga cttcgagtgg    660
ttgggcgagt tcgcaattga taacaatgaa gacaactacg tcattcgtac tcacatccct    720
```

```
gctgtagagg cactcaaggc agcgatcgat tcactggtcg acaccgttga gccacttcgt    780
gcagacgcta tcgctaagaa catcgaggct cagaagtctg acgttctggt tccccagctc    840
ttcctcgagc gtgcaactgc acagcgcgac accctgcgtg ttgtagaggc aatcttctct    900
acctctgctc gttacgttga actctacgag aacgtcgaga acgttaacgt tgagaacaag    960
acccttcgcc agcactactc ttccctgatc cctaacctct tcatcgcagc ggttggcaac   1020
atcaacgagc tcaacaatgc agatcaggct gcacgtgagc tcttcctcga ttgggacacc   1080
gacctcacca ccaacgatga ggacgaagct tactaccagg ctaagctcga cttcgctatc   1140
gagacctacg caaagatcct gatcaacggt gaagtttggc aggagccact cgcttacgtc   1200
cagaacctgg atgcaggcgc acgtcaggaa gcagctgacc gcgaagcaga gcgcgcagct   1260
gacgcagcat accgcgctga gcagctccgc atcgctcagg aagcagctga cgctcagaag   1320
gctctcgctg aggctcttgc taatgcaggc aacaacgaca acggtggcga caactcctcc   1380
gacgacaagg gaaccggttc ttccgacatc ggaacctggg gacctttcgc agcaattgca   1440
gctatcatcg cagcaatcgc agctatcttc ccattcctct ccggtatcgt taagttctaa   1500
```

<210> 68

<211> 499

<212> PRT

<213> Corynebacterium glutamicum

<400> 68

```
Met Phe Asn Asn Arg Ile Arg Thr Ala Ala Leu Ala Gly Ala Ile Ala
1               5                  10                  15

Ile Ser Thr Ala Ala Ser Gly Val Ala Ile Pro Ala Phe Ala Gln Glu
            20                  25                  30
Thr Asn Pro Thr Phe Asn Ile Asn Asn Gly Phe Asn Asp Ala Asp Gly
        35                  40                  45
Ser Thr Ile Gln Pro Val Glu Pro Val Asn His Thr Glu Glu Thr Leu
    50                  55                  60
Arg Asp Leu Thr Asp Ser Thr Gly Ala Tyr Leu Glu Glu Phe Gln Tyr
65                  70                  75                  80
Gly Asn Val Glu Glu Ile Val Glu Ala Tyr Leu Gln Val Gln Ala Ser
                85                  90                  95
Ala Asp Gly Phe Asp Pro Ser Glu Gln Ala Ala Tyr Glu Ala Phe Glu
            100                 105                 110
Ala Ala Arg Val Arg Ala Ser Gln Glu Leu Ala Ala Ser Ala Glu Thr
        115                 120                 125
Ile Thr Lys Thr Arg Glu Ser Val Ala Tyr Ala Leu Lys Ala Asp Arg
    130                 135                 140
Glu Ala Thr Ala Ala Phe Glu Ala Tyr Leu Ser Ala Leu Arg Gln Val
145                 150                 155                 160
Ser Val Ile Asn Asp Leu Ile Ala Asp Ala Asn Ala Lys Asn Lys Thr
                165                 170                 175
Asp Phe Ala Glu Ile Glu Leu Tyr Asp Val Leu Tyr Thr Asp Ala Asp
            180                 185                 190
Ile Ser Gly Asp Ala Pro Leu Leu Ala Pro Ala Tyr Lys Glu Leu Lys
        195                 200                 205
Asp Leu Gln Ala Glu Val Asp Ala Asp Phe Glu Trp Leu Gly Glu Phe
    210                 215                 220
Ala Ile Asp Asn Asn Glu Asp Asn Tyr Val Ile Arg Thr His Ile Pro
225                 230                 235                 240
Ala Val Glu Ala Leu Lys Ala Ala Ile Asp Ser Leu Val Asp Thr Val
                245                 250                 255
Glu Pro Leu Arg Ala Asp Ala Ile Ala Lys Asn Ile Glu Ala Gln Lys
            260                 265                 270
Ser Asp Val Leu Val Pro Gln Leu Phe Leu Glu Arg Ala Thr Ala Gln
        275                 280                 285
Arg Asp Thr Leu Arg Val Val Glu Ala Ile Phe Ser Thr Ser Ala Arg
    290                 295                 300
Tyr Val Glu Leu Tyr Glu Asn Val Glu Asn Val Asn Val Glu Asn Lys
305                 310                 315                 320
Thr Leu Arg Gln His Tyr Ser Ser Leu Ile Pro Asn Leu Phe Ile Ala
                325                 330                 335
Ala Val Gly Asn Ile Asn Glu Leu Asn Asn Ala Asp Gln Ala Ala Arg
            340                 345                 350
Glu Leu Phe Leu Asp Trp Asp Thr Asp Leu Thr Thr Asn Asp Glu Asp
        355                 360                 365
Glu Ala Tyr Tyr Gln Ala Lys Leu Asp Phe Ala Ile Glu Thr Tyr Ala
    370                 375                 380
Lys Ile Leu Ile Asn Gly Glu Val Trp Gln Glu Pro Leu Ala Tyr Val
```

```
385                 390                 395                 400
Gln Asn Leu Asp Ala Gly Ala Arg Gln Glu Ala Ala Asp Arg Glu Ala
                405                 410                 415
Glu Arg Ala Ala Asp Ala Ala Tyr Arg Ala Glu Gln Leu Arg Ile Ala
            420                 425                 430
Gln Glu Ala Ala Asp Ala Gln Lys Ala Leu Ala Glu Ala Leu Ala Asn
        435                 440                 445
Ala Gly Asn Asn Asp Asn Gly Gly Asp Asn Ser Ser Asp Asp Lys Gly
    450                 455                 460
Thr Gly Ser Ser Asp Ile Gly Thr Trp Gly Pro Phe Ala Ala Ile Ala
465                 470                 475                 480
Ala Ile Ile Ala Ala Ile Ala Ala Ile Phe Pro Phe Leu Ser Gly Ile
                485                 490                 495
Val Lys Phe
```

<210> 69

<211> 318

<212> DNA

<213> Corynebacterium glutamicum

<400> 69

```
atgtccctcg gaccatggga aattggaatc attgtcctgc tgatcatcgt gctgttcggc     60
gcgaagaagc tgcctgatgc agctcgttcc atcggccgtt ccatgcgcat cttcaagtct    120
gaagtcaaag aaatgaacaa ggacggcgat accccagaac aacagcagca gcctcagcag    180
cagattgcgc ccaaccagat cgaggctcct cagccaaact ttgagcagca ctaccaggga    240
cagcaggttc agcagcctca gaaccctcag acccctgact accgtcagaa ctacgaggat    300
ccaaaccgca cctcttaa                                                  318
```

<210> 70

<211> 105

<212> PRT

<213> Corynebacterium glutamicum

<400> 70

```
Met Ser Leu Gly Pro Trp Glu Ile Gly Ile Ile Val Leu Leu Ile Ile
1               5                   10                  15
Val Leu Phe Gly Ala Lys Lys Leu Pro Asp Ala Ala Arg Ser Ile Gly
            20                  25                  30
Arg Ser Met Arg Ile Phe Lys Ser Glu Val Lys Glu Met Asn Lys Asp
        35                  40                  45
Gly Asp Thr Pro Glu Gln Gln Gln Gln Pro Gln Gln Gln Ile Ala Pro
    50                  55                  60
Asn Gln Ile Glu Ala Pro Gln Pro Asn Phe Glu Gln His Tyr Gln Gly
65                  70                  75                  80
Gln Gln Val Gln Gln Pro Gln Asn Pro Gln Thr Pro Asp Tyr Arg Gln
                85                  90                  95
Asn Tyr Glu Asp Pro Asn Arg Thr Ser
            100                 105
```

<210> 71

<211> 471

<212> DNA

<213> Corynebacterium glutamicum

<400> 71

```
atgttttcta gcgtgggttg gggagagatc ttcctcttag tcgttgtggg ccttgttgtc     60
atcggcccgg aacggttgcc tcgtttgatc caggacgcac gcgctgcgct gctcgctgca    120
cgtaccgcta tcgacaatgc aaagcagtcg ttggacagtg attttggttc ggaatttgat    180
gaaatccgaa agccactaac ccaggttgca cagtacagcc ggatgagccc caagacggcc    240
atcactaagg cgttatttga taatgattcc tcgttcctgg atgactttga tccaaagaag    300
atcatggccg aaggaacaga aggcgaagct cagcgcaaca agcaggcagc tgacaacaat    360
gcgaatgtgg tggaacgtcc agctgatggt tccaccgcac gcccaacgca aaacgatcca    420
aaagacggcc cgaattactc aggtggcgtc tcttggaccg atattattta g             471
```

<210> 72

<211> 156

<212> PRT

<213> Corynebacterium glutamicum

<400> 72

```
Met Phe Ser Ser Val Gly Trp Gly Glu Ile Phe Leu Leu Val Val Val
1               5                  10                  15
Gly Leu Val Val Ile Gly Pro Glu Arg Leu Pro Arg Leu Ile Gln Asp
            20                  25                  30

Ala Arg Ala Ala Leu Leu Ala Ala Arg Thr Ala Ile Asp Asn Ala Lys
        35                  40                  45
Gln Ser Leu Asp Ser Asp Phe Gly Ser Glu Phe Asp Glu Ile Arg Lys
    50                  55                  60
Pro Leu Thr Gln Val Ala Gln Tyr Ser Arg Met Ser Pro Lys Thr Ala
65                  70                  75                  80
Ile Thr Lys Ala Leu Phe Asp Asn Asp Ser Ser Phe Leu Asp Asp Phe
                85                  90                  95
Asp Pro Lys Lys Ile Met Ala Glu Gly Thr Glu Gly Glu Ala Gln Arg
            100                 105                 110
Asn Lys Gln Ala Ala Asp Asn Asn Ala Asn Val Val Glu Arg Pro Ala
        115                 120                 125
Asp Gly Ser Thr Ala Arg Pro Thr Gln Asn Asp Pro Lys Asp Gly Pro
    130                 135                 140
Asn Tyr Ser Gly Gly Val Ser Trp Thr Asp Ile Ile
145                 150                 155
```

<210> 73

<211> 945

<212> DNA

<213> Corynebacterium glutamicum

<400> 73

```
atgtccattg ttgagcacat caaagagttt cgacgccgac ttcttatcgc tctggcgggc     60
atcctcgtgg gcaccattat cggctttatt tggtacgatt tctcattttg gcagatcccc    120
actttgggcg agctgctgag ggatccgtac tgttctctgc ctgctgaatc ccgctgggcc    180
atgagcgact cagaggaatg tcgactgctc gcaaccggcc cgtttgatcc attcatgctt    240
cgccttaaag tagcggcgtt ggtgggtatg gttcttggct cacccgtgtg gctgagccag    300
ctgtggggct ttatcacccc aggtttgatg aagaatgagc gccgttacac cgcaatcttc    360
gtcacgattg ctgttgtgct gtttgtcggc ggtgctgttc ttgcgtactt cgtcgttgca    420
tatggtttgg agttcctcct taccattggt ggagacaccc aggcagcggc cctgactggt    480
gataagtact tcggattctt gctcgcgttg ttggcgattt tcggcgtgag cttcgaagtt    540
ccactggtga tcggcatgct caacattgtg ggtatcttgc cttacgatgc cattaaagat    600
aagcgacgca tgatcatcat gattttgttc gtgttcgctg ctttcatgac acccggccag    660
gatcctttca ccatgttggt gttggcgctt tcactcaccg ttctggtaga gcttgccctg    720
cagttctgtc gtttcaacga caaacgccgg gacaagaagc gcccagaatg gcttgatggc    780
gatgacctct ctgcatcacc actggatact tctgctggtg gagaagatgc tccaagccca    840
gtcgaaaccc cagaggcggt ggagccttcg cggatgctga acccaagtgg ggaggcgtcg    900
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<211> 314

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<213> Corynebacterium glutamicum

<400> **74**

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            20                  25                  30
```

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Asp Phe Ser Phe Trp Gln Ile Pro Thr Leu Gly Glu Leu Leu Arg Asp
        35                  40                  45
Pro Tyr Cys Ser Leu Pro Ala Glu Ser Arg Trp Ala Met Ser Asp Ser
    50                  55                  60
Glu Glu Cys Arg Leu Leu Ala Thr Gly Pro Phe Asp Pro Phe Met Leu
65                  70                  75                  80
Arg Leu Lys Val Ala Ala Leu Val Gly Met Val Leu Gly Ser Pro Val
                85                  90                  95
Trp Leu Ser Gln Leu Trp Gly Phe Ile Thr Pro Gly Leu Met Lys Asn
            100                 105                 110
Glu Arg Arg Tyr Thr Ala Ile Phe Val Thr Ile Ala Val Val Leu Phe

        115                 120                 125
Val Gly Gly Ala Val Leu Ala Tyr Phe Val Val Ala Tyr Gly Leu Glu
    130                 135                 140
Phe Leu Leu Thr Ile Gly Gly Asp Thr Gln Ala Ala Ala Leu Thr Gly
145                 150                 155                 160
Asp Lys Tyr Phe Gly Phe Leu Leu Ala Leu Leu Ala Ile Phe Gly Val
                165                 170                 175
Ser Phe Glu Val Pro Leu Val Ile Gly Met Leu Asn Ile Val Gly Ile
            180                 185                 190
Leu Pro Tyr Asp Ala Ile Lys Asp Lys Arg Arg Met Ile Ile Met Ile
        195                 200                 205
Leu Phe Val Phe Ala Ala Phe Met Thr Pro Gly Gln Asp Pro Phe Thr
    210                 215                 220
Met Leu Val Leu Ala Leu Ser Leu Thr Val Leu Val Glu Leu Ala Leu
225                 230                 235                 240
Gln Phe Cys Arg Phe Asn Asp Lys Arg Arg Asp Lys Lys Arg Pro Glu
                245                 250                 255
Trp Leu Asp Gly Asp Asp Leu Ser Ala Ser Pro Leu Asp Thr Ser Ala
            260                 265                 270
Gly Gly Glu Asp Ala Pro Ser Pro Val Glu Thr Pro Glu Ala Val Glu
        275                 280                 285
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<210> 75

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<400> 75

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<210> 76

<211> 27

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<400> 76

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<400> 77

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<210> 78

<211> 34

<212> PRT

<213> Bacillus subtilis

<400> 78

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            20                  25                  30
Glu His
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<210> 79

<211> 30

<212> PRT

<213> Arthrobacter globiformis

<400> 79

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<221> misc-feature

<222> (1)..(1)

<223> Xaa can be any naturally occurring amino acid

<220>

<221> misc-feature

<222> (4)..(4)

<223> Xaa can be any naturally occurring amino acid

<400> 80

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<210> 81

<211> 5

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<210> 82

<211> 43

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<213> Corynebacterium glutamicum

<400> 82

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<210> 83

<211> 30

<212> PRT

<213> Corynebacterium glutamicum

<400> 83

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<210> 84

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<213> Corynebacterium stationis

<400> 84

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<210> 85

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<212> PRT

<213> Corynebacterium glutamicum

<400> 85

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            20                  25                  30
Thr Leu Arg Asp Leu Thr Asp Ser Thr Gly Ala Tyr Leu Glu Glu Phe
        35                  40                  45
Gln Tyr Gly Asn Val Glu Glu Ile Val Glu Ala Tyr Leu Gln Val Gln
    50                  55                  60
Ala Ser Ala Asp Gly Phe Asp Pro Ser Glu Gln Ala Ala Tyr Glu Ala

65                  70                  75                  80
Phe Glu Ala Ala Arg Val Arg Ala Ser Gln Glu Leu Ala Ala Ser Ala
                85                  90                  95
Glu Thr Ile Thr Lys Thr Arg Glu Ser Val Ala Tyr Ala Leu Lys Ala
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Asp Arg Glu Ala Thr Ala Ala Phe Glu Ala Tyr Leu Ser Ala Leu Arg
        115                 120                 125
Gln Val Ser Val Ile Asn Asp Leu Ile Ala Asp Ala Asn Ala Lys Asn
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Lys Thr Asp Phe Ala Glu Ile Glu Leu Tyr Asp Val Leu Tyr Thr Asp
145                 150                 155                 160
Ala Asp Ile Ser Gly Asp Ala Pro Leu Leu Ala Pro Ala Tyr Lys Glu
                165                 170                 175
Leu Lys Asp Leu Gln Ala Glu Val Asp Ala Asp Phe Glu Trp Leu Gly
            180                 185                 190
Glu Phe Ala Ile Asp Asn Asn Glu Asp Asn Tyr Val Ile Arg Thr His
        195                 200                 205
Ile Pro Ala Val Glu Ala Leu Lys Ala Ala Ile Asp Ser Leu Val Asp
    210                 215                 220
Thr Val Glu Pro Leu Arg Ala Asp Ala Ile Ala Lys Asn Ile Glu Ala
225                 230                 235                 240
Gln Lys Ser Asp Val Leu Val Pro Gln Leu Phe Leu Glu Arg Ala Thr
                245                 250                 255
Ala Gln Arg Asp Thr Leu Arg Val Val Glu Ala Ile Phe Ser Thr Ser
            260                 265                 270
Ala Arg Tyr Val Glu Leu Tyr Glu Asn Val Glu Asn Val Asn Val Glu
        275                 280                 285
Asn Lys Thr Leu Arg Gln His Tyr Ser Ser Leu Ile Pro Asn Leu Phe
    290                 295                 300
Ile Ala Ala Val Gly Asn Ile Asn Glu Leu Asn Asn Ala Asp Gln Ala
305                 310                 315                 320
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                325                 330                 335
Glu Asp Glu Ala Tyr Tyr Gln Ala Lys Leu Asp Phe Ala Ile Glu Thr
            340                 345                 350
Tyr Ala Lys Ile Leu Ile Asn Gly Glu Val Trp Gln Glu Pro Leu Ala
        355                 360                 365
Tyr Val Gln Asn Leu Asp Ala Gly Ala Arg Gln Glu Ala Ala Asp Arg
    370                 375                 380
Glu Ala Glu Arg Ala Ala Asp Ala Ala Tyr Arg Ala Glu Gln Leu Arg
385                 390                 395                 400
Ile Ala Gln Glu Ala Ala Asp Ala Gln Lys Ala Leu Ala Glu Ala Leu
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            420                 425                 430
Lys Gly Thr Gly Ser Ser Asp Ile Gly Thr Trp Gly Pro Phe Ala Ala
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Ile Ala Ala Ile Ile Ala Ala Ile Ala Ala Ile Phe Pro Phe Leu Ser
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<210> 86

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<400> 87
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<220>
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<210> 93
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<400> 93
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<210> 94

<211> 4

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<400> 94

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<210> 95

<211> 6

<212> PRT

<213> Artificial Sequence

<220>

<223> ProTEV

<400> 95

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1 5

<210> 96

<211> 708

<212> DNA

<213> Corynebacterium glutamicum

<400> 96

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ggcaatgaag ccctgaagat tgctcgtgag ttccgtccag acgcatacat cctcgatgtc 180
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atcggcgctg acgactacgt gaccaagcct ttctccctgg aagaagtaat cacccgcctg 360
cgcgtgattc ttcgtcgcgg tggagcagtt gaagaagaca cctcaacttc cctgcagtac 420
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tccaaggcaa agatcctgga taacgtgtgg cactacgatt ttggtggcga cggcaacgtc 600
gtggaatcct acatctccta cctgcgccgc aaggtggaca cccaggatcc gcagctaatt 660
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<210> 97

<211> 235

<212> PRT

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<400> 97

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Phe Ala Val Met Thr Ala Asn Asp Gly Asn Glu Ala Leu Lys Ile Ala
35 40 45
Arg Glu Phe Arg Pro Asp Ala Tyr Ile Leu Asp Val Met Met Pro Gly
50 55 60

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    50                  55                  60
Met Asp Gly Phe Glu Leu Leu Thr Lys Leu Arg Gly Glu Gly Leu Asp
65                  70                  75                  80
Ser Pro Val Leu Tyr Leu Thr Ala Lys Asp Ala Val Glu His Arg Ile
                85                  90                  95
His Gly Leu Thr Ile Gly Ala Asp Asp Tyr Val Thr Lys Pro Phe Ser
            100                 105                 110
Leu Glu Glu Val Ile Thr Arg Leu Arg Val Ile Leu Arg Arg Gly Gly
        115                 120                 125
Ala Val Glu Glu Asp Thr Ser Thr Ser Leu Gln Tyr Ala Asp Leu Thr
    130                 135                 140
Leu Asn Asp Glu Thr His Glu Val Thr Lys Ala Gly Glu Leu Ile Asp
145                 150                 155                 160
Leu Ser Pro Thr Glu Phe Asn Leu Leu Arg Tyr Leu Met Leu Asn Ala
                165                 170                 175
Glu Val Val Leu Ser Lys Ala Lys Ile Leu Asp Asn Val Trp His Tyr
            180                 185                 190
Asp Phe Gly Gly Asp Gly Asn Val Val Glu Ser Tyr Ile Ser Tyr Leu
        195                 200                 205
Arg Arg Lys Val Asp Thr Gln Asp Pro Gln Leu Ile Gln Thr Val Arg
    210                 215                 220
Gly Val Gly Tyr Val Leu Arg Thr Pro Arg Ser
225                 230                 235
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Patentkrav

1. Fremgangsmåde til fremstilling af et heterologt protein omfattende:
dyrkning af en coryneform bakterie med et genetisk konstrukt til sekretorisk ekspression af det heterologe protein; og
opsamling af det heterologe protein fremstillet ved sekretorisk produktion,
hvor den coryneforme bakterie er blevet modificeret, således at den rummer et *phoS*-gen, som koder for et mutant PhoS-protein,
hvor det mutante PhoS-protein er et PhoS-protein med en mutation af erstatning af en aminosyrerest svarende til tryptophanresten ved position 302 i SEQ ID NO: 4 med en lysinrest, alaninrest, valinrest, serinrest, cysteinrest, methioninrest, asparaginsyrerest eller asparaginrest i et vild-type PhoS-protein,
hvor det genetiske konstrukt, i retningen fra 5' til 3', omfatter en promotor-sekvens, som fungerer i den coryneforme bakterie, en nukleinsyresekvens som koder for et signalpeptid, som fungerer i den coryneforme bakterie, og en nukleinsyresekvens som koder for det heterologe protein, og
hvor det heterologe protein udtrykkes som et fusionsprotein med signalpeptidet.

2. Fremgangsmåden ifølge krav 1, hvor vild-type PhoS-proteinet er et protein defineret i (a), (b) eller (c) nævnt nedenfor:
(a) et protein omfattende aminosyresekvensen af SEQ ID NO: 4, 54, 55, 56, 57 eller 58;
(b) et protein omfattende aminosyresekvensen af SEQ ID NO: 4, 54, 55, 56, 57 eller 58, men som inkluderer substitution, deletion, insertion eller addition af 1 til 10 aminosyrerester;
(c) et protein omfattende en aminosyresekvens med en identitet på 90% eller højere i forhold til aminosyresekvensen af SEQ ID NO: 4, 54, 55, 56, 57 eller 58.

3. Fremgangsmåden ifølge krav 1 eller 2, hvor signalpeptidet er et Tat-afhængigt signalpeptid.

4. Fremgangsmåden ifølge krav 3, hvor det Tat-afhængige signalpeptid er valgt fra gruppen bestående af et TorA-signalpeptid, SufI-signalpeptid, PhoD-signalpeptid, LipA-signalpeptid og IMD-signalpeptid.

5. Fremgangsmåden ifølge krav 1 eller 2, hvor signalpeptidet er et Sec-afhængigt signalpeptid.

6. Fremgangsmåden ifølge krav 5, hvor det Sec-afhængige signalpeptid er valgt fra gruppen bestående af et PS1-signalpeptid, PS2-signalpeptid og SlpA-signalpeptid.

7. Fremgangsmåden ifølge et hvilket som helst af kravene 1 til 6, hvor den coryneforme bakterie er en bakterie tilhørende slægten *Corynebacterium,* fortrinsvis hvor den coryneforme bakterie er *Corynebacterium glutamicum.*

8. Coryneform bakterie,

som er blevet modificeret, således at den rummer et *phoS*-gen, som koder for et mutant PhoS-protein,

hvor det mutante PhoS-protein er et PhoS-protein med en mutation af erstatning af en aminosyrerest svarende til tryptophanresten ved position 302 i SEQ ID NO: 4 med en lysinrest, alaninrest, valinrest, serinrest, cysteinrest, methioninrest, asparaginsyrerest eller asparaginrest i et vild-type PhoS-protein.

9. Den coryneforme bakterie ifølge krav 8, hvor vild-type PhoS-proteinet er et protein defineret i (a), (b) eller (c) nævnt nedenfor:

(a) et protein omfattende aminosyresekvensen af SEQ ID NO: 4, 54, 55, 56, 57 eller 58;

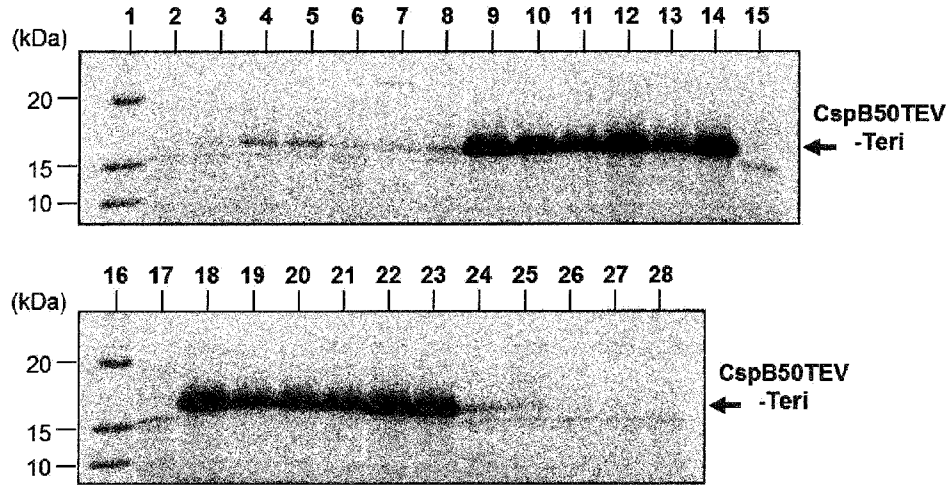
(b) et protein omfattende aminosyresekvensen af SEQ ID NO: 4, 54, 55, 56, 57 eller 58, men som inkluderer substitution, deletion, insertion eller addition af 1 til 10 aminosyrerester;

(c) et protein omfattende en aminosyresekvens med en identitet på 90% eller højere i forhold til aminosyresekvensen af SEQ ID NO: 4, 54, 55, 56, 57 eller 58.

10. Den coryneforme bakterie ifølge krav 8 eller 9, hvor den coryneforme bakterie er en bakterie tilhørende slægten *Corynebacterium,*
fortrinsvis hvor den coryneforme bakterie er *Corynebacterium glutamicum.*

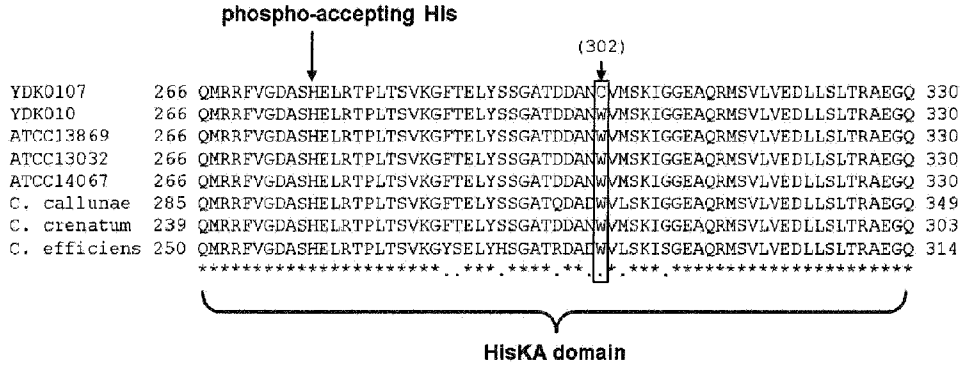
DRAWINGS

[Fig. 1]

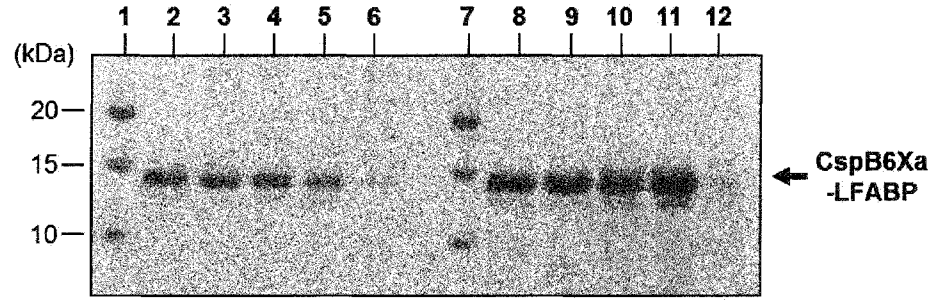


1,	Marker (XL-Ladder Broad, APRO science)
2,	YDK010/pPK4 (negative control)
3-8,	YDK010/pPKK50TEV-Teri
9-14,	YDK0107/pPKK50TEV-Teri
15,	YDK0107/pPK4 (negative control)
16,	Marker (XL-Ladder Broad, APRO science)
17,	YDK010::phoS(W302C)/pPK4 (negative control)
18-23,	YDK010::phoS(W302C)/pPKK50TEV-Teri
24-27,	YDK010ΔphoS/pPKK50TEV-Teri
28,	YDK010ΔphoS(W302C)/pPK4 (negative control)

[Fig. 2]

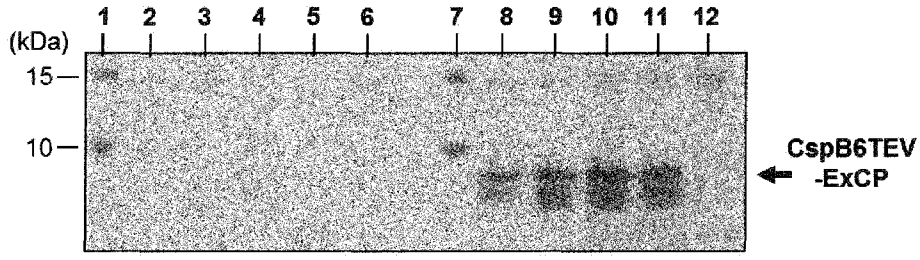

phospho-accepting His
(302)
YDK0107 266 QMRRFVGDASHELRTPLTSVKGFTELYSSGATDDANCVMSKIGGEAQRMSVLVEDLLSLTRAEGQ 330
YDK010 266 QMRRFVGDASHELRTPLTSVKGFTELYSSGATDDANWVMSKIGGEAQRMSVLVEDLLSLTRAEGQ 330
ATCC13869 266 QMRRFVGDASHELRTPLTSVKGFTELYSSGATDDANWVMSKIGGEAQRMSVLVEDLLSLTRAEGQ 330
ATCC13032 266 QMRRFVGDASHELRTPLTSVKGFTELYSSGATDDANWVMSKIGGEAQRMSVLVEDLLSLTRAEGQ 330
ATCC14067 266 QMRRFVGDASHELRTPLTSVKGFTELYSSGATDDANWVMSKIGGEAQRMSVLVEDLLSLTRAEGQ 330
C. callunae 285 QMRRFVGDASHELRTPLTSVKGFTELYSSGATQDADWVLSKIGGEAQRMSVLVEDLLSLTRAEGQ 349
C. crenatum 239 QMRRFVGDASHELRTPLTSVKGFTELYSSGATDDANWVMSKIGGEAQRMSVLVEDLLSLTRAEGQ 303
C. efficiens 250 QMRRFVGDASHELRTPLTSVKGYSELYHSGATRDADWVLSKISGEAQRMSVLVEDLLSLTRAEGQ 314
HisKA domain

[Fig. 3]



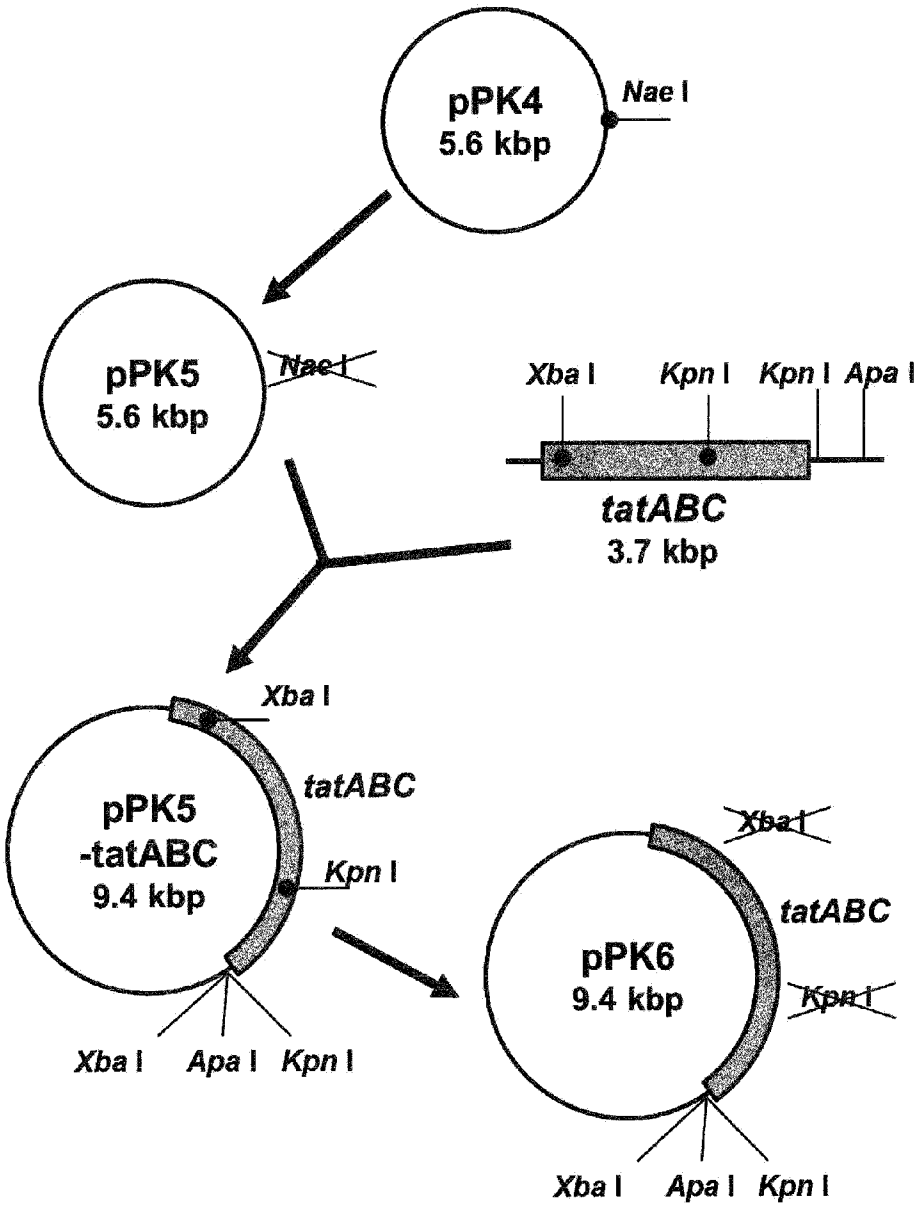
1, Marker (XL-Ladder Broad, APRO science)
2-5, YDK010/pPK4_CspB6Xa-LFABP
6, YDK010/pPK4 (negative control)
7, Marker (XL-Ladder Broad, APRO science)
8-11, YDK010::phoS(W302C)/pPK4_CspB6Xa-LFABP
12, YDK010::phoS(W302C)/pPK4 (negative control)

[Fig. 4]

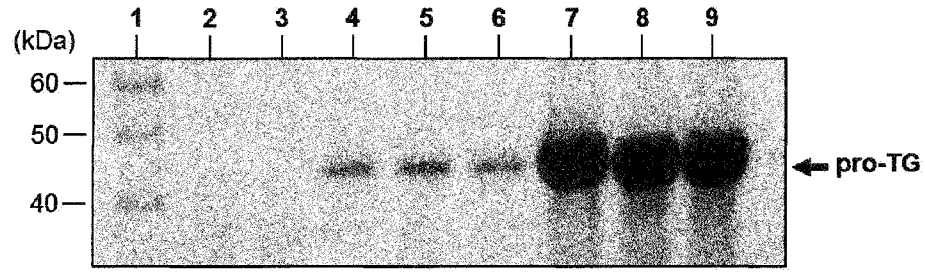


1, Marker (XL-Ladder Broad, APRO science)
2-5, YDK010/pPK4_CspB6TEV-ExCP
6, YDK010/pPK4 (negative control)
7, Marker (XL-Ladder Broad, APRO science)
8-11, YDK010::phoS(W302C)/pPK4_CspB6TEV-ExCP
12, YDK010::phoS(W302C)/pPK4 (negative control)

[Fig. 5]

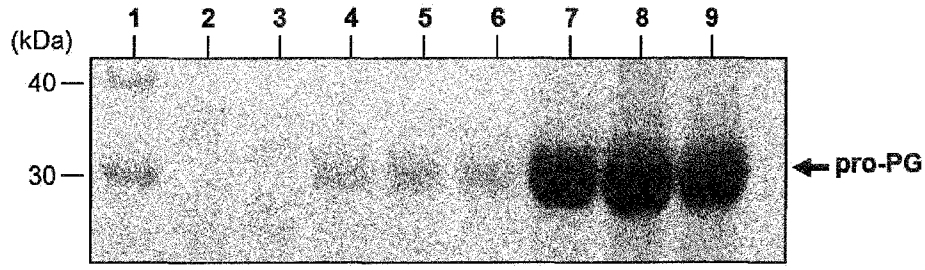

pPK4
5.6 kbp
Nae I
pPK5
5.6 kbp
Nae I
Xba I
Kpn I
Kpn I
Apa I
tatABC
3.7 kbp
Xba I
tatABC
pPK5
-tatABC
9.4 kbp
Kpn I
Xba I
Apa I
Kpn I
Xba I
tatABC
pPK6
9.4 kbp
Kpn I
Xba I
Apa I
Kpn I

[Fig. 6]



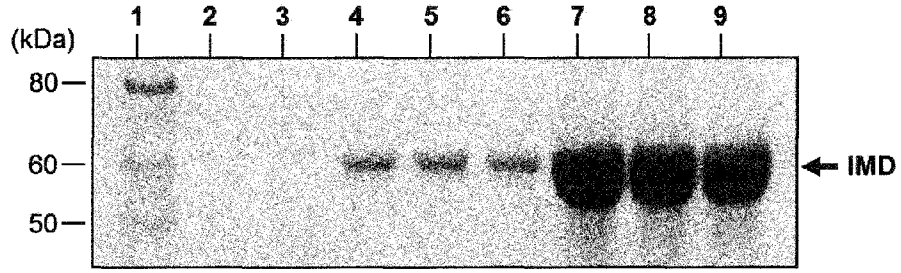
1, Marker (XL-Ladder Broad, APRO science)
2, YDK010/pPK6 (negative control)
3, YDK010::phoS(W302C)/pPK6 (negative control)
4-6, YDK010/pPK6_T_PTG
7-9, YDK010::phoS(W302C)/pPK6_T_PTG

[Fig. 7]



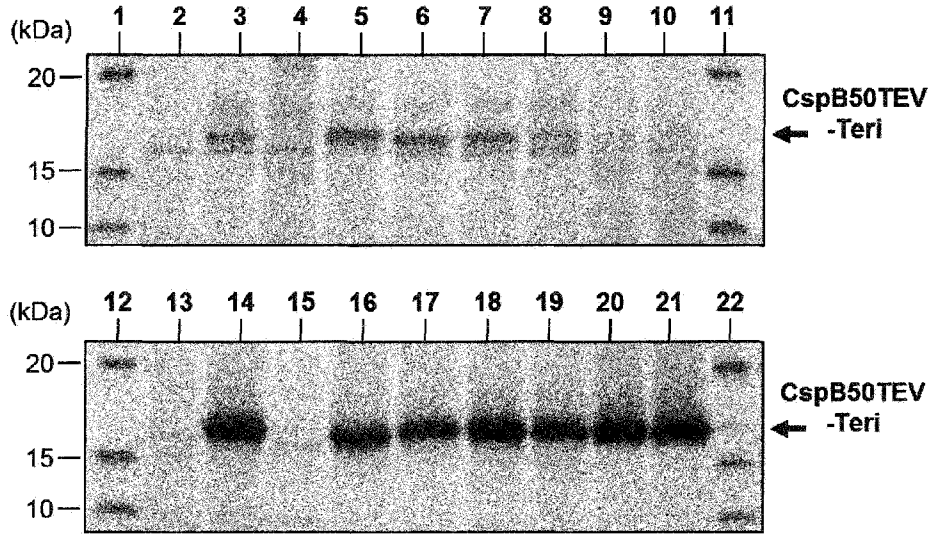
1, Marker (XL-Ladder Broad, APRO science)
2, YDK010/pPK6 (negative control)
3, YDK010::phoS(W302C)/pPK6 (negative control)
4-6, YDK010/pPK6_T_PPG
7-9, YDK010::phoS(W302C)/pPK6_T_PPG

[Fig. 8]



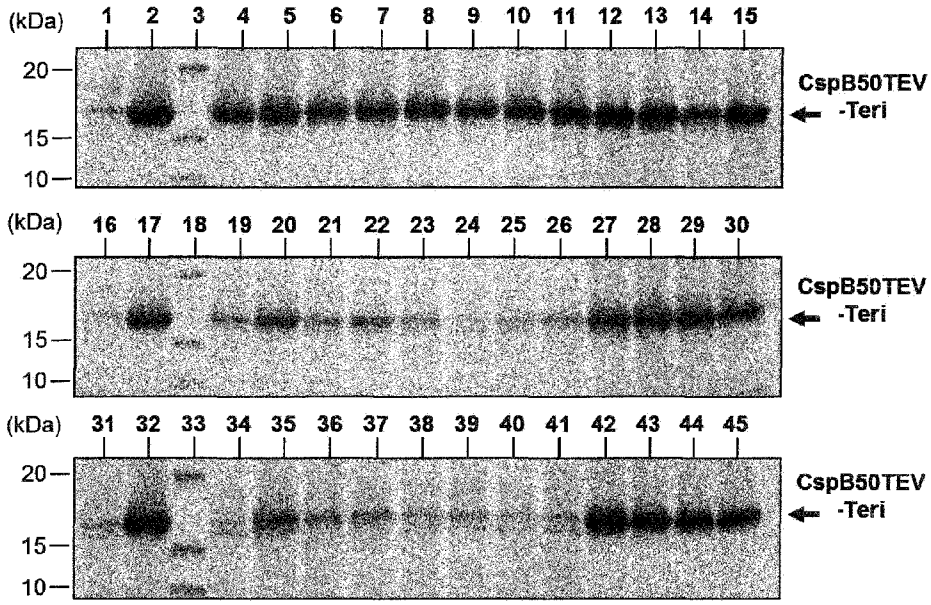
1, Marker (XL-Ladder Broad, APRO science)
2, YDK010/pPK6 (negative control)
3, YDK010::phoS(W302C)/pPK6 (negative control)
4-6, YDK010/pPK6_I_IMD
7-9, YDK010::phoS(W302C)/pPK6_I_IMD

[Fig. 9]



1,	Marker (XL-Ladder Broad, APRO science)
2,	YDK010/pPK4
3,	YDK010/pPKK50TEV-Teri (positive control)
4,	YDK010ΔphoS/pVphoS(WT)/pPK4
5-10,	YDK010ΔphoS/pVphoS(WT)/pPKK50TEV-Teri
11,	Marker (XL-Ladder Broad, APRO science)
12,	Marker (XL-Ladder Broad, APRO science)
13,	YDK010::phoS(W302C)/pPK4
14,	YDK010::phoS(W302C)/pPKK50TEV-Teri (positive control)
15,	YDK010ΔphoS/pVphoS(W302C)/pPK4
16-21,	YDK010ΔphoS/pVphoS(W302C)/pPKK50TEV-Teri
22,	Marker (XL-Ladder Broad, APRO science)

[Fig. 10]

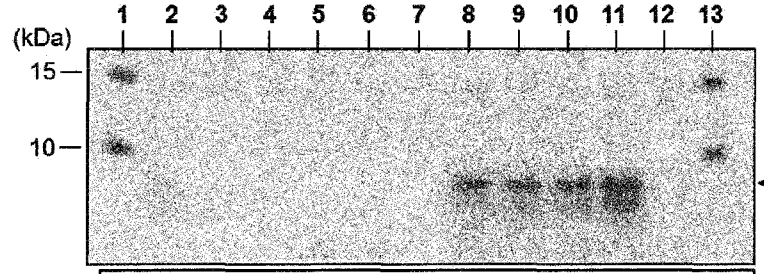


1, YDK010/pPKK50TEV-Teri
2, YDK010::phoS(W302C)/pPKK50TEV-Teri
3, Marker (XL-Ladder Broad, APRO science)
4-7, YDK010ΔphoS/pVphoS(W302S)/pPKK50TEV-Teri
8-11, YDK010ΔphoS/pVphoS(W302A)/pPKK50TEV-Teri
12-15, YDK010ΔphoS/pVphoS(W302V)/pPKK50TEV-Teri

16, YDK010/pPKK50TEV-Teri
17, YDK010::phoS(W302C)/pPKK50TEV-Teri
18, Marker (XL-Ladder Broad, APRO science)
19-22, YDK010ΔphoS/pVphoS(W302M)/pPKK50TEV-Teri
23-26, YDK010ΔphoS/pVphoS(W302F)/pPKK50TEV-Teri
27-30, YDK010ΔphoS/pVphoS(W302D)/pPKK50TEV-Teri

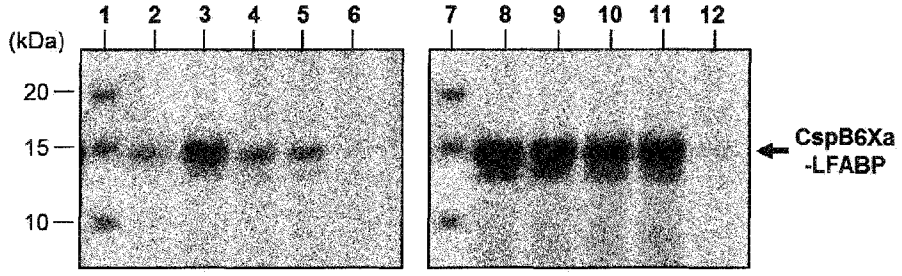
31, YDK010/pPKK50TEV-Teri
32, YDK010::phoS(W302C)/pPKK50TEV-Teri
33, Marker (XL-Ladder Broad, APRO science)
34-37, YDK010ΔphoS/pVphoS(W302N)/pPKK50TEV-Teri
38-41, YDK010ΔphoS/pVphoS(W302H)/pPKK50TEV-Teri
42-45, YDK010ΔphoS/pVphoS(W302K)/pPKK50TEV-Teri

[Fig. 11]



1, Marker (XL-Ladder Broad, APRO science)
2-6, ATCC13869/pPK4_CspB6TEV-ExCP
7, ATCC13869/pPK4 (negative control)
8-11, ATCC13869::phoS(W302C)/pPK4_CspB6TEV-ExCP
12, ATCC13869::phoS(W302C)/pPK4 (negative control)
13, Marker (XL-Ladder Broad, APRO science)

[Fig. 12]



1, Marker (XL-Ladder Broad, APRO science)
2-5, ATCC13869ΔcspB/pPK4_CspB6Xa-LFABP
6, ATCC13869ΔcspB/pPK4 (negative control)
7, Marker (XL-Ladder Broad, APRO science)
8-11, ATCC13869::phoS(W302C)ΔcspB/pPK4_CspB6Xa-LFABP
12, ATCC13869::phoS(W302C)ΔcspB/pPK4 (negative control)