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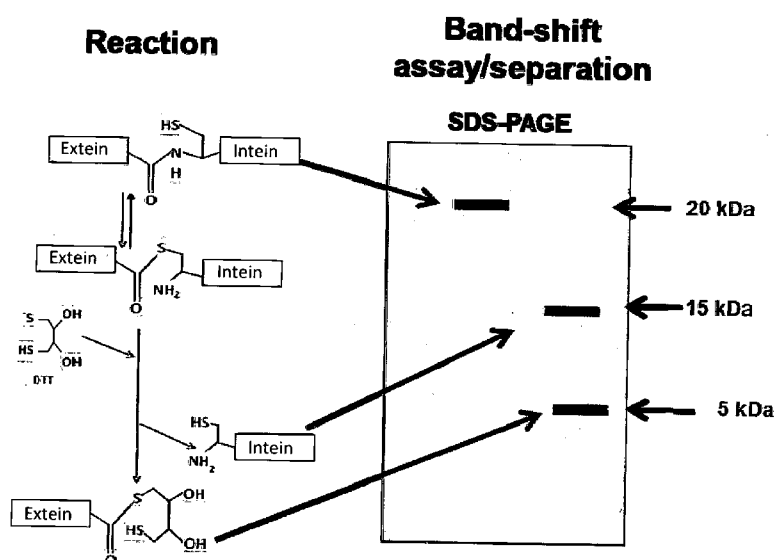
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[Continued on next page]

(54) Title: CORYNEFORM BACTERIUM AND METHOD FOR PRODUCING HETEROLOGOUS FUSION PROTEINS

FIG. 4

(57) **Abstract:** The present invention provides a coryneform bacterium having an ability to produce a heterologous fusion protein by secretory production, which has been modified to express a genetic construct for secretory production of the heterologous fusion protein encoding at least a heterologous fusion protein comprising an extein and an intein having an activity of acyl rearrangement. The method for producing proteins modified at the C-terminus is also provided.



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DESCRIPTION**CORYNEFORM BACTERIUM AND METHOD FOR PRODUCING
HETEROLOGOUS FUSION PROTEINS****Background of the Invention***Field of the Invention*

The present invention relates to the microbiological industry, and specifically to a coryneform bacterium which efficiently produces a heterologous fusion protein by secretory production and a method for secretory production of a heterologous fusion protein using the modified coryneform bacterium.

Description of the Related Art

Inteins are proteins which are capable of catalyzing their own excision from a precursor protein with the concomitant joining of the flanking protein sequences, known as exteins (Vila-Perelló M. and Muir T.W., Biological applications of protein splicing, *Cell*, 2010, 143(2):191-200). An intermediate forms during the intein-mediated protein splicing, which can be further modified chemically to obtain a recombinant protein or a peptide. In particular, the intermediate can be modified to produce proteins which are ligated, circularized or conjugated with radioisotopes or chemical drugs. Antibodies can also be site-specifically modified using an intein-mediated protein splicing. Various kinds of post-translational modifications of antibodies such as conjugation with polyethylene glycol (PEG), toxins, or radioisotopes have been investigated to enhance the efficacy of the antibody drugs, or add novel functions to the antibody drugs. For example, certolizumab pegol (Cimzia, UCB), a tumor-necrosis factor blocker which is a Fab-PEG conjugate, was approved by the FDA in 2008 for the treatment of adult patients with moderate-to severe Crohn's disease. Conjugation with PEG may enhance plasma half-life. Another example of modification of antibodies is a conjugation of an antibody or an antibody-related molecule with a toxin moiety (so called antibody-drug conjugates, ADC).

A method for protein engineering known as the «expressed protein ligation» has been developed which is suitable for modification of the C-terminus of a target protein of any size by a nucleophilic group-containing compounds (Muir T.W. et al., Expressed protein ligation: A general method for protein engineering, *Proc. Natl. Acad. Sci. USA*, 1998, 95:6705-6710). The method of «expressed protein ligation» was successfully applied for modification of the protein tyrosine kinase C-terminal Src kinase (Csk) at the C-terminus through the expression of a fusion protein and replacement of the intein with a synthetic phosphotyrosine peptide under the thiolysis conditions. The method for ligation of the expressed proteins is disclosed, which utilizes inteins such as the R1R1 intein from *Methanobacterium thermotrophicum* (US patent No. 6,849,428 B1; Evans T.C. Jr. et al., The in vitro ligation of bacterially expressed proteins using an intein from *Methanobacterium thermoautotrophicum*, *J. Biol. Chem.*, 1999, 274(7):3923-9326). The method for amidation of recombinant peptides expressed as fusion proteins containing inteins is also disclosed (Cottingham I.R. et al., A method for the amidation of recombinant peptides expressed as intein fusion proteins in *Escherichia coli*, *Nat. Biotechnol.*, 2001, 19:974-977). Most of the launched pharmaceutical peptides are amidated at their C-terminus to prolong the half-life *in vivo*. The peptide amidation technology utilizing combination of expression of the recombinant fusion proteins and intein-mediated amidation reaction is also an important technology to obtain C-terminal amidated peptides. The intein-mediated modifications can also be used for conjugation of proteins with labeled compounds such as radioisotopes or fluorescent compounds, biotin, etc. (Möhlmann S. et al., Site-specific modification of ED-B-targeting antibody using intein-fusion technology, *BMC Biotechnol.*, 2011, 11:76) and circularization of proteins (Sudheer P.D. et al., Cyclization tag for the detection and facile purification of backbone-cyclized proteins, *Anal. Biochem.*, 2013, 436:137-141).

To facilitate the intein-mediated protein modification, the target protein may be expressed as a fusion protein with an appropriate intein. In general, the target protein-intein fusion proteins are expressed in *Escherichia coli*. The IMPACT system from New England Biolabs (catalog No. N6951S) utilizing the

pTWIN vector is the most published commercial intein system in *E. coli*. Formation of an insoluble inclusion body may occur often when the target protein-intein fusion protein is expressed in *E. coli*. Therefore, one may have to establish a refolding process to obtain the active form of the target protein-intein fusion protein recovered from the insoluble fraction. Expression of a target protein-intein fusion protein into a culture medium is more favorable due to the convenience for recovering the folded target protein-intein fusion protein from the culture medium. Expression of a target protein-intein fusion protein into culture media has been demonstrated using a mammalian cell expression system (Möhlmann S. et al., Site-specific modification of ED-B-targeting antibody using intein-fusion technology, *BMC Biotechnol.*, 2011, 11:76).

Methods for obtaining fused target proteins from the culture supernatant using bacterial expression systems are known. For, example, a method for expression and secretion of the V_α , V_β and single chain V_α - V_β fragments of murine T-cell receptors fused with a signal peptide was reported for Gram-negative bacteria cells (US Patent No. 6399368 B1). A chimeric pro-region from *Streptomyces cinnamoneus* transglutaminase was used to attain a high level expression of *Streptomyces mobaraensis* transglutaminase as a fusion protein in *Corynebacterium glutamicum* (Date M. et al., *J. Biotechnol.*, 2004, 110(3):219-326). A method for the extracellular production of target proteins fused with an outer membrane protein OmpF and an oligopeptide for cleavage by a proteolytic enzyme utilizing *E. coli* as an expression host is known (US Patent No. 7491528 B2). Signal sequences from genes of *Pseudomonas fluorescens* and *Bacillus coagulans* for efficient secretion of correctly folded proteins from Gram-negative bacteria as expression hosts are described (EP 2108047 B1). Also, a process for the secretory production of an exogenous protein by using a coryneform bacterium is disclosed (WO 2001023591 A1). A target exogenous protein was expressed and excreted by a coryneform bacterium to a culture medium as a fusion protein with a signal peptide. The target protein was obtained by treating the extracellularly released fusion protein with a protease to cleave and eliminate the pro-part (WO 2001023591 A1).

Data demonstrating a secretory production of the target protein-intein fusion proteins (also referred to as the heterologous fusion proteins as described in the present invention) using a coryneform bacterium has not been previously reported.

Brief Summary of the Invention

An object of the present invention is to develop a novel technique for a secretory production of a heterologous fusion protein by a coryneform bacterium, and thereby to provide a coryneform bacterium that produces a heterologous fusion protein by secretory production and a method for secretory production of a heterologous fusion protein using the bacterium.

The authors of the present invention conducted various researches in order to achieve the aforementioned object, and as a result, they found that, in a method for producing a heterologous fusion protein utilizing a coryneform bacterium as an expression host, an ability of the coryneform bacterium to produce a heterologous fusion protein by secretory production could be imparted to the bacterium of the present invention by expressing a nucleic acid sequence encoding the heterologous fusion protein, and a method for producing a modified protein using the heterologous fusion protein.

The present invention thus provides the followings.

An aspect of the present invention is to provide a coryneform bacterium having an ability to produce a heterologous fusion protein by secretory production, which has been modified to express a genetic construct for secretory production of the heterologous fusion protein encoding at least a heterologous fusion protein, wherein the heterologous fusion protein comprises an extein and an intein having an activity of acyl rearrangement.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the extein further comprises a target protein.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the extein further comprises a linker,

which is linked to the C-terminus of the target protein in the extein and is in between the target protein and the intein.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the linker is a sequence of one or more amino acid residues.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the linker has the $\text{-NH-CH(R1)-CO-NH-CH(R2)-CO-}$ motif at the C-terminus, where R1 and R2 are a side-chain group of a proteinogenic L-amino acid of the same or different kinds, if the linker has a length of two or more amino acid residues.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the R1 is the side-chain group of any proteinogenic L-amino acid and R2 is the side-chain group of L-cysteine, or R1 is hydrogen and R2 is the side-chain group of L-cysteine.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the target protein is a heterologous protein for the coryneform bacterium.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the target protein is selected from the group consisting of a bioactive protein, a receptor protein, an antigenic protein, and an enzyme.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the bioactive protein is selected from the group consisting of a growth factor, a hormone, a cytokine, and an antibody-related molecule.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the bioactive protein is the exenatide as defined in the following (A) or (B):

(A) a protein having the amino acid sequence of SEQ ID NO: 34,

(B) a variant protein having the amino acid sequence of SEQ ID NO: 34, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues and has activity according to the amino acid sequence of SEQ ID NO: 34.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the antibody-related molecule consists of one or more proteins selected from Fab, F(ab')₂, an Fc-fusion protein, and scFv.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the Fab is a Fab for the trastuzumab consisting of the heavy chain as defined in the following (C) or (D) and a Fab for the trastuzumab consisting of the light chain as defined in the following (E) or (F):

(C) a protein having the amino acid sequence of SEQ ID NO: 35,

(D) a variant protein having the amino acid sequence of SEQ ID NO: 35, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues and has activity according to the amino acid sequence of SEQ ID NO: 35.

(E) a protein having the amino acid sequence of SEQ ID NO: 36,

(F) a variant protein having the amino acid sequence of SEQ ID NO: 36, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues and has activity according to the amino acid sequence of SEQ ID NO: 36.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the intein is defined in the following (G) or (H):

(G) an amino acid sequence of SEQ ID NO: 37,

(H) a variant amino acid sequence of SEQ ID NO: 37, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues and has activity of acyl rearrangement.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the intein is defined in the following (I) or (J), if the Fab is the Fab for the trastuzumab consisting of the heavy chain as defined in the preceding (C) or (D):

(I) an amino acid sequence of SEQ ID NO: 38, 40, 41 or 42,

(J) a variant amino acid sequence of SEQ ID NO: 38, 40, 41 or 42, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues and has activity of acyl rearrangement.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the intein is defined in the following (K) or (L), if the Fab is the Fab for the trastuzumab consisting of the light chain as defined in the preceding (E) or (F):

(K) an amino acid sequence of SEQ ID NO: 37,

(L) a variant amino acid sequence of SEQ ID NO: 37, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues and has activity of acyl rearrangement.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the bacterium has been modified further so that activity of a penicillin-binding protein is reduced.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the activity of the penicillin-binding protein is reduced by attenuating expression of a gene encoding the penicillin-binding protein or disrupting the gene.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the penicillin-binding protein is PBP1a or PBP1b.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the penicillin-binding protein is defined in the following (M) or (N):

(M) an amino acid sequence of SEQ ID NO: 44 or 46,

(N) a variant protein having the amino acid sequence of SEQ ID NO: 44 or 46, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues and has activity that if the activity thereof is reduced in the coryneform bacterium, amount of the heterologous fusion protein produced by secretory production is increased compared with that observed for a non-modified strain.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the bacterium has been modified further so that activity of a cell surface layer protein is reduced.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the activity of the cell surface layer protein is reduced by attenuating expression of a gene encoding the cell surface layer protein or disrupting the gene.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the cell surface layer protein is PS1, CspB, or CspA.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the cell surface layer protein is defined in the following (O) or (P):

(O) an amino acid sequence of SEQ ID NO: 56, 57 or 58,

(P) a variant protein having the amino acid sequence of SEQ ID NO: 56, 57 or 58, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues and has activity that if the activity thereof is reduced in the coryneform bacterium, amount of the heterologous fusion protein produced by secretory production is increased compared with that observed for a non-modified strain.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the genetic construct for secretory production of the heterologous fusion protein further comprises a promoter that functions in the coryneform bacterium.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the genetic construct for secretory production of the heterologous fusion protein further comprises a signal peptide that functions in the coryneform bacterium.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the bacterium belongs to the genus *Corynebacterium* or *Brevibacterium*.

It is a further aspect of the present invention to provide the coryneform bacterium as described above, wherein the bacterium is *Corynebacterium glutamicum*.

It is an aspect of the present invention to provide a method for producing a heterologous fusion protein by secretory production comprising:

- cultivating the bacterium of as described above in a culture medium; and
- collecting the heterologous fusion protein produced by secretory production.

It is an aspect of the present invention to provide a method for producing a protein ligated to a substance, comprising:

- producing a heterologous fusion protein by the method as described above, and
- reacting the heterologous fusion protein with a reactant, wherein the reactant comprises the substance, or the method further comprises modifying the reactant with the substance.

It is a further aspect of the present invention to provide the method as described above, wherein the heterologous fusion protein comprises a thioester or an ester bond in between the extein and the intein, and the thioester or the ester bond is cleaved by reacting the heterologous fusion protein with a reactant comprising a nucleophilic group selected from amino group, thiol group and hydroxyl group.

It is a further aspect of the present invention to provide the method as described above, wherein the heterologous fusion protein comprises a thioester bond in between the extein and the intein, and the thioester bond is cleaved by reacting the heterologous fusion protein with the reactant comprising thiol group.

It is a further aspect of the present invention to provide the method as described above, wherein the heterologous fusion protein is reacted with the reactant in the presence of 2-mercaptoethansulfonic acid.

It is a further aspect of the present invention to provide the method as described above, wherein the reactant comprises a toxin.

It is a further aspect of the present invention to provide the method as described above, wherein the reactant comprises a drug.

It is a further aspect of the present invention to provide the method as described above, wherein the reactant comprises a polyethylene glycol, a radioisotope-labeled compound, or a second polypeptide.

It is an aspect of the present invention to provide a method for producing a protein, which is amidated at the C-terminus, comprising:

- producing a heterologous fusion protein by the method as described above, and
- reacting the heterologous fusion protein with ammonia or a salt thereof.

It is a further aspect of the present invention to provide the method for producing a protein, which is amidated at the C-terminus,

wherein the heterologous fusion protein is reacted with ammonia or a salt thereof, in the presence of a compound containing a nucleophilic thiol group.

The present invention is described in detail hereafter.

Brief Description of Drawings

FIG. 1 shows the structure of the EXC1 DNA-fragment.

FIG. 2 shows the structure of the MAG DNA-fragment.

FIG. 3 shows the structure of the *KasI*-MAG-HT-*NotI* DNA-fragment.

FIG. 4 shows the scheme for the SDS-PAGE band-shift assay.

FIG. 5 shows the FPLC profile of crude mixture of EXI1.

FIG. 6 shows the SDS-PAGE analysis of fractions of EXI1 (photograph).

FIG. 7 shows the SDS-PAGE analysis of activity of EXI1 (photograph).

FIG. 8 shows the SDS-PAGE analysis of expression of EXI1HT (photograph).

FIG. 9 shows the results of purification of EXI1HT from the YDK010[pEXC1-exe-MAG-HT#2] strain culture broth using IMAC (photograph).

FIG. 10 shows the SDS-PAGE analysis of expression and purification of EXI1HT using denaturing IMAC (photograph).

FIG. 11 shows the prolonged «equilibrium» refolding of EXI1HT (photograph).

FIG. 12 shows the fast «non-equilibrium» refolding of EXI1HT (photograph).

FIG. 13 shows the structure of the Int4 DNA-fragment.

FIG. 14 shows the structure of the Int5 DNA-fragment.

FIG. 15 shows the structure of the Int7 DNA-fragment.

FIG. 16 shows the structure of the Int18 DNA-fragment.

FIG. 17 shows the structure of the Int19 DNA-fragment.

FIG. 18 shows the SDS-PAGE analysis of purification and activity of FabH-TGC-MAG-HT (photograph).

FIG. 19 shows the SDS-PAGE analysis of expression of FabL-CGC-MAG-HT (photograph).

FIG. 20 shows the SDS-PAGE analysis of purification of FabL-CGC-MAG-HT (photograph).

FIG. 21 shows the SDS-PAGE analysis of activity of FabL-CGC-MAG-HT (photograph).

FIG. 22 shows the SDS-PAGE analysis of crude cell lysates of the YDK010ΔPBP1a strain harboring pPKSherFabH-FabL-CGC-IntX, X = 4, 5 or 7 (photograph).

FIG. 23 shows the SDS-PAGE analysis of expression of FabH-TGC-IntX-HT, X = 4, 5 or 7 (photograph).

FIG. 24 shows the reducing and non-reducing SDS-PAGE of FabH-TGC-IntX-HT, X = 4, 5 or 7 (photograph).

FIG. 25 shows the SDS-PAGE analysis of activity of FabH-TGC-IntX-HT, X = 4, 5 or 7 (photograph).

FIG. 26 shows the SDS-PAGE analysis of activity of FabH-TGC-IntX-HT, X = 18 or 19 (photograph).

FIG. 27 shows the structure of the CBD DNA-fragment.

FIG. 28 shows the SDS-PAGE analysis of activity of FabH-TGC-Int7-CBD (A) and FabH-TGC-Int18-CBD (B) (photograph).

FIG. 29 shows the SDS-PAGE analysis of expression of BLA-exe-MAG-CBD (photograph).

FIG. 30 shows the SDS-PAGE analysis of activity of BLA-exe-MAG-CBD (photograph).

FIG. 31 shows the scheme for pEXC1-CspB(50)-exe-MAG-CBD plasmid.

FIG. 32 shows the scheme for cleavage of intein from FabH_{TGC}-Int7-HT-FabL and ligation of FabH to Cys-Lys(Biotine).

FIG. 33 shows the SDS-PAGE and Western blotting analysis of thiolysis of FabH_{TGC}-Int7-HT-FabL and ligation to Cys-Lys(Biotin) under various concentrations of MESNa (photograph).

FIG. 34 shows the SDS-PAGE and Western blotting analysis of thiolysis of FabH_{TGC}-Int7-HT-FabL and ligation to Cys-Lys(Biotin) under various times of thiolysis (photograph).

FIG. 35 shows the chemical structure of dibenzocyclooctyne-fluor 488.

FIG. 36 shows the scheme for cleavage of intein from FabH_{TGC}-Int7-HT-FabL, ligation to Cys-CH₂CH₂CH₂N₃ and reaction with dibenzocyclooctyne-fluor 488.

FIG. 37 shows the SDS-PAGE, Western blotting and fluorescent analysis of thiolysis of FabH_{TGC}-Int7-HT-FabL, ligation to Cys-CH₂CH₂CH₂N₃ and reaction with dibenzocyclooctyne-fluor 488 (photograph).

FIG. 38 shows the scheme for synthesis of Cys-Lys(Biotin).

FIG. 39 shows the chemical structure for Boc-Cys(Trt)-CH₂CH₂CH₂N₃.

FIG. 40 shows the chemical structure for Cys-CH₂CH₂CH₂N₃.

Description of Sequences

- SEQ ID NO: 1 shows the EXC1 DNA-fragment
- SEQ ID NO: 2 shows the MAG DNA-fragment
- SEQ ID NO: 3 shows the primer P1
- SEQ ID NO: 4 shows the primer P2
- SEQ ID NO: 5 shows the Int4 DNA-fragment
- SEQ ID NO: 6 shows the Int5 DNA-fragment

SEQ ID NO: 7 shows the Int7 DNA-fragment
SEQ ID NO: 8 shows the Int18 DNA-fragment
SEQ ID NO: 9 shows the Int19 DNA-fragment
SEQ ID NO: 10 shows the primer P3
SEQ ID NO: 11 shows the primer P4
SEQ ID NO: 12 shows the primer P5
SEQ ID NO: 13 shows the primer P6
SEQ ID NO: 14 shows the primer P7
SEQ ID NO: 15 shows the primer P8
SEQ ID NO: 16 shows the primer P9
SEQ ID NO: 17 shows the primer P10
SEQ ID NO: 18 shows the primer P11
SEQ ID NO: 19 shows the primer P12
SEQ ID NO: 20 shows the primer P13
SEQ ID NO: 21 shows the primer P14
SEQ ID NO: 22 shows the primer P15
SEQ ID NO: 23 shows the primer P16
SEQ ID NO: 24 shows the primer P17
SEQ ID NO: 25 shows the primer P18
SEQ ID NO: 26 shows the primer P19
SEQ ID NO: 27 shows the primer P20
SEQ ID NO: 28 shows the primer P21
SEQ ID NO: 29 shows the primer P22
SEQ ID NO: 30 shows the primer P23
SEQ ID NO: 31 shows the primer P24
SEQ ID NO: 32 shows the primer P25
SEQ ID NO: 33 shows the primer P26
SEQ ID NO: 34 shows the exenatide, protein
SEQ ID NO: 35 shows the trastuzumab, heavy chain, protein
SEQ ID NO: 36 shows the trastuzumab, light chain, protein
SEQ ID NO: 37 shows the intein MAG, protein
SEQ ID NO: 38 shows the intein Int4, protein
SEQ ID NO: 39 shows the intein Int5, protein
SEQ ID NO: 40 shows the intein Int7, protein
SEQ ID NO: 41 shows the intein Int18, protein

SEQ ID NO: 42 shows the intein Int19, protein
SEQ ID NO: 43 shows the Cgl0278, gene
SEQ ID NO: 44 shows the Cgl0278, protein
SEQ ID NO: 45 shows the mrcB, gene
SEQ ID NO: 46 shows the MrcB, protein
SEQ ID NO: 47 shows the intein MAG, gene
SEQ ID NO: 48 shows the intein Int4, gene
SEQ ID NO: 49 shows the intein Int5, gene
SEQ ID NO: 50 shows the intein Int7, gene
SEQ ID NO: 51 shows the intein Int18, gene
SEQ ID NO: 52 shows the intein Int19, gene
SEQ ID NO: 53 shows the exenatide, gene
SEQ ID NO: 54 shows the trastuzumab, heavy chain, gene
SEQ ID NO: 55 shows the trastuzumab, light chain, gene
SEQ ID NO: 56 shows the PS1, protein
SEQ ID NO: 57 shows the PS2 (CspB), protein
SEQ ID NO: 58 shows the SlpA (CspA), protein
SEQ ID NO: 59 shows the PS2 (CspB), gene
SEQ ID NO: 60 shows the plasmid pPKSherFabHL
SEQ ID NO: 61 shows the primer P27
SEQ ID NO: 62 shows the primer P28
SEQ ID NO: 63 shows the primer P29
SEQ ID NO: 64 shows the primer P30
SEQ ID NO: 65 shows the CBD DNA-fragment
SEQ ID NO: 66 shows the chitin binding domain, protein
SEQ ID NO: 67 shows the primer P31
SEQ ID NO: 68 shows the primer P32
SEQ ID NO: 69 shows the primer P33
SEQ ID NO: 70 shows the primer P34
SEQ ID NO: 71 shows the primer P35
SEQ ID NO: 72 shows the primer P36
SEQ ID NO: 73 shows the primer P37
SEQ ID NO: 74 shows the pEXC1-CspB(50)-exe-MAG-CBD
SEQ ID NO: 75 shows the leader peptide of beta-lactamase

Detailed Description of the Invention

1. Coryneform bacterium

The present invention provides a coryneform bacterium having an ability to produce a heterologous fusion protein by secretory production, which has been modified to express the nucleic acid sequence encoding a heterologous fusion protein (henceforth also referred to as the «bacterium of the present invention» or the «coryneform bacterium of the present invention»).

In the present invention, the secretory production of a heterologous fusion protein means that the protein is synthesized by the bacterial cell and transported out of the bacterial cell into an extracellular environment, for example, a culture medium. In such a sense, the heterologous fusion protein is extracellularly transported by the bacterial cell. The secretory production of heterologous fusion proteins can also include a case where the molecules of the fusion proteins eventually exist in the medium in completely free forms, a case where the molecules of the fusion proteins exist in the cell surface layer, and a case where a part of the molecules of the fusion proteins exist in the medium and the reminder part of the molecules exist in the cell surface layer.

Therefore, in the present invention, the «ability to produce a heterologous fusion protein by secretory production» refers to an ability of the bacterium of the present invention to synthesize the heterologous fusion protein, secrete it into the medium and/or the cell surface layer, and accumulate it in the medium and/or the cell surface layer to such an extent that the heterologous fusion protein can be collected from the medium and/or the cell surface layer, when the bacterium is cultured in the medium. As for the accumulation amount, for example, the accumulation amount in the medium may be 10 µg/L or more, 1 mg/L or more, 100 mg/L or more, or 1 g/L or more. Also, as for the accumulation amount, for example, the accumulation amount in the cell surface layer may be to such an extent that if the heterologous protein in the cell surface layer is collected and suspended in the same volume of liquid as the medium, the concentration of

the heterologous protein in the suspension is 10 µg/L or more, 1 mg/L or more, or 100 mg/L or more.

Coryneform bacteria

In the present invention, the coryneform bacteria are aerobic gram-positive bacilli, and include *Corynebacterium* bacteria, *Brevibacterium* bacteria, *Microbacterium* bacteria, and so forth. The coryneform bacteria include bacteria which have previously been classified into the genus *Brevibacterium* but are presently united into the genus *Corynebacterium* (Liebl W. et al., *Int. J. Syst. Bacteriol.*, 1991, 41:255-260). The coryneform bacteria also include bacteria which have previously been classified into *Corynebacterium ammoniagenes* but are presently reclassified into *Corynebacterium stationis* by nucleotide sequence analysis of 16S rRNA and so forth (Bernard K.A. et al., *Int. J. Syst. Evol. Microbiol.*, 2010, 60:874-879). Advantages of use of the coryneform bacteria include the fact that they inherently secrete an extremely smaller amount of proteins in the outside of cells compared with fungi, yeasts, and *Bacillus* bacteria, which are conventionally used for secretory production of proteins, and therefore purification process of a heterologous fusion protein produced by secretory production can be simplified or eliminated, the fact 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.

Specific examples of such coryneform bacteria include the following species:

Corynebacterium acetoacidophilum

Corynebacterium acetoglutamicum

Corynebacterium alkanolyticum

Corynebacterium ammoniagenes (*Corynebacterium stationis*)

Corynebacterium callunae

Corynebacterium glutamicum

Corynebacterium lilium

Corynebacterium melassecola

Corynebacterium thermoaminogenes (*Corynebacterium efficiens*)

Corynebacterium herculis

Brevibacterium divaricatum

Brevibacterium flavum

Brevibacterium immariophilum

Brevibacterium lactofermentum (*Corynebacterium glutamicum*)

Brevibacterium roseum

Brevibacterium saccharolyticum

Brevibacterium thiogenitalis

Brevibacterium album

Brevibacterium cerinum

Microbacterium ammoniophilum

Specific examples of such coryneform bacteria include the following strains:

Corynebacterium acetoacidophilum ATCC 13870

Corynebacterium acetoglutamicum ATCC 15806

Corynebacterium alkanolyticum ATCC 21511

Corynebacterium ammoniagenes (*Corynebacterium stationis*) ATCC 6871, ATCC 6872

Corynebacterium callunae ATCC 15991

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

Corynebacterium lilium ATCC 15990

Corynebacterium melassecola ATCC 17965

Corynebacterium thermoaminogenes AJ12340 (FERM BP-1539)

Corynebacterium herculis ATCC 13868

Brevibacterium divaricatum ATCC 14020

Brevibacterium flavum ATCC 13826, ATCC 14067, AJ12418 (FERM BP-2205)

Brevibacterium immariophilum ATCC 14068

Brevibacterium lactofermentum ATCC 13869

Brevibacterium roseum ATCC 13825

Brevibacterium saccharolyticum ATCC 14066

Brevibacterium thiogenitalis ATCC 19240

Brevibacterium album ATCC 15111

Brevibacterium cerinum ATCC 15112

Microbacterium ammoniaphilum ATCC 15354

These strains are available from, for example, the American Type Culture Collection (ATCC) (Address: 12301 Parklawn Drive, Rockville, Maryland 20852, P.O. Box 1549, Manassas, VA 20108, United States of America). That is, each strain is given a unique registration number (www.atcc.org), and can be ordered by using this registration number. The registration number of each strain is listed in the catalogue of the ATCC.

In particular, the *C. glutamicum* AJ12036 strain (FERM BP-734), which was isolated from the wild-type strain, *C. glutamicum* ATCC 13869, as a streptomycin (Sm) resistant mutant strain, is predicted to have a mutation in the functional gene responsible for secretion of proteins, and shows an extremely high secretory production ability for proteins as high as about 2 to 3 times in terms of accumulated 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 (FERM BP-734) was originally deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (Currently, the incorporated administrative agency, National Institute of Technology and Evaluation, International Patent Organism Depositary, AIST Tsukuba Central 6, 1-1, Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) on March 26, 1984 as an international deposit, and assigned an accession number of FERM BP-734.

Moreover, a strain having an enhanced ability to produce a heterologous fusion protein by secretory production may be selected from coryneform bacteria obtained from such coryneform bacteria 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.

Furthermore, if a strain obtained by modifying such a strain as mentioned above so that it does not produce a cell surface layer protein as the host, purification of the heterologous fusion protein secreted in the medium becomes easy, and therefore it is particularly preferred. Such modification can be carried out by introducing a mutation into a coding region of the cell surface layer protein or an expression control region thereof on a 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).

The coryneform bacterium having an ability to produce a heterologous fusion protein by secretory production can be obtained by introducing a genetic construct for secretory expression of the heterologous fusion protein into such a coryneform bacterium as mentioned above so that the construct is harbored by the bacterium. That is, the bacterium of the present invention has a genetic construct for secretory expression of a heterologous fusion protein. The «genetic construct» for secretory expression of a heterologous protein and a method for introducing it will be explained later.

The bacterium of the present invention can be obtained by modifying a coryneform bacterium having an ability to produce a heterologous fusion protein by secretory production so that activity of a penicillin-binding protein and activity of a cell surface layer protein are reduced. Alternatively, the bacterium of the present invention can also be obtained by modifying a coryneform bacterium so that activity of a penicillin-binding protein and activity of a cell surface layer protein are reduced, and then imparting an ability to produce a heterologous fusion protein by secretory production to it. Furthermore, the bacterium of the present invention can also be obtained by modifying a coryneform bacterium of which activity of a cell surface layer protein has been intrinsically reduced so that the bacterium has an ability to produce a heterologous fusion protein and activity of a penicillin-binding protein is reduced. In the present invention, the modification and impartation of the ability for constructing the bacterium of the present

invention can be carried out in an arbitrary order. The bacterium of the present invention may be a bacterium obtained from a bacterium that can produce a heterologous fusion protein by secretory production before it is modified so that activity of a penicillin-binding protein and/or activity of a cell surface layer protein are reduced. In addition, the bacterium of the present invention may also be a bacterium obtained from a bacterium that cannot produce a heterologous fusion protein by secretory production even when it has a genetic construct for secretory expression of a heterologous fusion protein before it is modified so that activity of a penicillin-binding protein and/or activity of a cell surface layer protein are reduced, which comes to be able to produce the heterologous fusion protein by secretory production as a result of such modification that the activity of the penicillin-binding protein and/or activity of a cell surface layer protein are reduced. In addition, the bacterium of the present invention may be further modified so that expression of a gene encoding a protease or a gene encoding a protein having a region homologous to a motif of a protease is increased.

Protein

The phrase «protein» produced by secretory production refers to a concept of protein including those called peptide such as oligopeptide or polypeptide. Exemplary, an oligopeptide can be a peptide consisting of between 2 and about 20 amino acid residues and may include dipeptides, tripeptides, tetrapeptides, pentapeptides, etc.; and polypeptide can be a peptide consisting of more than about 20 amino acid residues. The phrases «protein» and «peptide» are those concepts which can be unambiguously understood by the person skilled in the art.

Heterologous protein

The phrase «heterologous protein» refers to an exogenous protein for the coryneform bacterium that synthesizes 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 amino acid

sequence is artificially designed. The heterologous protein may be a monomer protein or a multimer protein. The multimer 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 or by non-covalent bonds such as hydrogen bonds, ionic bonds, van der Waals forces, and hydrophobic interactions, or linked by a combination of these. The multimer may comprise 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 multimer 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 the subunits may be heterologous. It is also possible that a part of a naturally endogenous protein for the coryneform bacterium is artificially designed thus constituting a heterologous part of the protein. A naturally endogenous protein for the coryneform bacterium or a part of that can be covalently linked through the peptide bond to a heterologous protein or a part of that, or an artificially designed amino acid sequence. Such complex protein also contributes to the phrase «heterologous protein». 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 secretory production of the heterologous protein includes secretory production of one or all the subunits constituting a desired heterologous protein, as well as secretory production of only a part of the subunits constituting a desired heterologous protein. Specific example of the heterologous protein is a heterologous fusion protein.

Fusion protein

The phrase «fusion protein» refers to a protein expressed from a fusion gene, which is created by joining two or more genes. That is, one gene is ligated upstream of another one or more genes encoding the proteins

constituting a fusion protein. A fusion protein consisting of two proteins, which expressed from the common nucleic acid sequence comprising the genes encoding said proteins, is a particular example of the presently disclosed subject-matter. So long as the fusion protein consists of two or more proteins, the terminal proteins of the molecule are referred to as the N-terminal and the C-terminal parts of the fusion protein. That is, the N-terminal part of a fusion protein is the protein located at the N-terminus of the amino acid sequence constituting the fusion protein; and the C-terminal part of a fusion protein is the protein located at the C-terminus of the amino acid sequence constituting the fusion protein. It is generally accepted that the N-terminus of the amino acid sequence is counted from the terminal amino group (H_2N-) of the sequence. Contrary, the C-terminus of the amino acid sequence is counted from the terminal carboxylic group ($-COOH$) of the sequence. Therefore, the fusion protein can consist merely of one N-terminal and one C-terminal part. However, if the fusion protein is encoded by more than two genes, said protein may have one or more «internal proteins» linked to the N-terminal and C-terminal parts. In such fusion protein, the N-terminus of the internal protein is linked to the C-terminus of the N-terminal protein, and the C-terminus of the internal protein is linked to the N-terminus of the C-terminal protein or the N-terminus of the second internal protein of which the C-terminus is linked to the N-terminus of the third internal protein or the N-terminus of the C-terminal protein. A fusion protein having merely one N-terminal and one C-terminal part is a particular example.

The fusion protein consisting of two or more proteins may include two structural regions, hereafter referred to as the «extein region» (also referred to as the «extein») and the «intein region» (also referred to as the «intein»). In the fusion protein consisting of two or more proteins, the N-terminal part *per se* or the N-terminal part linked to one or more internal proteins is referred to as the «extein». The C-terminal part *per se* or the C-terminal part linked to one or more internal proteins of the fusion protein consisting of two or more proteins is referred to as the «intein». Structural and functional features can be applied to an intein as described hereafter. Therefore, as soon as the

intein is determined as being in compliance with structural and functional requirements for inteins, the remaining part of the fusion protein can be determined as the extein.

Further, the heterologous fusion protein to be produced by secretory production may be a protein containing a pro-structure part (proprotein) within the extein region. In the case where the heterologous fusion protein to be produced by secretory production is a proprotein, the heterologous fusion protein to be eventually obtained by the method of the present invention may be the proprotein or may not be the proprotein. That is, the proprotein may be made into a mature fusion protein by cleavage of the pro-structure part in the extein region. The cleavage can be attained with, for example, a protease. When a protease is used, in view of the activity of the protein to be eventually obtained, the proprotein is generally cleaved preferably at a position substantially the same as that of the natural protein, or more preferably at a position exactly the same as that of the natural protein to obtain the same mature protein as the natural mature protein. Therefore, 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- and/or C-termini of the heterologous fusion 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 fusion protein to be produced, a protein having the N- and/or C-termini longer or shorter by one or more 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 can be used in an unpurified state, or may also be used after purification to an appropriate purity as required.

Extein

The extein can be, for example, a heterologous protein of a monomer structure (also referred to as the «monomer heterologous protein») or a

subunit of the heterologous protein having a multimer structure (also referred to as a subunit of the «multimer heterologous protein»), or a part of these shortened by one or more amino acid residues from the N- and/or C-terminus of the amino acid sequence. It is also possible that a monomer heterologous protein or a subunit of the multimer heterologous protein is extended by one or more amino acid residues from the N- and/or C-terminus of the amino acid sequence. Of course, a monomer heterologous protein or a subunit of the multimer heterologous protein can be shortened and extended by one or more amino acid residues from the N- and/or C-terminus concurrently. That is, when the N-terminus (C-terminus) of the protein is shortened by one or more amino acid residues or even unmodified, the C-terminus (N-terminus) of the protein can be extended by one or more amino acid residues or even unmodified. It is obvious that the amino acid sequence of an extein can be chosen from the naturally occurring amino acid sequences, or the reasonably modified natural amino acid sequences, or the artificially designed by the demand amino acid sequences. Furthermore, the N- and C-termini of an extein can also be modified, for example, through the shortening and/or extending by one or more amino acid residues, or even left unmodified. Therefore, an extein may be referred to as the «target protein». Specific examples of the «target protein» will be mentioned later.

So long as the heterologous fusion protein includes an extein, the heterologous fusion protein eventually obtained by the method of the present invention may have a target protein as having the primary structure chosen from the naturally occurring amino acid sequences, or artificially designed as a part of the naturally occurring amino acid sequence by the substitution, deletion, insertion and/or addition of one or more amino acid residues, or artificially designed as a complete amino acid sequence. Although the number of the «one or more» amino acid residues may differ depending on the full length or structure of the objective heterologous protein, specifically, it is 1 to 30, 1 to 20, 1 to 10, or 1 to 5.

Although the extein used for the present invention is not particularly limited so long as secretory production of the heterologous fusion protein is attained, it may contain a linker so that the linker is located in between a

target protein and an intein in the structure of a heterologous fusion protein. That is, a target protein and an intein can be joined through the linker consisting of one or more amino acid residues. The linker may have a length of 1, 2, 3, 4, 5, 10, 20, 50, 100 or more amino acid residues; specifically, it may be 1 to 3 amino acid residues. The N-terminus of the linker is linked to the C-terminus of a target protein and the C-terminus of the linker is linked to the N-terminus of an intein through the peptide bond. The linker can be ended at the C-terminus by the $\text{-NH-CH(R1)-CO-NH-CH(R2)-CO-}$ motif, where R1 and R2 are the side-chain groups of any proteinogenic L-amino acid of the same kind or different kinds, so long as the linker has a length of two or more amino acid residues. That is, R1 and R2 can be a side-chain group of L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine or L-valine; wherein R1 and R2 can be of the same chemical structure or different chemical structures. For example, R1 is the side-chain group of any proteinogenic L-amino acid and R2 is the side-chain group of L-cysteine, or R1 is hydrogen and R2 is the side-chain group of L-cysteine.

The phrase «peptide bond» refers to a covalent chemical bond -CO-NH- formed between two molecules when the carboxy part of one molecule, referred to as a carboxy component, reacts with the amino part of another molecule, referred to as an amino component, causing the release of a molecule. For example, proteinogenic L-amino acids can form the peptide bond upon joining with the release of a molecule of water. Therefore, proteins and peptides can be regarded as chains of amino acid residues held together by peptide bonds. A peptide bond can be also referred to as an «amide bond».

Intein

The inteins from various host organisms of Eukarya, Eubacteria, and Archaea can be used to eventually obtain a heterologous fusion protein by the method of the present invention. A collection of more than 115 kinds of inteins is provided by the InBase, The Intein Database (Perler F. InBase, the intein database, *Nucleic Acids Res.*, 2002, 30:383-384;

<http://tools.neb.com/inbase/list.php>). There are two kinds of inteins such as the classical inteins containing the DOD-type homing endonuclease and the mini-inteins. The DOD endonuclease-containing inteins and the mini-inteins are about 450-600 and about 134-300 amino acid residues in length, respectively. Both kinds of inteins have the N-terminal domain of 150-200 residues in length, C-terminal domain of 25-40 residues in length, and the internal domain which is a DOD endonuclease domain for the DOD endonuclease-containing inteins or a linker region domain for the mini-inteins (Starokadomskyy P.L. Protein splicing, *Mol. Biol.*, 2007, 41(2):278-293). The N-splicing domain of two kinds of inteins comprises the motifs A, N2, B, and N4. However, some inteins may lack of N4. The C-splicing domain of mini-inteins comprises the motifs F and G. Additional motif H precedes the motif F in DOD endonuclease-containing inteins. Motifs C, D, and E contribute to the structure of the DOD endonuclease domain and locate in between the motifs N4 and H.

Motif A is a short N-terminal sequence of 13 residues, of which the first and the second residues are highly conserved. Position 1 at the N-terminus of an intein is almost always occupied by Cys, Ala, Gln or Ser. Position 13 is occupied by Gly, Ala, Lys, Thr, Arg, Tyr or Asn. The N-terminal amino acid residue of intein (Cys, Ala, Gln or Ser) can be involved in acyl rearrangement. The more detailed explanation for the acyl rearrangement is given hereafter.

Motif N2 consists of 7 residues, of which Asp5 or Glu5 is highly conserved and is most often preceded by Gly.

Motif B consists of 14 residues. Position 10 is occupied by His in all known inteins. Position 7 is most often occupied by Thr.

Motif N4 consists of 16 residues including highly conserved Asp or Glu in position 11. As in motif N2, the Asp or Glu residue is usually preceded by Gly10.

Motifs C and E form a basis of the DOD endonuclease domain and have sequences of nine and ten residues. Both motifs are separated by a linker of 90-130 residues. The active center of the enzyme contains conserved Gly residues, which are in positions 3 and 9 of motif C and 4 and 10 of motif E. In addition, each motif harbors catalytically active Asn and Lys.

Motif D of eight residues in length is in the linker between motifs C and E. The Lys residue in position 2 may be required for an endonuclease activity of the DOD domain.

Motif H consists of 19 amino acid residues, of which Leu13 and Leu14 are rather conserved. Motifs C, D, E, and H form the DOD endonuclease domain.

Motifs F and G form the C-terminal domain. Motif F consists of 16 residues, half of which are highly conserved. Motif G is a short C-terminal sequence of seven residues. Motifs F and G are separated by a short linker, consisting of two to five residues. The C-terminal amino acid residue of the motif G is Asn, Gln or Asp; the last but one is His.

The documents Starokadomskyy P.L. Protein splicing, *Mol. Biol.*, 2007, 41(2):278-293 and Perler F. InBase, the intein database, *Nucleic Acids Res.*, 2002, 30:383-384 can be exclusively incorporated herein as references to the structural organization of inteins.

The inteins are not limited to those mentioned in the InBase, The Intein Database (<http://tools.neb.com/inbase/list.php>). The objective amino acid sequence can be searched against the intein database using the BLAST (Basic Local Alignment Search Tool) service to find a new intein or a homolog of the known intein. The BLAST service is provided by the InBase, The Intein Database (<http://tools.neb.com/~vincze/blast/index.php?blastdb=inbase>) or the National Center for Biotechnology Information (NCBI, <http://blast.ncbi.nlm.nih.gov>).

The following inteins as non-limiting examples can be used for the purposes of the present invention: Mmag Magn8951 BIL (for the sake of simplicity herein referred to as MAG) from *Magnetospirillum magnetotacticum* MS-1 (Amitai G. et al., *Mol. Microbiol.*, 2003, 47(1):61-73), Pvu PRP8 (PVU) from *Penicillium vulpinum* (formerly *P. claviforme*), Pab Pol-II (PAB) from *Pyrococcus abyssi*, Mxe GyrA (MXE) from *Mycobacterium xenopi* strain IMM5024, Mth R1R1 (MTH) from *Methanobacterium thermoautotrophicum* (Evans T.C. Jr. et al., *J. Biol. Chem.*, 1999, 274(7):3923-9326), Ctr VMA (SCE) from *Candida tropicalis* (nucleus), Ter ThyX (TER) from *Trichodesmium erythraeum* IMS101, Aha DnaE-c and Aha DnaE-n (SSP) from *Aphanothece*

halophytica, Tvu DnaE-c and Tvu DnaE-n (TVU) from *Thermosynechococcus vulcanus*, and so forth. Furthermore, the homologous intein sequences can be found using the BLAST analysis. For example, the inteins homologous to the Mmag Magn8951 BIL intein from *M. magnetotacticum* MS-1 can be found as iAAQ from *Aeromonas aquariorum* AAK1, as iBLA from *Brevibacillus laterosporus* LMG 15441, as iEKD from uncultured bacterium, as iSPH from *Microscilla marina*, and as iMIC from *Sphingobacterium* sp. 21 (refer to the Examples for details).

A protein is considered to contribute to the phrase «intein» so long as it has an «activity of acyl rearrangement» when linked to an extein through the peptide bond in the heterologous fusion protein produced by secretory production as described in the present invention. The «acyl rearrangement» proceeds in two stages, more specifically, two consecutive nucleophilic displacement reactions which result in trans-esterification reaction, that is, a shift of the extein C-terminus from the peptide bond linking extein and intein in the heterologous fusion protein to the nucleophilic group of a reactant. The mechanism of the acyl rearrangement is described in Perler F. InBase, the intein database, *Nucleic Acids Res.*, 2002, 30:383-384 and Starokadomskyy P.L. Protein splicing, *Mol. Biol.*, 2007, 41(2):278-293. The phrase «reactant» is explained hereafter.

At the first stage of the acyl rearrangement a nucleophilic group of the N-terminal amino acid residue of the intein attacks carbon atom of the neighboring peptide bond linking the extein and intein. Although, the kinds of the N-terminal amino acid residues of inteins are mentioned hereafter, the residues can be, for example, those residues which have thiol group (-SH) or hydroxyl group (-OH) terminating a side-chain group of a proteinogenic L-amino acid. When L-cysteine residue is located at the N-terminus of an intein, the thiol group (-SH) can be regarded as the nucleophilic group as set forth above. In this case, the first stage of the acyl rearrangement is referred to as «N-S shift». When L-serine or L-threonine residue is located at the N-terminus of an intein, the hydroxyl group (-OH) can be regarded as the nucleophilic group as set forth above. In this case, the first stage of the acyl rearrangement is referred to as «N-O shift». The N-S shift results in formation

of the thioester intermediate having the thioester bond (-CO-S-) in between extein and intein. The N-O shift results in formation of the ester intermediate having the ester bond (-CO-O-) in between extein and intein. As a result of the first stage of the acyl rearrangement, the C-terminus of extein shifts from the peptide bond linking extein and intein to the side-chain group of the amino acid residue located at the N-terminus of intein. At the second stage of the acyl rearrangement, a nucleophilic group of a reactant attacks carbon atom of the thioester bond or ester bond in between extein and intein.

As the «reactant», any organic or inorganic molecule can be used so long as it has one or more nucleophilic groups having a nucleophilic atom such as carbon atom, nitrogen atom, oxygen atom, and so forth, and can react with the thioester bond or ester bond, which is obtained as a result of the first stage in between extein and intein, in nucleophilic substitution reaction to attain the nucleophilic acyl substitution (March J., *Advanced Organic Chemistry*, 4th ed., Wiley, New York (USA), 1992; Carey F.A., *Organic Chemistry*, 6th ed., McGraw-Hill, New York (USA), 2006).

The acyl rearrangement results in cleavage of the intein from the heterologous fusion protein produced by secretory production and formation of a molecule consisting of an extein and a reactant residue linked to the C-terminus of the extein. Thus, the phrase «an activity of acyl rearrangement» can mean a property of a protein to take part in the acyl rearrangement as described above. So long as an objective protein has the activity of acyl rearrangement, it can be referred to as the «intein». It may be the case that the protein initially is unable to take part in the acyl rearrangement, but due to one or more modifications introduced into the amino acid sequence of the protein, said protein can be imparted with an activity of acyl rearrangement. Therefore, any protein can be modified in such a way that it can take part in the acyl rearrangement and, thus, referred to as an intein. Means for introducing substitutions, deletions, insertions, and/or additions of one or several amino acid residues into a protein are explained hereafter. A heterologous fusion protein to be produced by secretory production may be referred to as the «protein having an activity of acyl rearrangement» so long as the intein of such protein has the activity of acyl rearrangement.

The amino acid residue at the N-terminus of an intein can be preferably L-cysteine, L-threonine, L-serine or L-alanine residue, more preferably L-cysteine, L-threonine or L-serine residue, and particularly preferably L-cysteine residue.

Amines, thiols, alcohols, and so forth can be used as «organic reactants». Primary amines and secondary amines having, respectively, one or two hydrogen atoms substituted with organic radicals can be used to react with the thioester bond or ester bond in between extein and intein. In such a case, amide bond (-CO-NH-) is formed in between the extein and the reactant residue, and the reaction is referred to as «amidation reaction». Thiols having one, two, three or more thiol groups linked to organic radicals can be used to react with the ester bond in between extein and intein. In such a case, thioester bond (-CO-S-) is formed in between the extein and the reactant residue, and the reaction is referred to as «thioesterification reaction». It is also possible to react thiols with the thioester bond in between extein and intein so long as conditions of the nucleophilic acyl substitution reaction, such as, for example, the kind of a reactant, are appropriately chosen so that the leaving group (i.e. the intein) can be substituted with a thiol residue. Alcohols having one, two, three or more hydroxyl groups linked to organic radicals can be reacted with the thioester bond in between extein and intein. In such a case, ester bond (-CO-O-) is formed in between the extein and the reactant residue, and the reaction is referred to as «esterification reaction». It is also possible to react alcohols with the ester bond in between extein and intein so long as conditions of the nucleophilic acyl substitution reaction, such as, for example, the kind of reactant, are appropriately chosen so that the leaving group (i.e. the intein) can be substituted with the alcohol residue. Alkyl, alkenyl, alkynyl, aryl, and so forth, and derivatives thereof having one or more hydroxyl groups, thiol groups, amino groups, aldehyde groups, carboxyl groups, halogen groups, and so forth can be used as organic radicals. Nucleosides, nucleotides, nucleic acids of different length, for example, from 2 to 10, or from 2 to 20, or from 2 to 50, or from 2 to 100, or from 2 to 200, or from 2 to 500, or from 2 to more than 500 nucleotides in length can also be used as organic radicals. Amino acids or peptides of

different length, for example, from 2 to 5, or from 2 to 10, or from 2 to 20, or from 2 to 50, or from 2 to 100, or from 2 to more than 100 amino acid residues in length can also be used as organic radicals. Proteins such as bioactive proteins, receptor proteins, antigenic proteins as described hereafter can also be used as organic radicals. Dyes, fatty acids, carbohydrates, heterocyclic compounds, biotin; low, medium and high molecular weight drugs; radiolabeled molecules; and so forth can be used as organic radicals. Ammonia, hydroxylamine, sodium sulfide, and so forth are particular examples of the «inorganic reactants».

The rate of the acyl rearrangement may vary depending on, for example, the primary, secondary, tertiary structure of an intein and/or extein comprising a heterologous fusion protein produced by secretory production; environmental conditions such as ionic strength, acidity (pH), temperature, medium composition; purity of a sample of the heterologous fusion protein; and so forth. Means for obtaining heterologous fusion proteins having the activity of acyl rearrangement are described in the Examples hereafter.

It is preferable that three-dimensional structure, function and/or activity of a heterologous fusion protein produced by secretory production, or the extein or intein parts of the heterologous fusion protein, or a molecule consisting of an extein and a reactant residue linked to the C-terminus of the extein produced as a result of the acyl rearrangement, or an intein produced as a result of the acyl rearrangement is/are not affected at the first stage or second stage, or both stages of the acyl rearrangement to such an extent that the three-dimensional structure and/or function is/are completely or partially disturbed, or the activity is completely or partially disappeared. More specifically, the activity may be 30% or more, 50% or more, 70% or more, 90% or more, or 100% of that observed in a heterologous fusion protein produced by secretory production, or the extein or intein parts of the heterologous fusion protein, or a molecule consisting of an extein and a reactant residue linked to the C-terminus of the extein produced as a result of the acyl rearrangement, or an intein produced as a result of the acyl rearrangement, subjected to the first stage or second stage, or both stages of the acyl rearrangement.

Although, inteins have a certain characteristic of sequence common over biological species, an intein that has an activity of acyl rearrangement as to a certain extein does not necessarily exhibit the same activity as to another extein. Therefore, when an intein having an activity of acyl rearrangement as to another extein is used, an intein that has an activity of acyl rearrangement as to an objective extein may be appropriately chosen. It is therefore acceptable that any intein can be used to obtain a heterologous fusion protein produced by secretory production as described in the present invention so long as the intein has an activity of acyl rearrangement when linked to an extein through the peptide bond in the heterologous fusion protein. The appropriate intein having an activity of acyl rearrangement can be found by the BLAST search in the InBase, The Intein Database. It is also possible to find one or more homologues of the intein using the BLAST analysis when searched in the InBase, The Intein Database or an amino acid sequences database such as the Protein Database (NCBI, www.ncbi.nlm.nih.gov/protein). The activity of acyl rearrangement of the intein selected as a result of the BLAST search can be determined as described hereafter.

The following inteins as non-limiting examples can be used for the purposes of the present invention.

The wild-type nucleic acid sequence of SEQ ID NO: 47 (NCBI accession No. NZ_AAAP01003875; version NZ_AAAP01003875.1, GI: 23016286; reverse complement, 514 bp – 936 bp) encodes the Mmag Magn8951 BIL (MAG) intein having the amino acid sequence of SEQ ID NO: 37 from *Magnetospirillum magnetotacticum* MS-1.

The wild-type nucleic acid sequence of SEQ ID NO: 48 (GenBank accession No. AM042016; version AM042016.1, GI: 94442880; 152 bp – 634 bp) encodes the Pvu PRP8 (PVU) intein having the amino acid sequence of SEQ ID NO: 38 from *Penicillium vulpinum*.

The wild-type nucleic acid sequence of SEQ ID NO: 49 (GenBank accession No. BA000022; version BA000022.2, GI: 47118304; 2323 bp – 2691 bp) encodes the Ssp DnaE-n (SSP) intein having the amino acid sequence of SEQ ID NO: 39 from *Aphanothece halophytica*.

The wild-type nucleic acid sequence of SEQ ID NO: 50 (NCBI accession No. NC_000868; version NC_000868.1, GI: 14518450; 2863 bp – 3417 bp) encodes the Pab Pol-II (PAB) intein having the amino acid sequence of SEQ ID NO: 40 from *Pyrococcus abyssi*.

The wild-type nucleic acid sequence of SEQ ID NO: 51 (NCBI accession No. NZ_AFRV01000005; version NZ_AFRV01000005.1, GI: 339009619; 4990 bp – 5388 bp) encodes the iBLA intein having the amino acid sequence of SEQ ID NO: 41 from *Brevibacillus laterosporus* LMG 15441.

The wild-type nucleic acid sequence of SEQ ID NO: 52 (NCBI accession No. AMFJ01011091; version AMFJ01011091.1, GI: 406968816; 3226 bp – 3612 bp) encodes the iEKD intein having the amino acid sequence of SEQ ID NO: 42 from uncultured bacterium ACD_28C00111.

Since there may be some differences in nucleotide sequence sequences between biological species or strains, the wild-type genes encoding inteins are not limited to the genes encoding inteins described in, for example, the InBase, The Intein Database or the Protein Database (NCBI), or the intein genes specifically shown in SEQ ID NOs: 47, 48, 49, 50, 51, and 52, but may include genes which are variant nucleotide sequences of or homologous to the aforementioned nucleotide sequences, and which encode variants of the wild-type inteins. The phrase «a wild-type intein» refers to a native intein naturally expressed in a wild-type biological species. A wild-type intein can be encoded by the wild-type, or non-modified, gene naturally occurring in genome of a biological species.

The phrase «a variant of the wild-type intein» (also referred to as «a variant intein») can mean an intein which has one or several changes in the amino acid sequence compared with the wild-type sequence of the intein, whether they are substitutions, deletions, insertions, and/or additions of one or several amino acid residues, but still maintains an activity of acyl rearrangement similar to that of the wild-type intein. The number of changes in the variant intein depends on the position or the kind of amino acid residues in the three-dimensional structure of the intein. For example, one or more variant inteins can be obtained for an intein having the amino acid

sequence of SEQ ID NO: 37, 38, 39, 40, 41 or 42 by introducing one or several changes into the sequence.

Although the number of the «one or several» amino acid residues may differ depending on the position in the three-dimensional structure or kinds of amino acid residues of the intein, specifically, the changes in the amino acid sequence can be, but are not strictly limited to, 1 to 30, in another example 1 to 15, in another example 1 to 10, and in another example 1 to 5, in the amino acid sequence corresponding to the wild-type intein. These changes in the variant intein can occur in regions of the intein that are not critical for the activity of the intein. This is because some amino acids have high homology to one another so that the activity is not affected by such a change, or the three-dimensional structure of the variant intein is not significantly changed relative to the wild-type or non-modified intein. For example, as the motifs A, N2, B, N4, C, D, E, H, F, and G of an intein may be required for activity, the intein regions corresponding to these motifs can be, therefore, subjected to the modification to the low extent or even left unmodified. Quite the contrary, the intein regions linking said motifs are less conservative and, therefore, can be subjected to one or more changes. For the clarity reason, the iBLA intein (SEQ ID NO: 41) and the iEKD intein (SEQ ID NO: 42) can be homologues of the MAG intein (SEQ ID NO: 37) (Tables 7 and 8). That is, the iBLA and iEKD inteins can be obtained from the MAG intein by introducing one or several changes in the amino acid sequence of the MAG intein.

Therefore, the variant inteins encoded by the intein genes may have a homology, defined as the parameter «identity» when using the computer program BLAST, of not less than 20%, not less than 30%, not less than 40%, not less than 50%, not less than 60%, not less than 70%, not less than 80%, not less than 90%, not less than 95%, or not less than 98% with respect to the entire amino acid sequences of the wild-type inteins so long as the activity of the variant inteins is maintained.

The exemplary substitution, deletion, insertion, and/or addition of one or several amino acid residues can be a conservative mutation(s). The representative conservative mutation is a conservative substitution. The

conservative substitution can be, but is not limited to a substitution, wherein substitution takes place mutually among Phe, Trp and Tyr, if the substitution site is an aromatic amino acid; among Ala, Leu, Ile and Val, if the substitution site is a hydrophobic amino acid; between Glu, Asp, Gln, Asn, Ser, His and Thr, if the substitution site is a hydrophilic amino acid; between Gln and Asn, if the substitution site is a polar amino acid; among Lys, Arg and His, if the substitution site is a basic amino acid; between Asp and Glu, if the substitution site is an acidic amino acid; and between Ser and Thr, if the substitution site is an amino acid having hydroxyl group. Examples of conservative substitutions include 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 Asn, Glu or Gln for Asp, substitution of Ser or Ala for Cys, substitution Asn, Glu, Lys, His, Asp or Arg for Gln, substitution Asn, Gln, Lys or Asp for Glu, substitution of Pro for Gly, substitution 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 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.

The exemplary substitution, deletion, insertion, and/or addition of one or several amino acid residues can also be a non-conservative mutation(s) so long as the mutation(s) is/are compensated by one or more secondary mutations in the different position(s) of amino acids sequence so that the activity of the variant intein is maintained and similar to that of the wild-type intein.

To evaluate the degree of intein or DNA homology, several calculation methods can be used, such as BLAST search, FASTA search and ClustalW method. The BLAST (Basic Local Alignment Search Tool, www.ncbi.nlm.nih.gov/BLAST/) search is the heuristic search algorithm employed by the programs blastp, blastn, blastx, megablast, tblastn, and tblastx; these programs ascribe significance to their findings using the statistical methods of Samuel K. and Altschul S.F. («Methods for assessing

the statistical significance of molecular sequence features by using general scoring schemes» *Proc. Natl. Acad. Sci. USA*, 1990, 87:2264-2268; «Applications and statistics for multiple high-scoring segments in molecular sequences». *Proc. Natl. Acad. Sci. USA*, 1993, 90:5873-5877). The computer program BLAST calculates three parameters: score, identity and similarity. The FASTA search method is described by Pearson W.R. («Rapid and sensitive sequence comparison with FASTP and FASTA», *Methods Enzymol.*, 1990, 183:63-98). The ClustalW method is described by Thompson J.D. et al. («CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice», *Nucleic Acids Res.*, 1994, 22:4673-4680).

Moreover, the intein gene can be a variant nucleotide sequence. The phrase «a variant nucleotide sequence» can mean a nucleotide sequence which encodes «a variant intein» using any synonymous amino acid codons according to the standard genetic code table (see, e.g., Lewin B., «Genes VIII», 2004, Pearson Education, Inc., Upper Saddle River, NJ 07458).

The phrase «a variant nucleotide sequence» can also mean, but is not limited to a nucleotide sequence which hybridizes under stringent conditions with the nucleotide sequence complementary to the sequence encoding the wild-type intein. Furthermore, the phrase «a variant nucleotide sequence» can also mean a nucleotide sequence which hybridizes under stringent conditions with a probe which can be prepared from the nucleotide sequence under stringent conditions so long as it encodes an active intein. «Stringent conditions» include those under which a specific hybrid, for example, a hybrid having homology, defined as the parameter «identity» when using the computer program BLAST, of not less than 20%, not less than 30%, not less than 40%, not less than 50%, not less than 60%, not less than 70%, not less than 80%, not less than 90%, not less than 95%, not less than 96%, not less than 97%, not less than 98%, or not less than 99% is formed, and a non-specific hybrid, for example, a hybrid having homology lower than the above is not formed. For example, stringent conditions can be exemplified by washing one time or more, or in another example, two or three times, at a salt concentration of 1×SSC (standard sodium citrate or standard sodium

chloride), 0.1% SDS (sodium dodecyl sulphate), or in another example, 0.1×SSC, 0.1% SDS at 60°C or 65°C. Duration of washing depends on the type of membrane used for blotting and, as a rule, should be what is recommended by the manufacturer. For example, the recommended duration of washing for the Amersham Hybond™-N+ positively charged nylon membrane (GE Healthcare) under stringent conditions is 15 minutes. The washing step can be performed 2 to 3 times. As the probe, a part of the nucleotide sequence complementary to the sequence encoding the wild-type intein may also be used. Such a probe can be produced by PCR using oligonucleotides as primers prepared on the basis of the wild-type intein sequence and a DNA-fragment containing the nucleotide sequence as a template. The length of the probe is recommended to be >50 bp; it can be suitably selected depending on the hybridization conditions, and is usually 100 bp to 1 kbp. For example, when a DNA-fragment having a length of about 300 bp is used as the probe, the washing conditions after hybridization can be exemplified by 2×SSC, 0.1% SDS at 50 °C, 60°C or 65°C.

As the genes encoding the wild-type inteins have already been elucidated (see above), the variant nucleotide sequences encoding variant inteins of the wild-type inteins can be obtained by PCR (polymerase chain reaction; refer to White T.J. et al., The polymerase chain reaction, *Trends Genet.*, 1989, 5:185-189) utilizing primers prepared based on the nucleotide sequence of the wild-type inteins and genome DNA as a template; or the site-directed mutagenesis method by treating a DNA containing the wild-type intein gene *in vitro*, for example, with hydroxylamine; or chemically synthesized as full-length gene structure.

In addition, the aforementioned explanations concerning variants of genes and inteins can also be applied *mutatis mutandis* to arbitrary proteins such as an extein, a target protein, a linker in between an extein and an intein, a penicillin-binding protein, a cell surface layer protein, and a heterologous fusion protein to be produced by secretory production in the present invention, and genes encoding them.

Target protein

The non-limiting examples of the target protein produced by secretory production by the method of the present invention include, for example, bioactive proteins, receptor proteins, antigenic proteins to be used as vaccine, and enzymes. Examples of the enzymes include, for example, transglutaminases, proteases, endopeptidases, exopeptidases, aminopeptidases, carboxypeptidases, collagenases, chitinases, and so forth.

Examples of the bioactive proteins include, for example, growth factors, hormones, cytokines, antibody-related molecules. A bioactive 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 bioactive peptide, which consists of 34 amino acid residues of N-terminus of parathyroid hormone (PTH).

Specific examples of the growth factor include, for example, epidermal growth factor (EGF), insulin-like growth factor (IGF), transforming growth factor (TGF), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), vesicular endothelial growth factor (VEGF), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage-colony stimulating factor (GM-CSF), platelet-derived 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).

Specific examples of the hormone include, for example, insulin, glucagon, somatostatin, human growth hormone (hGH), parathyroid hormone (PTH), exenatide, and calcitonin. Exenatide is a glucagon-like peptide-1 agonist (GLP-1 agonist) of 39 amino acid residues in length. The nucleic acid sequence of SEQ ID NO: 53 (GenBank accession No. U77613; version U77613.1, GI: 1916066; 142 bp – 258 bp) encodes the exenatide protein having the amino acid sequence of SEQ ID NO: 34 from *Heloderma suspectum*.

Specific examples of the cytokine include, for example, interleukins, interferons, tumor necrosis factors (TNFs).

Growth factors, hormones, and cytokines may not be strictly distinguished from each other. For example, a bioactive protein may be a protein belonging to a single group selected from growth factor, hormone, and cytokine, or may be a protein belonging to a plurality of groups selected from those.

The antibody-related molecule refers to a protein comprising a molecular species consisting of a single domain or a combination of two or more domains selected from domains that constitute a complete antibody. Examples of the domains that constitute a complete antibody include VH, CH1, CH2, and CH3, which are domains of a heavy chain, and VL and CL, which are domains of a light chain. The antibody-related molecule may be a monomer protein or a multimer protein so long as comprising the above-mentioned molecular species. In the case where the antibody-related molecule is a multimer protein, the antibody-related molecule 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 molecule include, for example, complete antibodies, Fab, F(ab'), F(ab')₂, Fc, dimer consisting of the heavy chain (H chain) and the light chain (L chain), Fc-fusion proteins, the heavy chain (H chain), the light chain (L chain), single chain Fv (scFv), sc(Fv)₂, disulfide-linked Fv (sdFv), and diabody.

Fab (fragment, antigen binding) is a part of a complete antibody except for the Fc region of the H chain, and it is an antibody fragment consisting only of an antigen-binding region. Fab is a dimer consisting of one molecule of the Fab moiety of the H chain and one molecule of L chain, and they aggregate by a disulfide bond at the C-terminus. The complete antibody is an H2L2 tetramer, and has a huge molecular weight of about 150 kDa, whereas Fab has a small molecular weight of about 50 kDa, and therefore Fab is thought to show superior permeability for an objective tissue. Since Fab does not have the Fc region, it has neither complement activity nor crystallization ability, but since it has antigen-binding ability, it is mainly used for the purpose of neutralizing an antigen. Among the antibody drugs, Fab especially attracts attention in recent years.

Specific example of Fab includes, for example, a Fab for the trastuzumab consisting of the H chain of 228 amino acid residues in length and the L chain of 214 amino acid residues in length. The nucleic acid sequences of SEQ ID NOs: 54 and 55 encode the trastuzumab Fab H and L chains having amino acid sequences of SEQ ID NOs: 35 and 36, respectively.

F(ab') is a part of a complete antibody except for the Fc' region of the H chain. F(ab') is a dimer consisting of one molecule of the F(ab') moiety of the H chain and one molecule of the L chain, and they aggregate by a disulfide bond at the C-terminus. The remainder moiety of the H chain in F(ab') is longer than the remainder moiety of the H chain in Fab, and hence, in F(ab'), the disulfide bond moiety linking the H chains remains. Therefore, two molecules of F(ab') can form F(ab')₂ by a disulfide bond. F(ab') and F(ab')₂ can also be used as antibody drugs like a Fab fragment.

Fc (fragment, crystallizable) is an antibody fragment consisting only of the Fc region that participates in the complement activity and crystallization ability. A protein consisting of the Fc region of the H chain and another functional protein fused to each other is called an Fc-fusion protein.

Specific examples of the monomer protein include, for example, transglutaminases and the insulin-like growth factor 1 (IGF-1). Examples of transglutaminase gene include genes of secretory transglutaminases of actinomycetes such as *Streptoverticillium mobaraense* IFO 13819, *Streptoverticillium cinnamoneum* IFO 12852, *Streptoverticillium griseocarneum* IFO 12776, *Streptomyces lydicus* [WO9606931], filamentous fungi such as *Oomycetes* [WO96/22366], and so forth. In addition, specific examples of the monomer protein further include monomer proteins as the antibody-related molecules, for example, the heavy chain (H chain), the light chain (L chain), scFv, and sdFv.

Further, specific examples of the multimer protein include, for example, the vascular endothelial growth factor (VEGF), insulin, interleukin-5, interferon- γ , tumor necrosis factors (TNFs). In addition, specific examples of the multimer protein further include multimer proteins such as the antibody-related molecules, for example, complete antibodies, Fab, F(ab'), F(ab')₂, Fc, dimer consisting of the heavy chain (H chain) and the light chain (L chain),

Fc-fusion proteins, sc(Fv)₂, and diabody. Among these, Fab, F(ab')₂, and Fc-fusion proteins.

The receptor protein is not particularly limited, and can be, for example, a receptor protein for any of the bioactive proteins and other bioactive substances. Examples of other bioactive substances include, for example, neurotransmitters such as dopamine. In addition, the receptor protein can also be an orphan receptor, of which ligand has not been identified.

The antigenic protein to be used as vaccine is not particularly limited so long as it is a protein which causes an immune response, and the antigenic protein can be appropriately chosen according to the intended target of the immune response.

Genes encoding the heterologous fusion 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 be modified so that the proteins include substitution, deletion, insertion, and/or addition of one or several amino acid residues. The explanations concerning variants of the penicillin-binding proteins, the cell surface layer proteins or the inteins, or a combination of them, and the genes encoding them explained herein can also be applied *mutatis mutandis* to the heterologous fusion protein to be produced by secretory production by the method of the present invention and the gene encoding it. Further, in the genes encoding these proteins, any 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.

Genetic construct

Although the genetic construct used for the present invention is not particularly limited so long as secretory production of the heterologous fusion protein is attained, it preferably contains a promoter sequence that functions in a coryneform bacterium, a nucleic acid sequence encoding a signal peptide that is ligated downstream from the promoter sequence and functions in the coryneform bacterium, and a nucleic acid sequence encoding the heterologous fusion protein that is ligated downstream from the nucleic acid

sequence encoding the signal peptide. The nucleic acid sequence encoding a signal peptide may be ligated downstream from the promoter sequence so that the signal peptide is expressed under control by the promoter. The nucleic acid sequence encoding the heterologous fusion 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. As the heterologous fusion protein includes extein and intein regions located at the N- and C-terminal parts of the protein, respectively, the nucleic acid sequence encoding the heterologous fusion protein encodes an extein and an intein in such a way that the nucleic acid sequence encoding the extein is ligated downstream from the sequence encoding the signal peptide and the nucleic acid sequence encoding the intein is ligated downstream from the sequence encoding the extein.

The genetic construct used for the present invention may also contain a control sequence (operator, terminator, etc.) effective for expression of the heterologous fusion protein gene in a coryneform bacterium at such an appropriate position that it can function. The genetic construct used for the present invention may also contain the nucleic acid sequence encoding a protein tag such as the affinity tag, the solubilization tag, the chromatography tag, the epitope tag, or the fluorescence tag. As to an affinity tag, the chitin binding protein (CBP), maltose binding protein (MBP), glutathione-S-transferase (GST), and the poly(His) tag can be used to purify the heterologous fusion protein from the crude medium using an affinity technique.

The promoter used in the present invention is not particularly limited so long as a promoter that functions in a coryneform bacterium is chosen, and it may be a promoter derived from a coryneform bacterium, or a heterogeneous promoter. The «promoter that functions in a coryneform bacterium» refers to a promoter that shows a promoter activity in a coryneform bacterium. Specific examples of the heterogeneous promoter include, for example, promoters derived from *E. coli* such as tac promoter, lac promoter, trp promoter, rplK promoter, gapA promoter, and araBAD

promoter. Among these, potent promoters such as tac promoter, and inducible promoters such as araBAD promoter may be used.

Examples of the promoter derived from a coryneform bacterium include, for example, promoters 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 deoxyarabinoheptulosonate phosphate (DAHP) synthetase gene of the aromatic amino acid biosynthesis systems such as those of tryptophan, tyrosine, and phenylalanine, the phosphoribosyl pyrophosphate (PRPP) amidotransferase gene of the nucleic acid biosynthesis systems such as those of inosinic acid and guanylic acid, the inosinic acid dehydrogenase gene, and the guanylic acid synthetase gene.

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 method for evaluating strength of a promoter and strong promoters are described in the paper of Goldstein M.A. and Doi R.H. Prokaryotic promoters in biotechnology, *Biotechnol. Annu. Rev.*, 1995, 1:105-1285, and so forth. Furthermore, it is known that substitution of several nucleotides in the Shine-Dalgarno (SD) sequence, and/or in the spacer between the SD sequence and the start codon, and/or a sequence immediately upstream and/or downstream from the start codon in the ribosome-binding site greatly affects the translation efficiency of mRNA. For

example, a 20-fold range in the expression levels was found, depending on the nature of the three nucleotides preceding the start codon (Gold L. et al., *Annu. Rev. Microbiol.*, 1981, 35:365-403; Hui A. et al., *EMBO J.*, 1984, 3:623-629).

A nucleic acid sequence of the genetic construct used for the present invention can be optimized to express the genetic construct in a coryneform bacterium as a host microorganism. Expression of the genetic construct can be optimized by substituting rare and/or low-usage codons for synonymous middle- or high-usage codons, where codon usage can be defined as the number of times (frequency) a codon is translated per unit time in the cell of an organism or an average codon frequency of the sequenced protein-coding reading frames of an organism (Zhang S.P. et al., *Gene*, 1991, 105(1):61-72). The codon usage per organism can be found in the Codon Usage Database, which is an extended web-version of the CUTG (Codon Usage Tabulated from GenBank) (www.kazusa.or.jp/codon; Nakamura Y. et al., Codon usage tabulated from the international DNA sequence databases: status for the year 2000, *Nucl. Acids Res.*, 2000, 28(1):292). The substitution of low-usage codons for synonymous high-usage codons can be preferable. The substituting rare and/or low-usage codons for synonymous middle- or high-usage codons may be combined with co-expression of the genes which encode rare tRNAs recognizing rare codons.

Signal peptide

The signal peptide used in the present invention is not particularly limited so long as a signal peptide that functions in the coryneform bacterium is chosen, and it may be a signal peptide derived from the coryneform bacterium, or it may be a heterogeneous signal peptide. The «signal peptide that functions in the 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. The signal peptide is preferably a signal peptide of a secretory protein of the coryneform bacterium as the host, more preferably a signal peptide of a cell surface layer protein of the coryneform bacterium. 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 is shown in SEQ ID NO: 56, the amino acid sequence of the signal peptide of PS2 (CspB) is shown in SEQ ID NO: 57, and the amino acid sequence of the signal peptide of SlpA (CspA) is shown in SEQ ID NO: 58. 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.

Although signal peptides have a certain characteristic of sequence common over biological species, a signal peptide that exhibits a secretory function in a certain biological species does not necessarily exhibit a secretory function in another biological species. Therefore, when a heterogeneous signal peptide is used, a signal peptide that functions in the coryneform bacterium may be appropriately chosen. Whether a certain signal peptide functions in the coryneform bacterium can be confirmed by, for example, expressing the objective protein as a fusion protein with that signal peptide, and confirming whether the protein is secreted or not.

The signal peptide may have a part of N-terminus amino acid sequence of the secretory protein from which the signal peptide is derived. The signal sequence is generally cleaved by a signal peptidase, when the translation product is secreted out of the cell. In addition, 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.

Secretory protein

It is known that a secretory protein is generally translated as a preprotein (also referred to as prepeptide) or 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-part 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-part thereof is further cleaved by a protease, and the proprotein thereby becomes a mature protein. Hence, in the method of the present invention, it is preferable to use a signal peptide for the secretory production of a heterologous fusion protein. In the present invention, 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 existing at the N-terminus of a secretory protein precursor, and usually does not exist in a natural mature protein. More specifically, the signal peptide is joined to the N-terminus of an extein through the peptide bond.

Penicillin-binding protein

In general, the penicillin-binding proteins (PBPs) refer to proteins that bind with β -lactam type antibiotics, of which enzymatic function is inhibited by binding with β -lactam type antibiotics. The penicillin-binding proteins include high molecular weight PBPs (HMW-PBPs) and low molecular weight PBPs (LMW-PBPs). The high molecular weight PBPs include class A high molecular weight PBPs (class A HMW-PBPs) and class B high molecular weight PBPs (class B HMW-PBPs). The class A HMW-PBPs has both a transpeptidase activity domain having the transpeptidase activity for crosslinking peptidoglycan moieties and a transglycosylase activity domain having the transglycosylase activity for forming a polysaccharide chain from disaccharides. The class B HMW-PBPs has a transpeptidase activity domain. For example, as for *C. glutamicum*, PBP1a and PBP1b can be mentioned as the class A HMW-PBPs. As for *C. glutamicum*, FtsI, PBP2a, and PBP2b can be mentioned as the class B HMW-PBPs.

A coryneform bacterium of the present invention can be modified so that activity of a protein that is a penicillin-binding protein has a property that if the activity thereof is reduced in a coryneform bacterium, amount of a heterologous fusion protein to be produced by secretory production is increased compared with that observed for a non-modified strain. As such a

penicillin-binding protein, for example, one selected from PBP1a, PBP1b, class B HMW-PBPs, and LMW-PBPs, or one selected from PBP1a, PBP1b, and class B HMW-PBPs, or one selected from PBP1a and PBP1b can be used.

The «property that if activity of the protein is reduced in a coryneform bacterium, amount of a heterologous fusion protein to be produced by secretory production is increased compared with that observed for a non-modified strain» refers to a property that if activity of the protein is reduced in a coryneform bacterium, an ability to produce a heterologous fusion protein by secretory production in an amount larger than that observed for a non-modified strain such as wild strain or parent strain is imparted to the coryneform bacterium. Although degree of increase of amount of the heterologous fusion protein to be produced by secretory production is not particularly limited so long as the amount of the heterologous fusion protein produced by secretory production increases compared with that observed for a non-modified strain, to produce a heterologous fusion protein by secretory production in an amount larger than that observed for a non-modified strain may mean, for example, to produce the heterologous fusion protein by secretory production in an amount larger than that observed for a non-modified strain by 10% or more, 20% or more, 30% or more, or 100% or more, in terms of the accumulation amount in the medium and/or the cell surface layer. In addition, to produce a heterologous fusion protein by secretory production in an amount larger than that observed for a non-modified strain may mean that whereas the heterologous fusion protein cannot be detected when non-concentrated culture supernatant of a non-modified strain is applied to SDS-PAGE and stained with CBB, the heterologous fusion protein can be detected when non-concentrated culture supernatant of a modified strain is applied to SDS-PAGE and stained with CBB.

Also, the «property that if activity of the protein is reduced in a coryneform bacterium, amount of a heterologous fusion protein to be produced by secretory production is increased compared with that observed for a non-modified strain» regarding a penicillin-binding protein includes a property that if activity of the protein is reduced in a strain in which activity of a cell surface layer protein is not reduced, the ability of the strain to

produce a heterologous fusion protein by secretory production is not increased, however, if activity of the protein is reduced in a strain in which activity of a cell surface layer protein is reduced, the ability of the strain to produce a heterologous fusion protein by secretory production is increased.

Whether a protein has a property that if activity of the protein is reduced in a coryneform bacterium, amount of a heterologous fusion protein to be produced by secretory production is increased compared with that observed for a non-modified strain can be confirmed by preparing a strain from a strain belonging to the coryneform bacteria by such modification that activity of the protein is reduced, quantifying amount of the heterologous fusion protein produced by secretory production observed when the modified strain is cultured in a medium, and comparing that amount with amount of the heterologous fusion protein produced by secretory production when the strain not modified (unmodified strain) is cultured in the medium.

The *Cgl0278* gene encoding the PBP1a protein of the *C. glutamicum* ATCC 13032 corresponds to a sequence complementary to the sequence of the 294001 to 296388 positions in the genome sequence registered at the NCBI database as GenBank accession BA000036 (version BA000036.3 GI: 42602314). Also, the PBP1a protein of the *C. glutamicum* ATCC 13032 is registered as GenBank accession NP_599531 (version NP_599531.1 GI: 19551529, locus_tag=«NCgl0274»). The nucleotide sequence of the *Cgl0278* gene of *C. glutamicum* ATCC 13032 and the amino acid sequence of the PBP1a protein encoded by this gene are shown in SEQ ID NOs: 43 and 44, respectively.

The *Cgl2986* gene encoding the PBP1b protein of the *C. glutamicum* ATCC 13032 corresponds to a sequence complementary to the sequence of the 3160346 to 3162508 positions in the genome sequence registered at the NCBI database as GenBank accession BA000036 (VERSION BA000036.3 GI: 42602314). Also, the PBP1b protein of the *C. glutamicum* ATCC 13032 is registered as GenBank accession YP_227236 (version YP_227236.1 GI: 62391834, locus_tag=«cg3313»). The nucleotide sequence of the *Cgl2986* gene of *C. glutamicum* ATCC 13032 and the amino acid sequence of the PBP1b

protein encoded by this gene are shown in SEQ ID NOs: 45 and 46, respectively.

Since the gene encoding the penicillin-binding protein may differ depending on species or strain to which the coryneform bacterium belongs, the penicillin-binding protein may have one or more homologues or variant proteins. In such a case, the variant proteins encoded by the penicillin-binding protein gene may have a homology, defined as the parameter «identity» when using the computer program BLAST, of not less than 80%, not less than 90%, not less than 95%, or not less than 98% with respect to the entire amino acid sequence of the wild-type protein so long as the activity of the variant proteins is maintained, or the three-dimensional structure of the variant proteins is not significantly changed relative to the wild-type proteins. Therefore, the variant nucleotide sequences of the penicillin-binding protein may have a homology, defined as the parameter «identity» when using the computer program BLAST, of not less than 80%, not less than 90%, not less than 95%, not less than 96%, not less than 97%, not less than 98%, or not less than 99% with respect to the entire nucleotide sequences of the wild-type protein genes so long as the activity of the variant proteins encoded by the genes is maintained, or the three-dimensional structure of the variant proteins is not significantly changed relative to the wild-type proteins.

Cell surface layer protein

The cell surface layer proteins are proteins constituting the cell surface layers (S-layer) of bacteria and archaea. Examples of the cell surface layer proteins of coryneform bacteria include PS1 and PS2 (also referred to as CspB) of *C. glutamicum* and SlpA (also referred to as CspA) of *C. stationis*. Among them, it is preferred that the activity of PS2 protein is reduced.

The nucleotide sequence of the *cspB* gene of *C. glutamicum* ATCC 13869 and the amino acid sequence of the PS2 protein encoded by this gene are shown in SEQ ID NOs: 59 and 57, respectively.

Also, for example, amino acid sequences of CspB homologues regarding 28 strains of *C. glutamicum* have been reported (Hansmeier N. et al., Classification of hyper-variable *Corynebacterium glutamicum* surface-layer

proteins by sequence analyses and atomic force microscopy, *J. Biotechnol.*, 2004, 112:177-193). These 28 strains of *C. glutamicum* and the GenBank accession numbers of the *cspB* gene homologues in NCBI database are exemplified hereafter (the GenBank accession numbers are shown in the parentheses).

- C. glutamicum* ATCC13058 (AY524990)
- C. glutamicum* ATCC13744 (AY524991)
- C. glutamicum* ATCC13745 (AY524992)
- C. glutamicum* ATCC14017 (AY524993)
- C. glutamicum* ATCC14020 (AY525009)
- C. glutamicum* ATCC14067 (AY524994)
- C. glutamicum* ATCC14068 (AY525010)
- C. glutamicum* ATCC14747 (AY525011)
- C. glutamicum* ATCC14751 (AY524995)
- C. glutamicum* ATCC14752 (AY524996)
- C. glutamicum* ATCC14915 (AY524997)
- C. glutamicum* ATCC15243 (AY524998)
- C. glutamicum* ATCC15354 (AY524999)
- C. glutamicum* ATCC17965 (AY525000)
- C. glutamicum* ATCC17966 (AY525001)
- C. glutamicum* ATCC19223 (AY525002)
- C. glutamicum* ATCC19240 (AY525012)
- C. glutamicum* ATCC21341 (AY525003)
- C. glutamicum* ATCC21645 (AY525004)
- C. glutamicum* ATCC31808 (AY525013)
- C. glutamicum* ATCC31830 (AY525007)
- C. glutamicum* ATCC31832 (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)

Since 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 the aforementioned nucleotide sequence so long as the gene encodes a protein having a property that if activity of the protein is reduced in a coryneform bacterium, amount of a heterologous fusion protein to be produced by secretory production is increased compared with that observed for a non-modified strain. For example, the gene encoding a cell surface layer protein may be a gene encoding a protein having the aforementioned amino acid sequence 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 having a property that if activity of the protein is reduced in a coryneform bacterium, amount of a heterologous fusion protein to be produced by secretory production is increased compared with that observed for a non-modified strain. The aforementioned explanations concerning variants of a penicillin-binding protein and a gene encoding it can also be applied *mutatis mutandis* to variant proteins of a cell surface layer protein and a gene encoding it.

Also, the «property that if activity of the protein is reduced in a coryneform bacterium, amount of a heterologous fusion protein to be produced by secretory production is increased compared with that observed for a non-modified strain» regarding a cell surface layer protein includes a property that if activity of the protein is reduced in a strain in which activity of a penicillin-binding protein is not reduced, the ability of the strain to produce a heterologous fusion protein by secretory production is not increased, however, if activity of the protein is reduced in a strain in which activity of a penicillin-binding protein is reduced, the ability of the strain to produce a heterologous fusion protein by secretory production is increased.

In the present invention, the expression «activity of a cell surface layer protein is reduced» includes a case where a coryneform bacterium has been modified so that activity of a cell surface layer protein is reduced and a case where activity of a cell surface layer protein has been intrinsically reduced in a coryneform bacterium. The «case where activity of a cell surface layer

protein has been intrinsically reduced in a coryneform bacterium» includes a case where a coryneform bacterium is intrinsically deficient in a cell surface layer protein. That is, examples of a coryneform bacterium in which activity of a cell surface layer protein is reduced include a coryneform bacterium which intrinsically deficient in a cell surface layer protein. Examples of the «case where a coryneform bacterium is intrinsically deficient in a cell surface layer protein» include a case where a coryneform bacterium is intrinsically deficient in a gene encoding a cell surface layer protein. The expression «a coryneform bacterium is intrinsically deficient in a cell surface layer protein» can mean that a coryneform bacterium is intrinsically 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 intrinsically deficient in a cell surface layer protein» can mean that a *C. glutamicum* strain is intrinsically 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 which intrinsically deficient in a cell surface layer protein include *C. glutamicum* ATCC 13032, which is intrinsically deficient in the *cspB* gene.

Means for reducing activity of a protein

The phrase «activity of a protein is reduced» can mean that activity of the protein is decreased compared with that of a non-modified strain such as a wild-type strain or a parent strain, which includes a case where the activity completely disappears. Specifically, the phrase «activity of a protein is reduced» can mean that number of molecules of the protein per cell is reduced, and/or function of each molecule of the protein is reduced compared with those of a non-modified strain. That is, the phrase «activity» regarding the phrase «activity of a protein is reduced» can refer to the transcription amount (the amount of mRNA) of a gene encoding the protein or the amount of the protein, as well as the catalytic activity of the protein. In addition, the case where «number of molecules of the protein per cell is reduced» includes a case where the protein does not exist at all. Further, the

case where «function of each molecule of the protein is reduced» includes a case where function of each molecule of the protein completely disappears.

The modification for reducing activity of a protein can be attained by, for example, reducing expression of a gene encoding the protein. «Reduction of gene expression» is also referred to as «attenuation of gene expression». The reduction of gene expression may be induced by, for example, reduction of transcription efficiency, reduction of translation efficiency, or a combination of them. Reduction of expression of a gene can be attained by modifying an expression control sequence of the gene such as a promoter, an enhancer, an attenuator, a ribosome-binding site, etc. When an expression control sequence is modified, preferably one nucleotide or more, more preferably two nucleotides or more, particularly preferably three nucleotides or more, of the expression control sequence are modified. Moreover, a part or the entire expression control sequence may be deleted. Reduction of gene expression can also be attained 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.), and so forth.

The modification for reducing activity of a protein can also be attained by, for example, disrupting the gene encoding the protein. Disruption of a gene can be attained by, for example, deleting a part or the entire coding region of the gene on a chromosome. Furthermore, the total 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 a 3'-end region, an internal region, or a 5'-end region, so long as reduction of the activity of the protein is to be attained. Deletion of a longer region can usually more surely inactivate the gene. Further, it is preferred that reading frames of the sequences upstream and downstream from the region to be deleted are not the same.

Disruption of a gene can also be attained by, for example, introduction of 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 nucleotides into the coding region of the gene on a chromosome, or the like (Qiu Z. and Goodman M.F., *J. Biol. Chem.*, 1997, 272:8611-8617; Kwon D.H. et al., *J. Antimicrob. Chemother.*, 2000, 46:793-796; Yanø T. et al., *Proc. Natl. Acad. Sci. USA*, 1998, 95:5511-5515).

Disruption of a gene can also be attained by, for example, inserting another sequence into the coding region of the gene on a chromosome. Site of the insertion may be 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 activity of the encoded protein is chosen, and examples include, for example, a marker gene such as antibiotic resistance genes, a gene useful for production of a heterologous protein, a transcription termination signal, and so forth.

Such modification of a gene on a chromosome as described above can be attained by, for example, preparing a deficient type gene in which a partial sequence of the gene is deleted so that it cannot produce a protein that can normally function, and transforming a bacterium with a recombinant DNA containing the deficient type gene to cause homologous recombination between the deficient type gene and the gene on a chromosome and thereby substitute the deficient type gene for the gene on the chromosome. In such a case, if a marker gene selected according to characteristics of the host such as auxotrophy is included in the recombinant DNA, the operation becomes easy. The protein encoded by the deficient type gene has a conformation different from that of a 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 been already established, and there are a method called «Red driven integration» (Datsenko, K.A, and Wanner, B.L., *Proc. Natl. Acad. Sci. USA*, 2000, 97:6640-6645), a method of using a linear DNA such as a method utilizing the Red driven integration in combination with an excision system derived from λ phage (referred to as «Red/ET-driven integration» or « λ Red/ET-mediated integration» (Cho, E.H., Gumpert, R.I., Gardner, J.F., *J. Bacteriol.*, 2002, 184:5200-5203),

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 replication origin which functions in a host (U.S. Patent No. 6,303,383, Japanese Patent Laid-open (Kokai) No. 05-007491), and so forth.

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

Reduction of activity of a protein can be confirmed by measuring activity of the protein. In the case of a penicillin-binding protein, whether activity of the protein has been reduced can be confirmed by, for example, measuring the transpeptidase activity and/or the transglycosylase activity depending on the class to which the protein belongs. The transpeptidase activity and/or the transglycosylase activity can be measured by, for example, a method well known to those skilled in the art. Specifically, for example, the transpeptidase and transglycosylase activities of PBP1a can be measured by measuring the reaction of oligomerizing lipid II to glycan strands and forming peptide cross-links (Born P., et al., *J. Biol. Chem.*, 2006, 281(37):26985-26993). Specifically, activity of a protein is preferably decreased to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0%, of that observed in a non-modified strain.

Reduction of expression of a gene can be confirmed by confirming reduction of transcription amount of the gene or reduction of amount of the protein expressed from the gene. Reduction of transcription amount of a gene can be confirmed by comparing amount of mRNA transcribed from the gene with that observed in a non-modified strain. Examples of the method for measuring amount of mRNA include Northern hybridization, quantitative reverse transcription polymerase chain reaction (RT-PCR), and so forth (Molecular Cloning, Cold spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001). The amount of mRNA is preferably decreased to, for example,

50% or less, 20% or less, 10% or less, 5% or less, or 0%, of that observed in a non-modified strain.

Decrease in amount of a protein can be confirmed by Western blotting using antibodies that bind to the protein (Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001). The amount of the protein is preferably decreased to, for example, 50% or less, 20% or less, 10% or less, 5% or less, or 0%, of that observed in a non-modified strain.

Disruption of a gene can be confirmed by determining nucleotide sequence or restriction enzyme map of a part of the gene or a full length of the gene, or the like depending on the means used for the disruption. Presence or absence of the gene can be measured by, for example, restricting the chromosomal DNA followed by Southern blotting using a probe based on the gene sequence, fluorescence *in situ* hybridization (FISH), and the like.

The methods mentioned above for reducing activity of a protein can also be applied *mutatis mutandis* to arbitrary proteins and genes encoding them as well as for reducing activity of a penicillin-binding protein and reducing activity of a cell surface layer protein.

Means for introducing the genetic construct

The method for introducing the genetic construct used for the present invention into the coryneform bacterium is not particularly limited. In the bacterium of the present invention, the genetic construct used for the present invention may exist on a vector that autonomously replicates out of the chromosome such as a plasmid, or may be incorporated into the chromosome. In addition, as described above, for constructing the bacterium of the present invention, modifications such as introduction of the genetic construct used for the present invention, impartation or enhancement of ability to produce a protein by secretory production, reduction of activity of a penicillin-binding protein, and reduction of activity of a cell surface layer protein can be performed in an arbitrary order.

The genetic construct used for the present invention can be introduced into a host by using, for example, a vector containing the genetic construct. The vector is not particularly limited so long as a vector autonomously

replicable in the coryneform bacterium is chosen, and 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. As the vector, for example, a plasmid derived from a coryneform bacterium is preferred. Specific examples of vector autonomously replicable in coryneform bacteria include pHM1519 (Miwa K. et al., *Agric. Biol. Chem.*, 1984, 48(11):2901-2903); pAM330 (Miwa K. et al., *Agric. Biol. Chem.*, 1984, 48(11):2901-2903); 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; pVC7 described in Japanese Patent Laid-open (Kokai) No. 9-070291; and so forth.

Further, an artificial transposon and so forth can also be used. When a transposon is used, a heterologous protein gene is introduced into a chromosome by homologous recombination or translocation ability of the transposon itself. Other examples of the introduction method utilizing homologous recombination include, for example, the 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 which functions in a host, and so forth. In addition, when a heterologous protein gene is introduced into a chromosome, so long as the genetic construct used for the present invention is constituted on the chromosome, either one or both of a promoter sequence and a nucleic acid sequence encoding the signal peptide contained in the genetic construct may be one or those originally existing in the host chromosome. Specifically, for

example, by using a promoter sequence and a nucleic acid sequence encoding the signal peptide ligated downstream from the promoter sequence originally existing in the host chromosome as they are, and replacing only the gene ligated downstream from the nucleic acid sequence encoding the signal peptide with the objective heterologous protein gene, the genetic construct used in the present invention is also constituted on the chromosome, and the bacterium of the present invention can be thereby constructed.

Also, in the case where two or more kinds of proteins are expressed, genetic constructs for secretory expression of the proteins may be harbored by the bacterium of the present invention so that secretory expression of the heterologous fusion protein(s) can be attained. Specifically, for example, all of the genetic constructs for secretory expression of the proteins may be harbored on a single vector, or may be harbored on a chromosome. Further, the genetic constructs for secretory expression of the proteins may be harbored separately on a plurality of vectors, or may be harbored separately on a single or a plurality of vectors and a chromosome. The «case where two or more kinds of proteins are expressed» includes, for example, the case where two or more kinds of heterologous fusion proteins are produced by secretory production, or the case where a hetero-multimer protein is produced by secretory production.

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 (Miwa K. et al., *Gene*, 1985, 39:281-286), the electroporation method (Dunican L.K. and Shivanian E., *Nat. Biotechnol.*, 1989, 7:1067-1070), and so forth can be used.

Methods for manipulation with recombinant molecules of DNA and molecular cloning such as preparation of plasmid DNA, digestion, ligation and transformation of DNA, selection of an oligonucleotide as a primer, incorporation of mutations, and the like may be ordinary methods well-known to the person skilled in the art. These methods are described, for example, in Sambrook J., Fritsch E.F. and Maniatis T., «Molecular Cloning: A Laboratory Manual», 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 1989; Green M.R. and Sambrook J.R., «Molecular

Cloning: A Laboratory Manual», 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2012); Glick B.R., Pasternak J.J. and Cheryl L.P., «Molecular Biotechnology: principles and applications of recombinant DNA», 4th ed., ASM Press, Washington, DC (USA), 2009).

2. Method for producing heterologous fusion protein

The present invention provides a method for producing a heterologous fusion protein comprising culturing the bacterium of the present invention and collecting the heterologous fusion protein produced by secretory production (henceforth also referred to as the «method of the present invention» or the «method for producing a heterologous fusion protein of the present invention»).

The bacterium of the present invention can be cultured according to usually used method and conditions. For example, the bacterium 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.

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 for 1 to 7 days under aerobic conditions. Further, the culture conditions for L-amino acid production by coryneform bacteria and other conditions described in the methods for producing a protein using a signal peptide of the Sec type or the Tat type can be used (refer to WO01/23591 and WO2005/103278). Further, when an inducible promoter is used for expression of the heterologous fusion protein, culture may also be performed with adding a promoter-inducing 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, the produced heterologous fusion protein is secreted out of the cells, and therefore a protein that may be lethal if it is accumulated in a large amount in cells of microorganisms, such as transglutaminases, can also be continuously produced without lethal effect.

The heterologous fusion protein secreted in the medium according to the method of the present invention 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. Further, 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 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. Further, in a certain case, the protein secreted in the cell surface layer may be used as, for example, as immobilized enzyme, without solubilizing it.

Secretory production of the objective heterologous fusion protein can be confirmed by performing SDS-PAGE for the culture supernatant and/or a fraction containing the cell surface layer as a sample thereby confirming the molecular weight of the separated protein bands. In addition, secretory production of the objective heterologous fusion protein can 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). Further, secretory production of the objective heterologous protein can be confirmed by determination of N-terminus amino acid sequence using protein sequencer. Furthermore, secretory production of the objective

heterologous fusion protein can be confirmed by measuring its mass using mass spectrometer. Also, when the objective heterologous fusion protein is an enzyme or a protein having some kind of bioactivity that can be measured, secretory production of the objective heterologous fusion protein can be confirmed by measuring enzyme activity or bioactivity of the protein in the culture supernatant and/or a fraction containing the cell surface layer as a sample.

So long as the heterologous fusion protein is produced by secretory production using a coryneform bacterium, the activity of acyl rearrangement of an intein can be confirmed by using known techniques. For example, the activity can be confirmed by determining a product of the acyl rearrangement such as an intein or a molecule consisting of an extein and a reactant residue linked to the C-terminus of the extein. The product of acyl rearrangement can be determined using any conventional method which can distinguish between the heterologous fusion protein produced by secretory production and the products of acyl rearrangement. For example, spectrophotometric methods such as infrared spectroscopy can be used to determine formation of a new chemical bond in between an extein and a reactant residue linked to the C-terminus of the extein. Other methods of the spectrophotometric analysis of organic compounds are known to the person skilled in the art. Also, a chemical modification can be used to determine a product of acyl rearrangement. For example, a treatment of the heterologous fusion protein produced by secretory production, which is active in the acyl rearrangement, in the culture supernatant or a medium with a reactant having a nucleophilic group results in trans-esterification reaction to occur. Amines, thiols, alcohols, and so forth can be used to attain acyl rearrangement. For example, an extein can be carboxy amidated by reacting a heterologous fusion protein with hydroxylamine or ammonium bicarbonate as described in Cottingham I.R. et al., A method for the amidation of recombinant peptides expressed as intein fusion proteins in *Escherichia coli*, *Nat. Biotechnol.*, 2001, 19:974-977; Xu M.-Q. and Perler F.B. The mechanism of protein splicing and its modulation by mutation, *EMBO J.*, 1996, 15(19):5146-5153. In another example, a cleavage of the thioester bond in

between an extein and intein can be induced using a thioesterification reaction by reacting a heterologous fusion protein with chemical compounds containing nucleophilic thiol (-SH) group such as dithiothreitol (DTT) (Chong S. et al., Utilizing the C-terminal cleavage activity of a protein splicing element to purify recombinant proteins in a single chromatographic step, *Nucleic Acids Res.*, 1998, 26(22): 5109-5115). Furthermore, a cleavage of the thioester bond in between an extein and intein can be induced by reacting a heterologous fusion protein with other chemical compounds containing nucleophilic thiol (-SH) group such as thiophenol or 2-mercaptoethanesulfonic acid (MES) or a salt thereof such as sodium salt MESNa) to obtain extein thioesters. Thus obtained extein thioesters can be modified by a chemical protein ligation (Muir T.W. et al., Expressed protein ligation: A general method for protein engineering, *Proc. Natl. Acad. Sci. USA*, 1998, 95:6705-6710; Evans T.C. Jr. et al., The in vitro ligation of bacterially expressed proteins using an intein from *Methanobacterium thermoautotrophicum*, *J. Biol. Chem.*, 1999, 274(7):3923-9326). Cysteine or cysteine derivatives can also be chemical compounds containing nucleophilic thiol group, which can be used to obtain a ligation product of extein and cysteine or cysteine derivatives (Kurpiers T. and Mootz H.D., Site-specific chemical modification of proteins with a prelabelled cysteine tag using the artificially split Mxe GyrA intein, *Chem. Bio. Chem.*, 2008, 9:2317-2325). The products of the nucleophilic acyl substitution reaction can be determined by any conventional method such as, for example, mass-spectroscopy, ultraviolet/visible spectroscopy, and so forth. The acyl rearrangement results in intein removal and, hence, the decrease of a molecular weight and a change of charge of the heterologous fusion protein produced by secretory production. Therefore, the products of acyl rearrangement such as an intein and a molecule consisting of an extein and a reactant residue linked to the C-terminus of the extein can be determined by protein gel electrophoresis (Simpson R.J., Electrophoresis of peptides (Tricine-SDS-PAGE), 2006, *CSH Protoc.*).

3. Method for producing modified protein

The present invention also provides a method for producing a modified protein using the heterologous fusion protein. The modified protein produced by the method of this aspect may be a protein ligated to a substance, or a protein amidated at the C-terminus. The term "protein" refers to a concept of protein including those called peptide such as oligopeptide or polypeptide. Examples of the substance include toxin, drug, fluorophore, chromophore dye, polyethylene glycols (PEG), radioisotope-labeled compounds, a second polypeptide, and magnetic particles.

The present invention provides a method for producing a protein ligated to a substance, comprising producing the heterologous fusion protein of the present invention produced by the method of the present invention for producing a heterologous fusion protein; and reacting the heterologous fusion protein with a reactant, wherein the reactant comprises the substance or the method further comprises modifying the reactant with the substance.

As described with respect to the method for producing a heterologous fusion protein of the present invention, the heterologous fusion protein can be cleaved and the extein can be ligated to a reactant by using the activity of acyl rearrangement of an intein upon reacting the heterologous fusion protein of the present invention with the reactant. The heterologous fusion protein can comprise a thioester bond or an ester bond in between the extein and the intein, which bond is obtained after the first stage of the acyl rearrangement as a result of, respectively, N-S shift or N-O shift as explained above. The obtained thioester or ester bond can be cleaved by reacting the heterologous fusion protein with a reactant containing a nucleophilic group such as, for example, amino group, thiol group or hydroxyl group, such that the reactant can ligate to the C-terminus of the extein comprising thioester or ester bond to produce a ligated reactant. As a result, a protein ligated to the reactant through the amide, thioester or ester bond is produced. Reactants containing amino, thiol and hydroxyl groups are exemplified above.

Specifically, the thioester bond in between the extein and the intein, which is obtained after the first stage of the acyl rearrangement as a result of N-S shift, can be cleaved by reacting the heterologous fusion protein with a thiol group-containing reactant such as, for example, dithiothreitol (DTT),

thiophenol or 2-mercaptoethanesulfonic acid (MES) or a salt thereof such as sodium salt (MESNa), such that the reactant can ligate to the C-terminus of the extein comprising the thioester bond to produce a ligated reactant. As a result, a protein ligated to the reactant through the thioester bond is produced.

The ligating of a reactant to the C-terminus of extein of a heterologous fusion protein can be performed by the further ordinary methods. For example, when an extein thioester is obtained as a result of the first stage of the acyl rearrangement, thus obtained extein thioester can be modified by a chemical protein ligation (Muir T.W. et al., 1998; Evans T.C. Jr. et al., 1999). Cysteine or cysteine derivatives can also be the chemical compounds containing nucleophilic thiol group, which can be used to produce a ligation product of extein and cysteine or cysteine derivatives (Kurpiers T. and Mootz H.D., 2008). Polypeptides containing cysteine residue at its N-terminus can be also ligated to thus obtain extein thioester.

Two or more reactants may be used in combination. When plural reactants are used, at least one reactant can comprise the substance or be modified with the substance. An additional reactant may be one which accelerates the reaction of the heterologous fusion protein with a reactant which comprises the substance or which is to be modified with the substance. Examples of the additional reactant include dithiothreitol and 2-mercaptoethanesulfonic acid.

The protein, which is ligated to reactant and produced by the method of the present invention, can be modified further. For example, a reactant may also contain one or more reactive groups, so that the reactive groups can react with other molecules. In one non-limiting example, a reactant may contain thiol group and azide group such as, for example, Cys-CH₂CH₂CH₂N₃. In such case, the thiol group of cysteine can react with the C-terminus of the extein, and the azide group (N₃) can react further with another compound such as, for example, toxin, drug, fluorophore or chromophore dye, and so forth. Thus the reactant can be modified with the substance.

The reactant may have or be modified to have a group which can be used for the ligation. Examples of the ligated substance include toxins, drugs,

polyethylene glycols (PEG), radioisotope-labeled compounds, a second polypeptide, and magnetic particles. When the extein comprises the target protein which can be biologically recognized by a cell or tissue, the protein ligated to the substance can be used for targeting. Examples of such the target protein include an antibody, an antigen-binding fragment thereof, and receptor ligands such as hormones. The ligated substance can be delivered to the targeted cell or tissue depending on the kind of the target protein.

Yet specifically, as described with respect to the method for producing a heterologous fusion protein of the present invention, extein of the heterologous fusion protein can be amidated at its C-terminus by reacting the heterologous fusion protein with ammonia to obtain amidated peptides or proteins (Cottingham I.R. et al., A method for the amidation of recombinant peptides expressed as intein fusion proteins in *Escherichia coli*, *Nat. Biotech.*, 2001, 19:974-977).

The present invention also provide a method for producing a protein which is amidated at the C-terminus, comprising producing a heterologous fusion protein by the method for producing the heterologous fusion protein of the present invention, and reacting the heterologous fusion protein with ammonia or a salt thereof.

Examples of the salt of ammonia includes $(\text{NH}_4)_2\text{CO}_3$, NH_4Cl , and $(\text{NH}_4)_2\text{SO}_4$. The heterologous fusion protein may be reacted with ammonia or the salt thereof in the presence of a compound containing a nucleophilic thiol group.

Examples

The present invention will be more precisely explained hereafter with reference to the following non-limiting Examples.

Example 1.

Exenatide: Experimental part

1.1. Design and chemical synthesis of EXC1 and MAG DNA-fragments

The chemical synthesis of the EXC1 DNA-fragment (SEQ ID NO: 1) and MAG DNA-fragment (SEQ ID NO: 2) were ordered from ATG Service Gene

(Russian Federation, St.-Petersburg, www.service-gene.spb.ru). The EXC1 DNA-fragment encodes a regulatory part and a structural part, which corresponds to the first 37 amino acid residues, of the *cgr_2373* gene (NCBI Reference Sequence: NC_009342.1; gene ID 4992619; locus_tag=«*cgR_2373*»; nucleotides position from 2608342 to 2609838, complement) from *Corynebacterium glutamicum* R (NCBI Reference Sequence: NC_009342.1; GI: 145294042). Also, the EXC1 encodes a multi-cloning site *KasI-NotI* and a terminator of the *nusG* gene from *Corynebacterium glutamicum* R. The sequence of the EXC1 DNA-fragment was designed to remove *NdeI* site within regulatory part of *cgr_2373* by the T305G substitution and introduce *NdeI* site just before the ATG start codon by the TCT588-590CAT substitution. The structure of the EXC1 DNA-fragment is shown on FIG. 1.

The MAG DNA-fragment encodes an artificial open reading frame (ORF) having a TEV protease cleavage site, exenatide (NCBI Pub Chem, compound ID (CID): 56927919; SEQ ID NO: 34), and the intein Mmag Magn8951 BIL from *Magnetospirillum magnetotacticum* MS-1 (Amitai G. et al., *Mol. Microbiol.*, 2003, 47(1):61-73; MAG; <http://tools.neb.com/inbase/intein.php?name=Mmag+Magn8951+BIL>; SEQ ID NO: 37). The original sequence of the MAG encoding region was modified to delete *BamHI* site by the TTG389-391CTC substitution; the last triplet encoding asparagine (N) was deleted. The structure of the MAG DNA-fragment is shown on FIG. 2.

The EXC1 and MAG DNA-fragments were cloned into pUC57 cloning vector (GenBank: Y14837.1; GI: 2440162) into *EcoRV* site. Thus the pUC57-EXC1 and pUC57-MAG plasmids were constructed (ATG Service Gene).

1.2. Construction of pEXC1 plasmid

The EXC1 DNA-fragment was excised from pUC57-EXC1 plasmid using *KpnI* and *BamHI*, purified by electrophoresis in agarose gel, and ligated with pPK4/*KpnI-BamHI* vector. Thus the pEXC1 plasmid was obtained. The pPK4 plasmid was described in U.S. Patent No. 6,090,597 A.

1.3. Construction of pEXC1-exe-MAG plasmid.

The MAG DNA-fragment was excised from pUC57-MAG plasmid using *KasI* and *NotI*, purified by electrophoresis in agarose gel, and ligated with pEXC1/*KasI-NotI* vector. Thus the pEXC1-exe-MAG plasmid was obtained.

1.4. Construction of the pEXC1-exe-MAG-HT#2.

The MAG-HT DNA-fragment (903 bp, FIG. 3) was PCR-amplified using primers P1 (SEQ ID NO: 3), P2 (SEQ ID NO: 4), and plasmid pEXC1-exe-MAG as the template. The PCR protocol was as follows: (95°C, 10"/52°C, 15"/72°C, 140"; 25 cycles). The *KasI*-MAG-HT-*NotI* DNA-fragment harbors an artificial ORF having genes encoding the exenatide (exe), intein (MAG), and His6-tag (HT) (FIG. 3). The primer P1 is flanked by *NotI* site and the His6-tag locus. Therefore, the MAG-HT DNA-fragment contained the C-terminus-His6-tagged exenatide-MAG fused gene flanked by *KasI* and *NotI* sites. The MAG-HT DNA-fragment was digested with *KasI* and *NotI*, purified by electrophoresis in agarose gel, and ligated with pEXC1/*KasI-NotI* vector. Obtained ligation mixture was introduced into JM109 bacterial strain (Catalog No. P9751) using standard calcium chloride transformation procedure (Mandel M. and Higa A., Calcium-dependent bacteriophage DNA infection, *J. Mol. Biol.*, 1970, 53:159-162). Thus, about 500 kanamycin-resistant (Kn^R) colonies were obtained. Restriction analysis of plasmids isolated from twelve arbitrary chosen clones revealed eight plasmids with desired structure. Sequencing analysis of selected plasmids revealed seven plasmids with desired sequence. One plasmid pEXC1-exe-MAG-HT with desired structure, conditionally referred to as pEXC1-exe-MAG-HT#2, was used in subsequent experiments.

1.5. Cultivation of *C. glutamicum* plasmid strains

All strains and plasmids used are described in Table 1. Electroporation of *C. glutamicum* strains was performed according to the method described in Bonnassie S. et al., Transfer of plasmid DNA to *Brevibacterium lactofermentum* by electrotransformation, *J. Gen. Microbiol.*, 1990, 136:2107-2112). Routine cultivation of *C. glutamicum* plasmid strains was performed in 2YT broth or 2YT-agar media (Sambrook J., Fritsch E.F. and Maniatis T., «Molecular Cloning: A Laboratory Manual», 2nd ed., Cold Spring Harbor

Laboratory Press, Cold Spring Harbor (USA), 1989) supplemented with kanamycin (25-50 µg/mL) or chloramphenicol (4-8 µg/mL).

The *C. glutamicum* YDK010 (WO2004/029254) strain was used as a host strain for production of the heterologous fusion proteins. The YDK010 strain is a cell surface protein PS2 deficient strain of the *C. glutamicum* AJ12036 strain (FERM BP-734). The modified YDK010 strain was cultivated in a MM-medium. The MM-medium was prepared from the components A, B, and C:

Component A:

Glucose	60 g/L
MgSO ₄ · 7H ₂ O	1 g/L
FeSO ₄ · 7H ₂ O	0.01 g/L (add 1 mL/L-medium using 10 g/L stock solution in 0.1M HCl)
MnSO ₄ · 7H ₂ O	0.01 g/L (add 1 mL/L-medium using 10 g/L stock solution)

Component B:

(NH ₄) ₂ SO ₄	30 g/L
KH ₂ PO ₄	1.5 g/L
Thiamine hydrochloride	450 µg/L (add 0.45 mL/L-medium using 1 g/L stock solution)
Biotin	450 µg/L (add 0.45 mL/L-medium using 1 g/L stock solution in 70% EtOH)
DL-methionine	0.15 g/L

Component C:

CaCO ₃	50 g/L
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The A, B, and C components were sterilized separately and mixed just before fermentation. Cells of modified YDK010 were cultivated in a volume of 4 mL of MM-medium supplemented with kanamycin (50 µg/mL) in 20-mL test-tubes upon vigorous shaking (100 rpm) at 30°C for 48 hours. Then, cells

and residual CaCO_3 were precipitated by centrifugation at 13000 rpm at 4°C; and the resulting culture broth was filtered through 0.45 μm filter, aliquoted, and stored at -20°C.

1.6. SDS-PAGE analysis of proteins

A standard procedure was used for the routine analysis of proteins (Laemmli U.K. et al., Form-determining function of the genes required for the assembly of the head of bacteriophage T4, *J. Mol. Biol.*, 1970, 49:99-113). The polyacrylamide gel (PAG) having 30%T : 3%C was stained with Coomassie R250. The minor proteins in the matrix of PAG were visualized using Dodeca Silver Stain Kit (BIO-RAD, USA, catalog No. 161-0480).

The Tricine-SDS-PAGE was used for analysis of peptides (Schagger H. and von Jagow G., Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, *Anal. Biochem.*, 1987, 166:368-379; Schagger H., Tricine-SDS-PAGE, *Nat. Prot.*, 2006, 1:16-22; Simpson R.J., Electrophoresis of peptides: Tricine-SDS-PAGE, 2006, *CSH Protoc.* The pre-incubation in 2% solution of glutaraldehyde and silver staining were used to fix small peptides in PAG such as exenatide.

The «DTT plus» sample buffer for SDS-PAGE contained 50 mM Tris-HCl pH 6.8, 30% (v/v) glycerol, 1% SDS, 100 mM DTT. The «DTT minus» sample buffer was of the same composition but without DTT. Either buffer was added to a protein solution in relation of 1:5 (1 part of buffer to 5 parts of the protein solution) and incubated at 95 °C for 5-10 minutes.

1.7. Activity assay

The activity was studied using a band-shift assay. A scheme of the band-shift assay is described on FIG. 4. On the FIG. 4, the DTT (dithiothreitol) is a particular, non-limiting example of a nucleophilic compound. A test reaction mixture contained 100 mM Tris-HCl pH 8.0, 100 mM DTT, and a preparation of the objective protein. Control reaction was of the same composition but without DTT. Reaction mixtures were incubated at 4 °C for about 16 hours (overnight). Then, samples for SDS-PAGE were

prepared using «DTT plus» and «DTT minus» sample buffers and subjected to SDS-PAGE analysis as was described in Example 1.6. Incubation of a heterologous fusion protein with a nucleophilic compound DTT results in trans-thioesterification reaction and cleavage of the heterologous fusion protein. Therefore, the band corresponding to the heterologous fusion protein disappears and two additional protein bands corresponding to an extein and an intein appear.

1.8. Purification of the exenatide-MAG (EXI1) fusion protein

Purification of the exenatide-MAG heterologous fusion protein (EXI1) was performed by fast protein liquid chromatography (FPLC) using AKTA Purifier system (GE Healthcare) at 4 °C. A sample of about 50-80 mL of the culture broth obtained after cultivation of the YDK010[pEXC1-exe-MAG] strain for 48 hours (Example 1.5) was applied onto the Sephadex-G25 column (2.6 × 30 cm) equilibrated with 20 mM Tris-HCl pH 8.0 buffer. Isocratic elution was performed at a flow rate of 10 mL/min. Fractions containing proteins were pooled and applied onto the Source-15Q 4.6/100 PE column (GE Healthcare) equilibrated with 20 mM Tris-HCl pH 8.0 buffer. A liner gradient from 0 to 1 M in 15 column volumes of NaCl in the 20 mM Tris-HCl pH 8.0 buffer was applied for elution of bounded proteins. Fractions of 1.7 mL each were collected and analyzed by SDS-PAGE (Example 1.6). Fractions containing the EXI1 protein were pooled and stored at -20 °C. If necessary, fractions were desalted using PD10 desalting columns (GE Healthcare) equilibrated with 20 mM Tris-HCl pH 8.0 buffer.

1.9. Purification of the exenatide-MAG-HT#2 protein (EXI1HT)

Purification of the exenatide-MAG protein having the His6-tag (EXI1HT) was performed using immobilized metal ion affinity chromatography (IMAC). NaCl was added to a sample of 40 mL of the culture broth obtained after cultivation of the YDK010[pEXC1-exe-MAG-HT#2] strain for 48 hours (Example 1.5) to a final concentration of 500 mM. The solution was filtered through 0.45 µm filter and applied onto the HisTrap 1 mL Chelating HP column (GE Healthcare) equilibrated with HT-binding buffer (20 mM Tris-HCl,

500 mM NaCl, and 20 mM imidazole, pH 8.0). A linear gradient from 20 to 500 mM in 10-15 column volumes of imidazole in the 20 mM Tris-HCl, 500 mM NaCl, and 20 mM imidazole, pH 8.0 buffer was applied. Fractions of 1 mL each were collected and analyzed by SDS-PAGE (Example 1.6). Fractions containing the EXI1HT protein were collected, if necessary, desalted using PD10 column equilibrated with 20 mM Tris-HCl pH 8.0, and stored at -20 °C.

1.10. Large-scale purification of the exenatide-MAG-HT#2 (EXI1HT) protein

Large-scale purification of the exenatide-MAG-HT#2 (EXI1HT) protein was performed using the denaturing IMAC. A sample of about 500 mL of the culture broth was obtained after cultivation of the YDK010[pEXC1-exe-MAG-HT#2] strain for 48 hours (Example 1.5) in ten 750-mL flask each containing 50 mL of the culture broth (Example 1.5). The culture broth was treated with ammonium sulfate (65% of saturation) and boiled (100°C) for about 2-3 minutes until a protein precipitate was formed. Denatured proteins were harvested by centrifugation, dissolved in 40 mL of buffer D (20 mM Tris-HCl pH 8.0, 8 M urea, 500 mM NaCl) and incubated at 4°C for about 16 hours (overnight). Insoluble proteins were removed by centrifugation. The final protein preparation was applied onto the HisTrap 1 mL Chelating HP column (GE Healthcare) equilibrated with buffer D. Fractions of 100 µL each were collected and analyzed by SDS-PAGE (Example 1.6). Fractions containing the EXI1HT protein were collected, supplemented with DTT to a final concentration of 200 mM, and stored at -20°C.

1.11. *In vitro* refolding of EXI1HT

1.11.1. Prolonged «equilibrium» refolding

A sample (0.5 mL) of a protein preparation from Example 1.10 was dialyzed for 16 hours (overnight) at room temperature against 100 mL of buffer R1 (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 2 mM DTT). Then, the protein preparation was subjected to size exclusion chromatography (SEC) on the Superdex-75 10/300 column (GE Healthcare) equilibrated with the 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 2 mM DTT buffer. Isocratic elution was

performed at a flow rate of 0.25 mL/min. Fractions of 0.75 mL each were collected and analyzed by SDS-PAGE (Example 1.6). Fractions containing the EXI1HT protein were collected and stored at 4°C.

1.11.2. Fast «non-equilibrium» refolding

A sample (0.5 mL) of a protein preparation from Example 1.10 was subjected to size exclusion chromatography (SEC) on the Superdex-75 10/300 column (GE Healthcare) equilibrated with the 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 2 mM DTT buffer. Isocratic elution was performed at a flow rate of 0.25 mL/min. Fractions of 0.75 mL each were collected and analyzed by SDS-PAGE (Example 1.6). Fractions containing the EXI1HT protein were collected and stored at 4°C.

Exenatide: Results

1.12. Purification and activity of the exenatide-MAG (EXI1) fusion protein

The EXI1 protein was purified using FPLC as described in Example 1.8 and analyzed using SDS-PAGE as described in Example 1.6. The EXI1 protein was eluted at high concentration of NaCl in a broad elution peak (FIGs. 5 and 6). Activity of EXI1 in fractions Nos. 18 and 22 was investigated using SDS-PAGE analysis (Example 1.7; FIG. 7). The band corresponding to EXI1 from fractions Nos. 18 and 22 disappeared and the band corresponding to intein MAG appeared upon treatment of EXI1 with DTT. Thus, activity of the EXI1 fusion protein was confirmed.

1.13. Purification and activity of the exenatide-MAG-HT#2 (EXI1HT) fusion protein

The EXI1HT protein was purified using IMAC as described in Example 1.9 and analyzed using SDS-PAGE as described in Example 1.6. Comparative SDS-PAGE analysis of proteins profile in culture broths of the control YDK010[pEXC1] and test YDK010[pEXC1-exe-MAG-HT#2] strains revealed a protein band corresponding to the EXI1HT protein (FIG. 8, compare lanes 1 and 2). The EXI1HT protein did not show activity in the culture broth (FIG. 8, compare lanes 2 and 3).

Then, a purification of EXI1HT from a culture broth of the YDK010[pEXC1-exe-MAG-HT#2] strain was performed using IMAC (Example 1.9). Approximately only one third of total soluble EXI1HT was bounded to the column (FIGs. 8 and 9). Eluted EXI1HT did not show activity even after desalting using PD10 column. Therefore, the *in vitro* refolding of EXI1HT was used (Example 1.11).

The EXI1HT was refolded as follows. EXI1HT was completely denaturated by a precipitation from the culture broth using sulfate ammonia (65% of saturation) and heating (100°C) (Example 1.10). Precipitated proteins were dissolved in a buffer containing 8 M urea and purified using denaturing IMAC (Example 1.10). DTT was added to the resulting protein preparations up to 100 mM to accomplish a denaturation process. Comparison of reducing and non-reducing SDS-PAGEs of fractions eluted from the HisTrap column revealed a partial dimerization of EXI1HT (FIG. 10). We postulate, that EXI1HT can form a dimer through the intermolecular formation of a disulphide bond (S-S) between cysteine residues which required for the N-S shift.

The prolonged «equilibrium» refolding (Example 1.11.1) and fast «non-equilibrium» refolding (Example 1.11.2) were used to refold *in vitro* EXI1HT. The prolonged «equilibrium» refolding resulted in formation of a stable inactive dimer of the EXI1HT (FIG. 11). The fast «non-equilibrium» refolding resulted in formation of the EXI1HT dimer and a monomer of the EXI1HT (FIG. 12). The EXI1HT monomer showed activity (FIG. 12).

Example 2.

Trastuzumab: Experimental part

All plasmids and strains used are described in Tables 2 and 3.

2.1. Design and chemical synthesis of intein-encoding DNA-fragments

The chemical synthesis of the intein-encoding DNA-fragments such as Int4 DNA-fragment (SEQ ID NO: 5), Int5 DNA-fragment (SEQ ID NO: 6), Int7 DNA-fragment (SEQ ID NO: 7), Int18 DNA-fragment (SEQ ID NO: 8), and Int19 DNA-fragment (SEQ ID NO: 19) were ordered from ATG Service Gene (Russian Federation, St.-Petersburg, www.service-gene.spb.ru).

The Int4 DNA-fragment encodes an artificial ORF having a TEV protease cleavage site, exenatide (SEQ ID NO: 34), and the intein Int4 (Pvu PRP8; <http://tools.neb.com/inbase/intein.php?name=Pvu+PRP8>; SEQ ID NO: 38). The original sequence of the Int4 encoding region was modified so that the last nucleotide triplet encoding asparagine (N) was deleted. The structure of the Int4 DNA-fragment is shown on FIG. 13.

The Int5 DNA-fragment encodes an artificial ORF having a TEV protease cleavage site, exenatide (SEQ ID NO: 34), and the intein Int5 (Ssp DnaE-n; <http://tools.neb.com/inbase/intein.php?name=Ssp+DnaE-n>; SEQ ID NO: 39). The original sequence of the Int5 encoding region was modified so that the last nucleotide triplet encoding asparagine (N) was deleted. The structure of the Int5 DNA-fragment is shown on FIG. 14.

The Int7 DNA-fragment encodes an artificial ORF having a TEV protease cleavage site, exenatide (SEQ ID NO: 34), and the intein Int7 (Pab Pol-II; <http://tools.neb.com/inbase/intein.php?name=Pab+Pol-II>; SEQ ID NO: 40). The original sequence of the Int7 encoding region was modified so that the last nucleotide triplet encoding asparagine (N) was deleted. The structure of the Int7 DNA-fragment is shown on FIG. 15.

The Int18 DNA-fragment encodes an artificial ORF having the intein Int18 (iBLA; SEQ ID NO: 41) and His6-tag. The structure of the Int18 DNA-fragment is shown on FIG. 16.

The Int19 DNA-fragment encodes an artificial ORF having the intein Int19 (iEKD; SEQ ID NO: 42) and His6-tag. The structure of the Int19 DNA-fragment is shown on FIG. 17.

The intein-encoding DNA-fragments were cloned into pUC57 cloning vector (GenBank: Y14837.1; GI: 2440162) into *EcoRV* site. Thus the pUC57-Int4, pUC57-Int5, pUC57-Int7, pUC57-Int18(iBLA), and pUC57-Int19(iEKD) plasmids were constructed.

2.2. Construction of the pPKSherFabH-TGC-IntX(HT)-FabL plasmids

The technical details as to construction of pPKSherFabH-TGC-IntX(HT)-FabL plasmids harboring trastuzumab Fab heavy chain (H) fused with MAG, Int4, Int5, Int7, Int18 or Int19 inteins (Table 2) are explained hereafter.

The [*KpnI*-PS2-FabH-TGC-IntX(HT)-*BamHI*] DNA-fragments were constructed using the steps from I to III as follows:

Step I:

DNA-fragments were PCR-amplified using primers PR1 and PR2 (Table 4) in an amount of 10 pmoles each, and a template DNA (Table 4) in an amount of 100 ng. The PCR protocol was as follows: 94°C, 30"/50°C, 30"/72°C, time T1 (see Table 4); 25 cycles.

Step II:

The plasmid pPKSherFabHL (SEQ ID NO: 60) in an amount of 10 pmoles was added to an aliquot of the mixtures obtained from Step I, and the following PCR protocol was applied: 94°C, 30"/30°C, 30"/72°C, 4'; 12 cycles.

Step III:

The primers PR2 (Table 4) and P3 (SEQ ID NO: 10) were added to an aliquot of the mixtures obtained from Step II, and the following PCR protocol was applied: 94°C, 30"/50°C, 30"/72°C, time T2 (see Table 4); 12 cycles.

The primers P3 (SEQ ID NO: 10), P4 (SEQ ID NO: 11), P5 (SEQ ID NO: 12), P6 (SEQ ID NO: 13), P7 (SEQ ID NO: 14), P8 (SEQ ID NO: 15), P9 (SEQ ID NO: 16), P10 (SEQ ID NO: 17), P11 (SEQ ID NO: 18), P12 (SEQ ID NO: 19), P13 (SEQ ID NO: 20), P14 (SEQ ID NO: 21), and P15 (SEQ ID NO: 22) were used for construction of the pPKSherFabH-TGC-IntX(HT)-FabL plasmids.

The plasmid pPKSherFabH-TGC-MAG(HT)-FabL (Table 4) was constructed after construction of the plasmid pPKSherFabH-TGC-MAG-FabL. In this case, only Step I as described above was performed to synthesize the *KpnI*-*BamHI* fragment, which was directly cloned into pPKSherFabHL/*KpnI*-*BamHI* vector.

Resulting DNA-fragments were purified using electrophoresis in agarose gel, digested with *KpnI* and *BamHI*, and cloned into the pPKSherFabHL/*KpnI*-*BamHI* vector. Thus the plasmids were constructed (Table 2).

2.3. Construction of the pPKSherFabH-FabL-cgc-IntX(HT) plasmids

The auxiliary plasmid pPKSherFabHL-(*Xba*/*Nhe*) was constructed as follows. The 1333 bp DNA-fragment [*NheI*-*BglII*-PS2-FabL-*NotI*-*NheI*] was

PCR-amplified using primers P17 (SEQ ID NO: 24) and P18 (SEQ ID NO: 25), and the pPKSherFabHL plasmid as the template. The PCR protocol was as follows: 94°C, 30"/50°C, 30"/72°C, 240"; 25 cycles. Resulting DNA-fragment was digested with *NheI* and ligated with pPKSherFabHL/*XbaI* vector. Thus the plasmid pPKSherFabHL-(*Xba/Nhe*) was constructed.

The [*BglII*-PS2-FabL-*CGC*-IntX-*NotI*] DNA-fragments were constructed using the steps from I to III as follows:

Step I:

DNA-fragments were PCR-amplified using primers PR3 and PR4 (Table 5) in an amount of 10 pmoles each, and a template DNA (Table 5) in an amount of 100 ng. The PCR protocol was as follows: 94°C, 30"/50°C, 30"/72°C, time T3 (see Table 5); 25 cycles.

Step II:

The plasmid pPKSherFabHL (SEQ ID NO: 60) in an amount of 10 pmoles was added to an aliquot of the mixtures obtained from Step I, and the following PCR protocol was applied: 94°C, 30"/35°C, 30"/72°C, 4'; 10 cycles.

Step III:

The primers PR4 (Table 5) and P17 (SEQ ID NO: 24) were added to an aliquot of the mixtures obtained from Step II, and the following PCR protocol was applied: 94°C, 30"/35°C, 30"/72°C, time T4 (see Table 5); 10 cycles.

Resulting DNA-fragments were purified using electrophoresis in agarose gel, digested with *BglII* and *NotI*, and cloned into the pPKSherFabHL-(*Xba/Nhe*)/*BglII-NotI* vector. Thus the plasmids were constructed (Table 2).

2.4. Construction of the pPKSherFabH-FabL-*CGC*-MAG-HT plasmid

The DNA-fragment [FabL-*CGC*-MAG-HT] (1757 bp) was PCR-amplified using primers P16 (SEQ ID NO: 23) and P17 (SEQ ID NO: 24), and the plasmid pPKSherFabH-FabL-*CGC*-MAG as the template (Example 2.3). The PCR protocol was as follows: 95°C, 10"/52°C, 15"/72°C, 240"; 25 cycles). Obtained DNA-fragment was digested with *BglII* and *NotI*, purified by electrophoresis in agarose gel, and ligated with pPK-SherFabHL-(*Xba/Nhe*)/*BglII-NotI* vector. The resulting ligation mixture was introduced into JM109 strain (Promega, catalog No. P9751) calcium chloride transformation

procedure (Mandel M. and Higa A., Calcium-dependent bacteriophage DNA infection, *J. Mol. Biol.*, 1970, 53:159-162). Thus, about 200 kanamycin-resistant (Kn^R) colonies were obtained. Restriction analysis of plasmids isolated from twelve arbitrary chosen clones revealed twelve plasmids with desired structure.

2.5. Cultivation of *C. glutamicum* plasmid strains

The *C. glutamicum* YDK010 (WO2004/029254) and YDK010ΔPBP1a strains harboring plasmids (Table 2) were cultivated in MM-medium as described in Example 1.5. Construction of *C. glutamicum* YDK010ΔPBP1a strain is described in Auxiliary example 1. Cells were cultivated in 4 mL of MM-medium supplemented with kanamycin (50 µg/mL) in 20-mL test-tubes upon vigorous shaking (200-250 rpm) at 30°C for 48-72 hours. Then, cells and residual CaCO₃ were precipitated by centrifugation at 13000 rpm at 4°C; and the resulting culture broth was filtered through 0.45 µm filter, aliquoted, and stored at -20°C.

2.6. SDS-PAGE analysis of proteins

A standard procedure was used for the routine analysis of proteins (Laemmli U.K. et al., Form-determining function of the genes required for the assembly of the head of bacteriophage T4, *J. Mol. Biol.*, 1970, 49:99-113). The polyacrylamide gel (PAG) having 30%T : 3%C was stained with Coomassie R250. The minor proteins in the matrix of PAG were visualized using Dodeca Silver Stain Kit (BIO-RAD, USA, catalog No. 161-0480).

The «DTT plus» sample buffer for SDS-PAGE contained 50 mM Tris-HCl pH 6.8, 30% (v/v) glycerol, 1% SDS, 100 mM DTT. The «DTT minus» sample buffer was of the same composition but without DTT. Either buffer was added to a protein solution in relation of 1:5 (1 part of either buffer to 5 parts of the protein solution) and incubated at 95°C for 5-10 minutes.

Reaction mixture for the activity assay contained: 100 mM Tris-HCl pH 8-11, 100 mM DTT, and protein preparation. Control reaction contained all components with exception of DTT. All reactions were incubated at incubated at 4°C for about 16 hours (overnight) and then subjected to the SDS-PAGE band shift analysis.

2.7. Activity assay

The activity was studied using a band-shift assay as described in Example 1.7. A test reaction mixture contained 100 mM Tris-HCl pH 8.0, 100 mM DTT, and a preparation of the objective protein. Control reaction was of the same composition but without DTT. Reaction mixtures were incubated at 4 °C for about 16 hours (overnight). Then, samples for SDS-PAGE were prepared using «DTT plus» and «DTT minus» sample buffers and subjected to SDS-PAGE analysis as was described in Example 2.6.

2.8. Purification of the FabH-TGC-IntX-HT, X = 4, 5, 7, 18 or 19, and FabL-CGC-MAG-HT fusion proteins

Purification of the FabH-TGC-IntX-HT, X = 4, 5, 7, 18 or 19, and FabL-CGC-MAG-HT fusion proteins was performed using IMAC (Example 1.9). A sample of about 100 mL of the culture broth obtained after cultivation of the YDK010ΔPBP1a[pPKSherFabH-TGC-IntX(HT)-FabL] and YDK010ΔPBP1a[pPKSherFabH-FabL-CGC-MAG-HT] strains for 48 hours (Example 2.5) were subjected to treatment with ammonium sulfate (from 65% to 75% of saturation). Precipitated proteins were harvested by centrifugation, dissolved in 10-12 mL of HisTrap binding buffer (20 mM Tris-HCl, 500 mM NaCl, and 20 mM imidazole, pH 8.0), and applied onto the HisTrap 1 mL Chelating HP column (GE Healthcare). An isocratic elution was applied using the HT-binding buffer (20 mM Tris-HCl, 500 mM NaCl, and 20 mM imidazole, pH 8.0). Fractions of 100 µL each were collected and analyzed by SDS-PAGE (Example 2.6). Fractions containing the FabH-TGC-IntX-HT and FabL-CGC-MAG-HT proteins were collected and stored at -20 °C.

Trastuzumab: Results

2.9. Purification and activity of fusion proteins

The fusion proteins were purified from culture broths obtained after cultivation of the plasmid strains described in Table 3. The proteins profile of the final culture broths and activity of fusion proteins were analyzed using SDS-PAGE band-shift (Examples 2.6 and 2.7). The data for expression and

activity of the FabH-TGC-IntX(HT) and FabL-CGC-IntX(HT) fusion proteins, where X = 4, 5, 7, 18 or 19, or IntX = MAG, if X = 1 are shown on FIGs. 18 – 26 and summarized in Table 9.

2.9.1. FabH-TGC-MAG-HT

The FabH-TGC-MAG-HT fusion protein was purified using denaturing and non-denaturing IMAC. FIG. 8A shows results of SDS-PAGE analysis of fractions 8-14 eluted from HisTrap column under denaturing conditions. FIG. 18B shows results of SDS-PAGE analysis of fractions 8-11 eluted from HisTrap column under non-denaturing conditions. FIG. 18C shows the same SDS-PAGE analysis as on FIG. 18B, but all samples were prepared using a sample buffer without DTT. FIG. 18D shows results confirming activity of the FabH-TGC-MAG-HT fusion protein from fractions 9 and 10.

2.9.2. FabL-CGC-MAG-HT

FIG. 19 shows reducing SDS-PAGE analysis of culture broth for expression of FabL-CGC-MAG-HT fusion protein. The FabL-CGC-MAG-HT fusion protein was purified using denaturing and non-denaturing IMAC. FIG. 20A shows results of SDS-PAGE analysis of fractions 8-15 eluted from HisTrap column under denaturing conditions. FIG. 20B shows results of SDS-PAGE analysis of fractions 8-15 eluted from HisTrap column under non-denaturing conditions. Activity of FabL-CGC-MAG-HT was confirmed using reducing SDS-PAGE of mixtures containing FabL-CGC-MAG-HT from fractions 8-12 shown on FIG. 20 (FIG. 21).

2.9.3. FabH-TGC-IntX-HT, X = 4, 5 or 7

FIG. 22 shows reducing SDS-PAGE analysis of crude cell lysates of the YDK010ΔPBP1a strain harboring the plasmids pEXC1, pPKSherFabHL, pPKSherFabH-FabL-CGC-MAG-HT, pPKSherFabH-FabL-CGC-Int4, pPKSherFabH-FabL-CGC-Int5, and pPKSherFabH-FabL-CGC-Int7. FIG. 23 shows reducing SDS-PAGE analysis of culture broth for expression of FabH-TGC-Int4-HT, FabH-TGC-Int5-HT, and FabH-TGC-Int7-HT fusion proteins. Comparative analysis of the culture broths of the YDK010ΔPBP1a strain

harboring the plasmids pPKSherFabH-FabL-cgc-IntX, where X = 4, 5 or 7 was performed using reducing (FIG. 24A) and non-reducing (FIG. 24B) SDS-PAGE. Activity of FabH-TGC-IntX-HT, X = 4, 5 or 7 was confirmed using reducing SDS-PAGE of mixtures containing FabH-TGC-IntX-HT, X = 4, 5 or 7 (FIG. 25). The aliquots of culture broths of corresponding strains were used as protein preparations. Reactions were performed at 20°C for about 16 hours (overnight).

2.9.4. FabH-TGC-IntX-HT, X = 18 or 19

The inteins Int18 (iBLA) and Int19 (iEKD) were found by a homology search for the MAG intein using the BLAST service (Table 6). Two intein-like proteins such as iBLA (Table 7) and iEKD (Table 8) were selected for cloning. The FabH-TGC-IntX-HT, X = 18 or 19 fusion proteins were expressed, purified and analyzed as described for FabH-TGC-IntX-HT, X = 4, 5 or 7 proteins (Example 2.9.3). Activity of FabH-TGC-IntX-HT, X = 18 or 19 was confirmed using reducing SDS-PAGE of mixtures containing FabH-TGC-IntX-HT, X = 18 or 19 (FIG. 26). The aliquots of culture broths of corresponding strains were used as protein preparations. Reactions were performed at 20°C for about 16 hours (overnight).

Example 3.

Trastuzumab tagged with CBD

3.1. Construction of the pPKSher-CBD plasmid

The chemical synthesis of the CBD DNA-fragment (SEQ ID NO: 65) was ordered from ATG Service Gene (Russian Federation, St.-Petersburg, www.service-gene.spb.ru). The CBD DNA-fragment encodes a structural part of the chitin binding domain (CBD) of 52 amino acid residues in length (GenBank: AAD49604.1; SEQ ID NO: 66). The structure of the CBD DNA-fragment is shown on FIG. 27. The CBD DNA-fragment was cloned into pUC57 cloning vector (GenBank: Y14837.1; GI: 2440162) into *EcoRV* site. Thus the plasmid pUC57-CBD was constructed.

The CBD DNA-fragment was excised from pUC57-MAG plasmid using *KpnI* and *BamHI*, purified by electrophoresis in agarose gel, and ligated with

pPKSherFabHL vector (Table 2) into *KpnI*/*BamHI* restriction sites. Thus the pPKSher-CBD plasmid was constructed.

3.2. Construction of the pPKSherFabH-TGC-IntX(CBD)-FabL plasmids

The technical details as to construction of pPKSherFabH-TGC-IntX(CBD)-FabL plasmids harboring trastuzumab Fab heavy chain (H) fused with Int7 or Int18 inteins (Table 6; SEQ ID NOs: 40 and 41) are explained hereafter.

The *NotI*-PS2-FabH-TGC-Int7-*KasI* and *NotI*-PS2-FabH-TGC-Int18-*KasI* DNA-fragments were PCR-amplified using primers PR5 and PR6 (Table 10) in an amount of 10 pmoles each, and a template DNA (Table 10) in an amount of 100 ng. The PCR protocol was as follows: 94°C, 30"/50°C, 30"/72°C, 4'; 25 cycles.

Resulting DNA-fragments were purified using electrophoresis in agarose gel, digested with *NotI* and *KasI*, and cloned into the pPKSher-CBD/*NotI*-*KasI* vector. Thus the pPKSherFabH-TGC-Int7(CBD)-FabL and pPKSherFabH-TGC-Int18(CBD)-FabL plasmids were constructed.

3.3. DTT-dependent self-cleavage activity assay of the FabH-TGC-IntX-CBD fused proteins

The *C. glutamicum* YDK010 (WO2004/029254) strain harboring pPKSherFabH-TGC-Int7(CBD)-FabL and pPKSherFabH-TGC-Int18(CBD)-FabL plasmids were cultivated in MM-medium as described in Example 1.5. Cells were cultivated in 4 mL of MM-medium supplemented with kanamycin (50 µg/mL) in 20-mL test-tubes upon vigorous shaking (200-250 rpm) at 30°C for 48 hours. Then, cells and residual CaCO₃ were precipitated by centrifugation at 13000 rpm at 4°C; and the resulting 3 mL of culture broth was diluted in 1:1 ratio with 3 mL of buffer A (20 mM HEPES pH 8.0, 0.5 M NaCl, 0.1 mM EDTA).

Then, the 500 µL of Chitin Resin (New England BioLabs; catalog No. S6651L) equilibrated with buffer A was added to the 6 mL of diluted culture broth and incubated at room temperature (25°C) for 15 minutes with gentle agitation.

The Chitin Resin with absorbed proteins was harvested by centrifugation, decanted, and washed twice with 5 mL of buffer A.

DTT (up to final concentration of 50 mM) was added to the 40 μ L of Chitin Resin with absorbed proteins and incubated in 1.5-mL Eppendorf vials at room temperature (25°C) for 16 hours (overnight). The control reaction was the same but without DTT addition.

The control and test vials were punctured at the bottom by hypodermic needle, inserted into the new 1.5 mL vials, and centrifuged at 2000 rpm for 3 minutes thus allowing to separate the buffer with eluted proteins (as a solution penetrating through a micro-hole in the bottom of vials) and Chitin Resin (as a solid remaining in vials).

Aliquots of the resulting control and test preparations were subjected to SDS-PAGE analysis. Activity of FabH-TGC-IntX-CBD (X = 7 or 18) was confirmed using reducing SDS-PAGE of mixtures containing FabH-TGC-IntX-CBD, X = 7 or 18 fused proteins (FIG. 28).

Example 4.

Exenatide tagged with CBD

4.1. Construction of the pEXC1-CS-exe-MAG-CBD plasmid

The following procedure was applied to construct the pEXC1-CS-exe-MAG-CBD plasmid. A DNA-fragment was PCR-amplified using primers P34 (SEQ ID NO: 70) and P35 (SEQ ID NO: 71) in an amount of 10 pmoles each and plasmid pEXC1-CspB(50)-exe-MAG-CBD (FIG. 31, SEQ ID NO: 74) as the template (100 ng). The PCR protocol was as follows: 94°C, 5'; 94°C, 30"/53°C, 30"/72°C, 1'; 25 cycles.

Resulting DNA-fragment (0.8 kbp) was purified using electrophoresis in agarose gel, digested with *KasI* and *BamHI*, and ligated with pEXC1/*KasI*-*BamHI* vector (Table 2). Resulting ligated DNA was introduced into TG1 strain (Table 1) using the standard calcium-dependent transformation. The ampicillin-resistant (Ap^R) colonies were selected on L-agar-plates. The plasmid DNA was purified from ten arbitrary chosen colonies and subjected to *BamHI* and *EcoRI* digestion. Thus the pEXC1-CS-exe-MAG-CBD plasmid was constructed.

4.2. Construction of the pEXC1-BLA-exe-MAG-CBD plasmid

The *KasI*-*NotI* DNA-fragment harboring structural part (that is, the part lacking nucleotide sequence encoding a leader peptide having the sequence of MSIQHFRVALIPFFAAFCCLPVFA (SEQ ID NO: 75)) of the *bla* gene encoding beta-lactamase (TEM; YP_006952162) was PCR-amplified using primers P36 (SEQ ID NO: 72) and P37 (SEQ ID NO: 73) in an amount of 10 pmoles each and plasmid pET15(b+) (Novagen, Germany, catalog No. 69661-3) as the template (100 ng).

Resulting DNA-fragment (0.8 kbp) was purified using electrophoresis in agarose gel, digested with *KasI* and *NotI*, and ligated with pEXC1-CS-exe-MAG-CBD/*KasI*-*NotI* vector (Example 4.1). Resulting ligated DNA was introduced into TG1 strain using the standard calcium-dependent transformation. The Ap^R colonies were selected on L-agar-plates. The plasmid DNA was purified from ten arbitrary chosen colonies and subjected to *Bam*HI and *Eco*RI digestion. Thus the pEXC1-BLA-exe-MAG-CBD plasmid was constructed.

4.3. DTT-dependent self-cleavage activity assay of the BLA-exe-MAG-CBD fused protein

The *C. glutamicum* YDK010 (WO2004/029254) strain harboring pEXC1-BLA-exe-MAG-CBD plasmid was cultivated in MM-medium as described in Example 1.5. Cells were cultivated in 4 mL of MM-medium supplemented with kanamycin (50 µg/mL) in 20-mL test-tubes upon vigorous shaking (200-250 rpm) at 30°C for 48 hours. Then, cells and residual CaCO₃ were precipitated by centrifugation at 13000 rpm at 4°C, and 3 mL of the resulting culture broth was diluted in 1:1 ratio with 3 mL of buffer A (20 mM HEPES pH 8.0, 0.5 M NaCl, 0.1 mM EDTA). The culture broth was subjected to SDS-PAGE analysis for proteins profile (FIG. 29).

Then, the 500 µL of Chitin Resin (New England BioLabs; catalog No. S6651L) equilibrated with buffer A was added to the 6 mL of diluted culture broth and incubated at room temperature (25°C) for 15 minutes with gentle agitation of mix.

Then Chitin Resin with absorbed proteins was harvested by centrifugation, decanted, and washed twice with 5 mL of buffer A.

DTT (up to final concentration of 50 mM) was added to the 40 μ L of Chitin Resin with absorbed proteins and incubated in 1.5-mL Eppendorf vials at room temperature (25°C) for 16 hours (overnight). The control reaction was the same but without DTT addition.

The control and test vials were punctured at the bottom by hypodermic needle, inserted into the new 1.5 mL vials, and centrifuged at 2000 rpm for 3 minutes thus allowing to separate the buffer with eluted proteins (as a solution penetrating through a micro-hole in the bottom of vials) and Chitin Resin (as a solid remaining in vials).

Aliquots of the resulting control and test preparations were subjected to SDS-PAGE analysis. Activity of BLA-exe-MAG-CBD was confirmed using reducing SDS-PAGE of a mixture containing BLA-exe-MAG-CBD fused protein (FIG. 30).

Example 5.

Modification of C-terminus of Trastuzumab

5.1. Secretory production of FabHL and FabH-TGC-Int7-HT-FabL

The YDK010 Δ PBP1a strain (Auxiliary example 1.2) harboring pPKSherFabHL and pPKSherFabH-TGC-Int7-HT-FabL plasmids (Table 2) is inoculated in a volume of 3 mL of CM2G medium supplemented with kanamycin (25 mg/L), and cultivated in a test tube upon shaking (120 rpm) at 30°C for 16 hours. The composition of CM2G medium is as follows:

CM2G:

Glucose	5 g/L
Polypeptone	10 g/L
Yeast extract	10 g/L
NaCl	5 g/L
Adjusted to pH 7.0 with KOH	

The obtained culture (200 μ L) is inoculated in a volume of 4 mL of MMTG medium supplemented with kanamycin (25 mg/L), and cultivated in a

test tube upon shaking (120 rpm) at 30°C for 112 hours. The MMTG medium was prepared from the Components A, B and C:

Component A:

Glucose	120 g/L
MgSO ₄ ·7H ₂ O	3 g/L
FeSO ₄ ·7H ₂ O	0.03 g/L
MnSO ₄ ·5H ₂ O	0.03 g/L

Component B:

(NH ₄) ₂ SO ₄	30 g/L
KH ₂ PO ₄	1.5 g/L
Bean lysate (TN)	0.2 g/L
Thiamine hydrochloride	450 µg/L
Biotin	450 µg/L
DL-methionine	0.15 g/L

Component C:

CaCO ₃	50 g/L
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The A, B, and C components are sterilized separately and mixed just before cultivation. After the cultivation, cells are precipitated by centrifugation at 8,000 rpm, and culture supernatant is collected.

5.2. SDS-PAGE analysis of protein

A standard procedure was used for analysis of proteins (Laemmli U.K. et al., Form-determining function of the genes required for the assembly of the head of bacteriophage T4, *J. Mol. Biol.*, 1970, 49:99-113). Polyacrylamide gels were stained with CBB Stain One (Nacalai Tesque) or SYPRO® Ruby protein gel stain (Invitrogen). Visualization of the fluorescently-labeled proteins or proteins stained with SYPRO® Ruby protein gel stain was performed with Gel Ninja (Oriental Instruments, major wavelength: 500 nm).

For reducing-condition SDS-PAGE, a protein solution was incubated at 95°C for 1 minute and then mixed with 2×Laemmli Sample Buffer (BIO RAD)

and 10×NuPAGE® Sample Reducing Agent (Invitrogen) in a ratio of 4:5:1. The mixture was incubated at 98°C for 5 minutes, and the resultant sample was subjected to SDS-PAGE.

For non-reducing-condition SDS-PAGE, a protein solution, 2×Laemmli Sample Buffer (BIO RAD) and ultra-pure water were mixed in a ratio of 4:5:1, and the resultant sample was subjected to SDS-PAGE.

5.3. Western blotting analysis of proteins

Proteins in the polyacrylamide gel obtained by SDS-PAGE according to the method described in Example 5.2 were transferred to PVDF (polyvinylidene difluoride) membrane. The resultant PVDF membrane was immersed with shaking for 1 hour or more in TTBS (20 mM Tris-HCl pH 7.6, 500 mM NaCl, 0.2% Tween 20) supplemented with 2% skim milk. Then, a 1/1000 volume of Streptavidin-Alkaline phosphatase (R&D Systems) was added to TTBS (20 mM Tris-HCl pH7.6, 500 mM NaCl, 0.2% Tween 20) supplemented with 2% skim milk, and the PVDF membrane was left with shaking in the solution for 1 hour. Then, the PVDF membrane was immersed in 1-Step™ NBT/BCIP (Thermo Scientific) and alkaline phosphatase activity was detected.

5.4. Purification of the FabHL

The culture supernatant (120 mL), in which FabHL was secreted and expressed, was applied to an affinity chromatography column HiTrap ProteinG HP (GE Healthcare, CV of 5 mL, used in tandem) equilibrated with 20 mM Tris-HCl pH 8.0. The not adsorbed by the carrier proteins (non-adsorbed proteins) were washed out with 20 mM Tris-HCl pH 8.0. The adsorbed proteins were eluted with 0.1 M glycine pH 2.7. The 1-mL fractions were each collected in 96-deep well plate, the every well of which contained 40 µL of 2 M Tris-HCl pH 8.5. The resultant fractions were analyzed with non-reducing-condition SDS-PAGE according to the method described in Example 5.2.

The FabHL-containing fraction in a total volume of 11 mL was collected and mixed with ultra-pure water (100 mL). A solution of sodium acetate (50%,

w/v) was added to adjust pH to 5. The mixture was equally divided and applied onto an ion-exchange chromatography column RESOURCE S (GE Healthcare, CV of 6 mL) equilibrated with 20 mM sodium acetate pH 5.0. The non-adsorbed proteins were washed out initially with 20 mM sodium acetate pH 5.0 and finally with 20 mM sodium dihydrogen phosphate pH 6.0. The adsorbed proteins were eluted under linear gradient of 20 mM disodium hydrogen phosphate pH 8.0 in a range from 0 to 100%, in a total volume of 120 mL. The resultant fractions were analyzed with non-reducing-condition SDS-PAGE according to the method described in Example 5.2. The FabHL protein was obtained from the fractions corresponding to 30-100% of 20 mM disodium hydrogen phosphate pH 8.0.

Fractions containing FabHL were collected and concentrated with Amicon Ultra-15 10 kDa (Millipore). The resultant concentrate was applied onto a gel filtration chromatography column HiLoad 16/60 Superdex 200 pg (GE Healthcare, CV of 120 mL) equilibrated with 50 mM Tris-HCl pH 8.0, 200 mM NaCl and 1 mM EDTA pH 8.0, and eluted with the same buffer at a flow rate of 0.8 mL/min. The resultant fractions were analyzed with non-reducing-condition SDS-PAGE according to the method described in Example 5.2. The FabHL protein was obtained from the fractions corresponding to CV of 0.72. Fractions containing FabHL were collected, concentrated and ultrafiltrated with Amicon Ultra-15 10 kDa (Millipore) to replace the buffer with 20 mM Tris-HCl pH 7.6. The obtained solution was used as a FabHL solution.

5.5. Purification of the FabH-TGC-Int7-HT-FabL

The culture supernatant, in which FabH-TGC-Int7-HT-FabL was secreted and expressed, was concentrated and ultrafiltrated with Amicon Ultra-15 10 kDa (Millipore) to replace the buffer with 20 mM Tris-HCl pH 7.6, 300 mM NaCl and 10 mM imidazole. This solution was applied onto an affinity chromatograph column His TALON superflow 5 mL Cartridge (Clontech) equilibrated with 20 mM Tris-HCl pH 7.6, 300 mM NaCl and 10 mM imidazole. The non-adsorbed proteins were washed out with 20 mM Tris-HCl pH 7.6, 300 mM NaCl and 10 mM imidazole. The adsorbed proteins were

eluted with 20 mM Tris-HCl pH 7.6, 300 mM NaCl and 150 mM imidazole. The resultant fractions were analyzed with non-reducing-condition SDS-PAGE according to the method described in Example 5.2. To perform purification, the quantity of linked columns was varied depending on the volume of the culture supernatant.

Fractions containing FabH_{TGC}-Int7-HT-FabL were collected and concentrated with Amicon Ultra-15 10 kDa (Millipore). The resultant concentrate was applied onto a gel filtration chromatography column HiLoad 16/60 Superdex 200 pg (GE Healthcare, CV of 120 mL) equilibrated with 50 mM Tris-HCl pH 8.0, 200 mM NaCl and 1 mM EDTA pH 8.0, and eluted with the same buffer at a flow rate of 0.8 mL/min. The resultant fractions were analyzed with non-reducing-condition SDS-PAGE according to the method described in Example 5.2. The FabH_{TGC}-Int7-HT-FabL protein was obtained from the fractions corresponding to CV of 0.6. Fractions containing FabH_{TGC}-Int7-HT-FabL were collected, concentrated and ultrafiltrated with Amicon Ultra-15 10 kDa (Millipore) to replace the buffer with 20 mM Tris-HCl pH 7.6. The obtained solution was used as a FabH_{TGC}-Int7-HT-FabL solution.

5.6. Thiolysis of the FabH_{TGC}-Int7-HT-FabL and ligation to Cys-Lys(Biotin)

The FabH_{TGC}-Int7-HT-FabL protein solution was added to a reaction mixture {100 mM Tris-HCl pH 7.0, 500 mM NaCl, 1 mM EDTA pH 8.0, 1 mM TCEP (tris(2-carboxyethyl)phosphine) pH 8.0, 0 or 5 or 50 mM MESNa pH 7.0 and 5 mM Cys-Lys(Biotin) (also abbreviated as Cys-Lys(Biot)-NH₂, see Auxiliary example 2)} to a final concentration of FabH_{TGC}-Int7-HT-FabL 150 µg/mL and reacted in a volume of 50 µL at 37°C for 14 hours. As a control experiment, the FabHL protein solution was added to the same reaction mixture to a final concentration of 150 µg/mL and reacted under the same condition. The reaction scheme is shown on FIG. 32. After the reaction, the reaction mixture was ultrafiltrated with Amicon Ultra-0.5 3 kDa (Millipore) to replace the buffer with 20 mM Tris-HCl pH 7.6. The reducing-condition SDS-PAGE and the Western blotting analysis were performed according to the methods described in Examples 5.2 and 5.3. The cleavage of intein from

FabH-TGC-Int7-HT-FabL and ligation of FabH to Cys-Lys(Biotin) were observed under various concentrations of MESNa (FIG. 33).

The FabH-TGC-Int7-HT-FabL solution was added to a reaction mixture {100 mM Tris-HCl (pH7.0), 500 mM NaCl, 1 mM EDTA (pH8.0), 5 mM TCEP (pH8.0), 50 mM MESNa (pH7.0), 5 mM Cys-Lys(Biot)-NH₂} to a final concentration FabH-TGC-Int7-HT-FabL 150 µg/ml and reacted in a volume of 250 µl at 37°C. At each of specified time points, sampling was performed and the sampled reaction mixture was ultrafiltrated with Amicon Ultra-0.5 3 kDa (Millipore) to replace the buffer with 20 mM Tris-HCl (pH7.6). The reducing-condition SDS-PAGE and the Western blotting analysis were performed according to the methods described in Examples 5.2 and 5.3. The time-dependent cleavage of intein and ligation of FabH to Cys-Lys(Biot)-NH₂ were observed (FIG. 34).

5.7. Thiolysis of the FabH-TGC-Int7-HT-FabL, ligation to Cys-CH₂CH₂CH₂N₃ and reaction with dibenzocyclooctyne-fluor 488

The FabH-TGC-Int7-HT-FabL protein solution was added to a reaction mixture {100 mM Tris-HCl pH 7.0, 500 mM NaCl, 1 mM EDTA pH 8.0, 1 mM TCEP pH 8.0, 50 mM MESNa pH 7.0 and 5 mM Cys-CH₂CH₂CH₂N₃ (see Auxiliary example 3)} to a final concentration of FabH-TGC-Int7-HT-FabL 150 µg/mL and reacted in a volume of 100 µL at 37°C for 42 hours. As a control experiment, the FabHL protein solution was added to a final concentration of 150 µg/mL and reacted under the same condition. The reaction scheme is shown on FIG. 36. After thiolysis and ligation, the reaction mixture was ultrafiltrated with Amicon Ultra-0.5 3 kDa (Millipore) to replace the buffer with 20 mM Tris-HCl pH 7.6. An aliquote (20 µL) of the obtained solution was mixed with sodium phosphate buffer pH 7.0 (final concentration 100 mM) and dibenzocyclooctyne-fluor 488 (Sigma Aldrich, FIG. 35) (final concentration 1 mM). The copper-free click reaction was conducted in a volume of 25 µL at 25°C for 16 hours with shading. The reducing-condition SDS-PAGE was performed according to the method described in Example 5.2. The cleavage of intein from FabH-TGC-Int7-HT-FabL, ligation of FabH to Cys-

CH₂CH₂CH₂N₃ and reaction of the ligation product with dibenzocyclooctyne-fluor 488 was observed (FIG. 37).

Auxiliary example 1.

Construction of *C. glutamicum* deficient in penicillin-binding protein PBP1a

1.1. Construction of vector pBSΔCgl0278 for deleting *Cgl0278* gene encoding PBP1a

The genome sequence of *C. glutamicum* ATCC13032 and the nucleotide sequence of the *Cgl0278* gene encoding the penicillin-binding protein PBP1a have already been determined (GenBank accession BA000036 (version BA000036.3 GI: 42602314, locus_tag=«NCgl0274»). With reference to this sequence, the primers P27 (SEQ ID NO: 61), P28 (SEQ ID NO: 62), P29 (SEQ ID NO: 63), and P30 (SEQ ID NO: 64) were synthesized. By PCR using the chromosomal DNA of the *C. glutamicum* ATCC 13869 strain prepared in a conventional manner (Saito H. and Miura K.I., *Biochim. Biophys. Acta*, 1963, 72:619-629) as a template, and the primers of P27 and P28, and P29 and P30, about 1 kbp of 5' side upstream region and about 1 kbp of 3' side downstream region of *Cgl0278* encoding PBP1a were amplified, respectively. Then, by PCR using both the amplified DNA fragments as a template and primers P27 and P30, a DNA fragment of about 2 kbp consisting of both the fragments fused to each other was obtained. In the primers P27 and P30, recognition sequences for the restriction enzymes *Bam*HI and *Xba*I were designed, respectively. For PCR, Pyrobest DNA Polymerase (produced by Takara Bio) was used, and the reaction conditions were those of the protocol recommended by the manufacturer. This DNA fragment was treated with the restriction enzymes *Bam*HI and *Xba*I, and inserted into the *Bam*HI-*Xba*I site of pBS4 described in WO2005/113744 to obtain a vector pBSΔCgl0278 for deleting the *Cgl0278* gene. For the ligation reaction, DNA Ligation Kit Ver. 2.1 (produced by Takara Bio) was used, and the reaction conditions were those of the protocol recommended by the manufacturer.

1.2. Construction of PBP1a-deficient strain

The *C. glutamicum* YDK010 strain described in WO2004/029254 was transformed with the constructed vector pBSΔCgl0278. The *C. glutamicum* YDK010 strain is a cell surface layer protein PS2 deficient strain of the *C. glutamicum* AJ12036 strain (FERM BP-734) (WO2004/029254). Strain was selected from the obtained transformants according to the methods described in WO2005/113744 and WO2006/057450 to obtain YDK010ΔPBP1a strain deficient in the *Cgl0278* gene.

Auxiliary example 2.

Synthesis of Cys-Lys(Biotin)

The biotin-labeled peptide Cys-Lys(Biotin) (also abbreviated as Cys-Lys(Biot)-NH₂) was chemically synthesized using ordinary methods according to the scheme shown on FIG. 38.

Auxiliary example 3.

3.1. Synthesis of Boc-Cys(Trt)-CH₂CH₂CH₂N₃

To a solution of Boc-Cys(Trt)-OH 1.39 g (3.0 mmol) in dichloroethane (28 mL), 3-azido-1-propanamine 0.36 g (3.6 mmol), 1-hydroxy-7-azabenzotriazole 0.52 g (3.8 mmol), triethylamine 300 μL, and EDC-HCl salt (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) 0.73 g (3.8 mmol) were added. The mixture was stirred at room temperature for 75 hours. The obtained reaction mixture was washed twice with saturated aqueous ammonium chloride solution (20 mL), and the organic phase was collected and concentrated. The resultant oil was purified using liquid column chromatography (column: 20 cm × 3 cm I.D.; mobile phase: dichloromethane and methanol as 25:1, v/v; isocratic elution was applied.). Thus Boc-Cys(Trt)-CH₂CH₂CH₂N₃ was obtained (1.64 g, 3.0 mmol; FIG. 39).

3.2. Synthesis of Cys-CH₂CH₂CH₂N₃

To a mixture of trifluoroacetate (30 mL), triisopropylsilane (0.8 mL), and water (0.8 mL), Boc-Cys(Trt)-CH₂CH₂CH₂N₃ 1.64 g (3.0 mmol) was added. The mixture was stirred at room temperature for 2 hours. After the solvent

was evaporated under reduced pressure, dichloromethane (30 mL) and water (30 mL) were added to separate organic and aqueous layers. The aqueous phase was collected and washed with dichloromethane (30 mL) three times. The resultant aqueous phase was concentrated under reduced pressure, diluted with 0.05% (w/v) trifluoroacetate aqueous solution (10 mL), and purified using preparative liquid chromatography (column: Hydrosphere C18, 25 cm × 2 cm I.D.; mobile phase: from 0.05% (v/v) TFA in H₂O to 0.05% (v/v) TFA in CH₃CN; linear gradient from 0%-50% CH₃CN with 0.05% TFA over 30 min; flow rate: 8 mL/min). The fractions containing Cys-CH₂CH₂CH₂N₃ were collected and lyophilized. Thus Cys-CH₂CH₂CH₂N₃ was obtained (0.46 g, 2.3 mmol; FIG. 40).

¹H NMR (400 MHz, DMSO-*d*₆): δ 1.68–1.72(m, 2H), δ 2.89–2.93 (m, 2H), δ 3.10–3.30 (m, 2H), δ 3.35–3.45 (m, 2H), δ 3.88–3.92 (m, 1H), δ 8.15–8.35 (bs, 2H), δ 8.50–8.55 (m, 1H), δ 8.60–8.70 (bs, 1H).

While the invention has been described in detail with reference to preferred embodiments thereof, it will be apparent to one skilled in the art that various changes can be made, and equivalents employed, without departing from the scope of the invention. All the cited references herein are incorporated by reference as a part of this application.

Table 1.

Strain / Plasmid	Description	Reference
pPK4	Expression shuttle-vector	U.S. Patent No. 6,090,597 A
pEXC1	Expression shuttle-vector constructed by cloning the <i>KpnI</i> -EXC1- <i>BamHI</i> expression cassette (EXC1 contains promoter PS2 (<i>cspB</i>), multi-cloning site, and terminator of <i>nusG</i> gene) into pPK4/ <i>KpnI</i> - <i>BamHI</i> vector.	This work
pEXC1-exe-MAG	The DNA-fragment containing the exenatide-MAG fusion gene was cloned into pEXC1 vector.	
pEXC1-exe-MAG-HT#2	The C-terminus-His ₆ -tagged exenatide-MAG fusion gene cloned into pEXC1 vector.	
TG1	<i>E. coli supE hsdΔ5thi Δ(lac-proAB)F'</i> [<i>traD36 proAB⁺ lac⁺lacZΔM15</i>]	Gibson, T.J. (1984) Studies on the Epstein-Barr virus genome. Ph.D. thesis, University of Cambridge, UK.
JM109	<i>E. coli F (traD36, proAB⁺ lacI_q, (lacZ)M15) endA1 recA1 hsdR17(rk⁻, mk⁺) mcrA supE44 λ⁻ gyrA96 relA1(lac-proAB) thi-1</i>	Promega, catalog No. P9751
YDK010	<i>C. glutamicum</i> strain	WO2004/029254
YDK010 [pEXC1]	YDK010 harbouring plasmid designated in brackets.	This work
YDK010 [pEXC1-exe-MAG]		
YDK010 [pEXC1-exe-MAG-HT#2]		

Table 2.

Strain / Plasmid	Description	Reference
Auxiliary plasmids		
pPKSherFabHL	Expression shuttle-vector harboring genes encoding trastuzumab Fab heavy chain (H) and light chain (L) (FabHL).	This work
pEXC1	Expression shuttle-vector	This work
pEXC1-exe-MAG-HT#2	The C-terminus-His ₆ -tagged exenatide-MAG fusion gene cloned into pEXC1 vector.	This work
pPKSherFabHL- <i>Xba</i> I/ <i>Nhe</i> I	Auxiliary plasmid for cloning FabHL-G-MAG fusion gene.	This work
Plasmids harboring genes encoding inteins		
pUC57-MAG	Standard <i>E. coli</i> vector harboring chemically synthesized genes encoding Inteins MAG, Int4, Int5, Int7, Int18, and Int19.	ATG Service Gene
pUC57-Int4		
pUC57-Int5		
pUC57-Int7		
pUC57-iBLA(Int18)		
pUC57-iEKD(Int19)		
Inteins fused with FabL (Trastuzumab)		
pPKSherFabH-FabL-cgc-MAG-HT	Shuttle-vectors harboring genes encoding trastuzumab Fab heavy chain (H) (FabH) and light chain (L) (FabL) fused with an intein or a His ₆ -tagged (HT) intein through the Cys-Gly-Cys (CGC) linker.	This work
pPKSherFabH-FabL-cgc-MAG		
pPKSherFabH-FabL-cgc-Int4		
pPKSherFabH-FabL-cgc-Int5		
pPKSherFabH-FabL-cgc-Int7		
Inteins fused with FabH (Trastuzumab)		
pPKSherFabH-TGC-MAG-HT-FabL	Shuttle-vectors harboring genes encoding trastuzumab Fab heavy chain (H) (FabH) fused with an intein or a His ₆ -tagged (HT) intein through the Thr-Gly-Cys (TGC) linker and light chain (L) (FabL).	This work
pPKSherFabH-TGC-MAG-FabL		
pPKSherFabH-TGC-Int4-HT-FabL		
pPKSherFabH-TGC-Int5-HT-FabL		
pPKSherFabH-TGC-Int7-HT-FabL		
pPKSherFabH-TGC-Int18-HT-FabL		
pPKSherFabH-TGC-Int19-HT-FabL		

Table 3.

Strain / Plasmid	Description	Reference
YDK010	<i>C. glutamicum</i> strain	WO2004/029254
YDK010ΔPBP1a	<i>C. glutamicum</i> strain modified so that activity of a penicillin-binding protein is reduced	This work
YDK010ΔPBP1a [pPKSherFabH _{-TGC} -IntX(HT)-FabL]; X = 4, 5, 7, 18 or 19, or IntX = MAG, if X = 1	All constructed plasmids were introduced into YDK010ΔPBP1a strain using electroporation	This work
YDK010ΔPBP1a [pPKSherFabH- FabL _{-CGC} -IntX(HT)]; X = 4, 5, 7, 18 or 19, or IntX = MAG, if X = 1	All constructed plasmids were introduced into YDK010ΔPBP1a strain using electroporation	This work

Table 4.

Intein	Primer PR1	Primer PR2	Template DNA	Time T1 (min:sec)	Time T2 (min:sec)
MAG	P4	P5	pUC57-MAG	1:30	5:00
MAG-HT	P3	P6	pPKSherFabH _{-TGC} -MAG-FabL	5:00	-
Int4-HT	P7	P8	pUC57-Int4	2:00	5:00
Int5-HT	P9	P10	pUC57-Int5	2:00	5:00
Int7-HT	P11	P12	pUC57-Int7	2:00	5:00
Int18-HT	P13	P15	pUC57-Int18(iBLA)	1:30	5:00
Int19-HT	P14	P15	pUC57-Int19(iEKD)	1:30	5:00

Table 5.

Intein	Primer PR3	Primer PR4	Template DNA	Time T3 (min:sec)	Time T4 (min:sec)
MAG	P19	P20	pEXC1-exe-MAG	1:30	4:00
Int4	P21	P22	pUC57-Int4	1:30	4:20
Int5	P23	P24	pUC57-Int5	1:30	4:20
Int7	P25	P26	pUC57-Int7	1:30	4:20

Table 6.

Intein	Name / Prototype allele *	Abbreviation	Source
MAG	Mmag Magn8951 BIL	MAG	<i>Magnetospirillum magnetotacticum</i> MS-1
Int4	Pvu PRP8	PVU	<i>Penicillium vulpinum</i>
Int5	Aha DnaE-n	SSP	<i>Aphanothece halophytica</i>
Int7	Pab Pol-II	PAB	<i>Pyrococcus abyssi</i>
Int18	/Mmag Magn8951 BIL	iBLA	<i>Brevibacillus laterosporus</i> LMG 15441
Int19	/Mmag Magn8951 BIL	iEKD	Uncultured bacterium

* - Inteins' Names / Prototypes alleles are given in accordance with the intein database InBase (<http://tools.neb.com/inbase/list.php>).

Table 7.

iBLA

Wall-associated protein [Brevibacillus laterosporus LMG 15441]

Sequence ID: ref|ZP_08642447.1|Length: 1897 Number of Matches: 1

Range 1: 1664 to 1795

Alignment statistics for match #1						
Score	Expect	Method	Identities	Positives	Gaps	Frame
54.7 bits(130)	2e-06	Composition- based stats	51/151 (34%)	73/151 (48%)	30/151 (19%)	

```

Query   1      CFVAGTPVRMADGSEKAJETVEIGE QV-QGTDGT-----INEVIGFGRPRLDGRRLYALNS   55
          CF AGT V + D  EK IE +E+G++V  +D T      EV+G  + + D  ++Y ++
Sbjct  1664    CFTAGTKV-LTDEGEKPIEEIEVGDKVLSKSDETGVVAYKEVVGLFQKQSD--QIYNVHV   1720

Query   56      LD---FFVTADHPFLTSG-GWKSLDPDVTNRINPALNVTQLVIGDTLITVGGP-VDLRSIE   111
          D      TA+HPF  G GW                      V  L +GD L++ G  + +  IE
Sbjct  1721    GDEVLEATAEHFPWVDGKGWTF-----VKDLKVGDLLVSSSGTTLAIEKIE   1766

Query   112     SQDAPAE TVVYNLHLIGNNTYVAS--GYVH   140   (SEQ ID NO: 37)
          AP ET VYN  +   N+Y  S  G +VH
Sbjct  1767    K--APRETTVYNFEVKDFNSYFVSNLGIWVH   1795   (SEQ ID NO: 41)

```

Table 8.

iEKD

Hypothetical protein ACD_65C00277G0001, partial [uncultured bacterium]

Sequence ID: gb|EKD47815.1| Length: 1180 Number of Matches: 1

Range 1: 655 to 783

Alignment statistics for match #1						
Score	Expect	Method	Identities	Positives	Gaps	Frame
47.0 bits(110)	0.001	Composition-based stats	41/142 (29%)	67/142 (47%)	15/142 (10%)	
Query 1	CFVAGTPVRMADGSEKAIETVEIGEQQVQGTDTGTINEVIGFGRPRLDGRRLYALNSLDFFV					60
	CF GT + M+DGS K IE + G+ ++ +E++ G L + + V					
Sbjct 655	CFKEGTKILMSDGSYKNIEDIVAGDLIKTRPNEFDEILVTGE-----VLKTYEHVV					705
Query 61	TADHPFLTSGGWKSLDPDVTNRINPALNVTQ-LVIGDTLITVGG-PVDLRSIESQDAPAE					118
	+ +GG + P+ +N ++ + L +GD LI + G V + S E+ +AP					
Sbjct 706	ST--YISVNGGVLEVTPHEVIFLNDKWSLAENLKVGDYLINMDGEKVWVESAEINAP--					761
Query 119	TVVYNLHLIGNNTYVASGYVH					140 (SEQ ID NO: 37)
	T VYN + +TY A YVH					
Sbjct 762	TKVYNFEVEKYHTYFADNIYVH					783 (SEQ ID NO: 42)

Table 9.

Intein	Mw (kDa)	IP ¹⁾	Ch ²⁾ (pH 7)	Cys No. ³⁾	Fab Trastuzumab	
					Light chain (FabL)	Heavy chain (FabH)
MAG	15	4.61	-7.7	1	P/A ⁴⁾	P
Int4 (PVU)	18	4.83	-10.85	1	nd	P/A
Int5 (SSP)	14	4.35	-13.96	2	P	P
Int7 (PAB)	21	4.98	-10.76	1	P/nt	P/A
Int18 (iBLA)	15	4.66	-9.95	1	nt	P/A
Int19 (iEKD)	15	4.64	-10.87	1	nt	P/A

1) IP - isoelectric point;

2) Ch - protein charge at pH 7;

3) Cys - quantity of cysteine residues in protein;

4) P - production of a fusion protein was detected in culture broth of the corresponding plasmid strain; A - activity of the fusion protein was detected by using band-shift assay; nd - the fusion protein was not detected in the culture broth; nt - not tested.

Table 10.

DNA-fragment	Primer PR5	Primer PR6	Template DNA
FabH _{-TGC} -Int7-CBD	P31	P32	pPKSherFabH _{-TGC} -Int7(HT)-FabL
FabH _{-TGC} -Int18-CBD		P33	pPKSherFabH _{-TGC} -Int18(HT)-FabL

CLAIMS

1. A coryneform bacterium having an ability to produce a heterologous fusion protein by secretory production, which has been modified to express a genetic construct for secretory production of the heterologous fusion protein encoding at least a heterologous fusion protein, wherein said heterologous fusion protein comprises an extein and an intein having an activity of acyl rearrangement.
2. The coryneform bacterium according to claim 1, wherein said extein further comprises a target protein.
3. The coryneform bacterium according to claim 2, wherein said extein further comprises a linker, which is linked to the C-terminus of the target protein in the extein and is in between the target protein and the intein.
4. The coryneform bacterium according to claim 3, wherein said linker is a sequence of one or more amino acid residues.
5. The coryneform bacterium according to claim 4, wherein said linker has the $\text{-NH-CH(R1)-CO-NH-CH(R2)-CO-}$ motif at the C-terminus, where R1 and R2 are a side-chain group of a proteinogenic L-amino acid of the same or different kinds, if the linker has a length of two or more amino acid residues.
6. The coryneform bacterium according to claim 5, wherein said R1 is the side-chain group of any proteinogenic L-amino acid and R2 is the side-chain group of L-cysteine, or R1 is hydrogen and R2 is the side-chain group of L-cysteine.
7. The coryneform bacterium according to any of claims 1 and 2, wherein said target protein is a heterologous protein for the coryneform bacterium.
8. The coryneform bacterium according to claim 7, wherein said target protein is selected from the group consisting of a bioactive protein, a receptor protein, an antigenic protein, and an enzyme.
9. The coryneform bacterium according to claim 8, wherein said bioactive protein is selected from the group consisting of a growth factor, a hormone, a cytokine, and an antibody-related molecule.

10. The coryneform bacterium according to any of claims 8 and 9, wherein said bioactive protein is the exenatide as defined in the following (A) or (B):
 - (A) a protein having the amino acid sequence of SEQ ID NO: 34,
 - (B) a variant protein having the amino acid sequence of SEQ ID NO: 34, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues and has activity according to the amino acid sequence of SEQ ID NO: 34.
11. The coryneform bacterium according to claim 9, wherein said antibody-related molecule consists of one or more proteins selected from Fab, F(ab')₂, an Fc-fusion protein, and scFv.
12. The coryneform bacterium according to claim 11, wherein said Fab is a Fab for the trastuzumab consisting of the heavy chain as defined in the following (C) or (D) and a Fab for the trastuzumab consisting of the light chain as defined in the following (E) or (F):
 - (C) a protein having the amino acid sequence of SEQ ID NO: 35,
 - (D) a variant protein having the amino acid sequence of SEQ ID NO: 35, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues and has activity according to the amino acid sequence of SEQ ID NO: 35.
 - (E) a protein having the amino acid sequence of SEQ ID NO: 36,
 - (F) a variant protein having the amino acid sequence of SEQ ID NO: 36, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues and has activity according to the amino acid sequence of SEQ ID NO: 36.
13. The coryneform bacterium according to claim 10, wherein said intein is defined in the following (G) or (H):
 - (G) an amino acid sequence of SEQ ID NO: 37,
 - (H) a variant amino acid sequence of SEQ ID NO: 37, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues and has activity of acyl rearrangement.
14. The coryneform bacterium according to claim 12, wherein said intein is defined in the following (I) or (J), if the Fab is the Fab for the

trastuzumab consisting of the heavy chain as defined in the preceding (C) or (D):

(I) an amino acid sequence of SEQ ID NO: 38, 40, 41 or 42,

(J) a variant amino acid sequence of SEQ ID NO: 38, 40, 41 or 42, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues and has activity of acyl rearrangement.

15. The coryneform bacterium according to claim 12, wherein said intein is defined in the following (K) or (L), if the Fab is the Fab for the trastuzumab consisting of the light chain as defined in the preceding (E) or (F):

(K) an amino acid sequence of SEQ ID NO: 37,

(L) a variant amino acid sequence of SEQ ID NO: 37, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues and has activity of acyl rearrangement.

16. The coryneform bacterium according to claim 1, wherein said bacterium has been modified further so that activity of a penicillin-binding protein is reduced.

17. The coryneform bacterium according to claim 16, wherein said activity of the penicillin-binding protein is reduced by attenuating expression of a gene encoding the penicillin-binding protein or disrupting the gene.

18. The coryneform bacterium according to claim 17, wherein said penicillin-binding protein is PBP1a or PBP1b.

19. The coryneform bacterium according to claim 18, wherein said penicillin-binding protein is defined in the following (M) or (N):

(M) an amino acid sequence of SEQ ID NO: 44 or 46,

(N) a variant protein having the amino acid sequence of SEQ ID NO: 44 or 46, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues and has activity that if the activity thereof is reduced in the coryneform bacterium, amount of the heterologous fusion protein produced by secretory production is increased compared with that observed for a non-modified strain.

20. The coryneform bacterium according to any of claims 16 to 19, wherein said bacterium has been modified further so that activity of a cell surface layer protein is reduced.
21. The coryneform bacterium according to claim 20, wherein said activity of the cell surface layer protein is reduced by attenuating expression of a gene encoding the cell surface layer protein or disrupting the gene.
22. The coryneform bacterium according to claim 21, wherein said cell surface layer protein is PS1, CspB, or CspA.
23. The coryneform bacterium according to claim 22, wherein said cell surface layer protein is defined in the following (O) or (P):
(O) an amino acid sequence of SEQ ID NO: 56, 57 or 58,
(P) a variant protein having the amino acid sequence of SEQ ID NO: 56, 57 or 58, but which includes substitution, deletion, insertion, or addition of one or several amino acid residues and has activity that if the activity thereof is reduced in the coryneform bacterium, amount of the heterologous fusion protein produced by secretory production is increased compared with that observed for a non-modified strain.
24. The coryneform bacterium according to claim 1, wherein said genetic construct for secretory production of the heterologous fusion protein further comprises a promoter that functions in the coryneform bacterium.
25. The coryneform bacterium according to any of claims 1 and 24, wherein said genetic construct for secretory production of the heterologous fusion protein further comprises a signal peptide that functions in the coryneform bacterium.
26. The coryneform bacterium according to claim 1, wherein said bacterium belongs to the genus *Corynebacterium* or *Brevibacterium*.
27. The coryneform bacterium according to claim 26, wherein said bacterium is *Corynebacterium glutamicum*.
28. A method for producing a heterologous fusion protein by secretory production, comprising:
 - cultivating the bacterium of any of claims 1 to 27 in a culture medium;
 - and

- collecting the heterologous fusion protein produced by secretory production.
29. A method for producing a protein ligated to a substance, comprising:
- producing a heterologous fusion protein by the method of claim 28, and
 - reacting the heterologous fusion protein with a reactant, wherein the reactant comprises the substance, or the method further comprises modifying the reactant with the substance.
30. The method according to claim 29, wherein the heterologous fusion protein comprises a thioester or an ester bond in between the extein and the intein, and said thioester or said ester bond is cleaved by reacting the heterologous fusion protein with a reactant comprising a nucleophilic group selected from amino group, thiol group and hydroxyl group.
31. The method according to claim 29, wherein the heterologous fusion protein comprises a thioester bond in between the extein and the intein, and said thioester bond is cleaved by reacting the heterologous fusion protein with the reactant comprising thiol group.
32. The method according to claim 31, wherein the heterologous fusion protein is reacted with the reactant in the presence of 2-mercaptoethansulfonic acid.
33. The method according to claim 29, wherein the reactant comprises a toxin.
34. The method according to claim 29, wherein the reactant comprises a drug.
35. The method according to claim 29, wherein the reactant comprises a polyethylene glycol, a radioisotope-labeled compound, or a second polypeptide.
36. A method for producing a peptide or a protein, which is amidated at the C-terminus, comprising:
- producing a heterologous fusion protein by the method of claim 28, and
 - reacting the heterologous fusion protein with ammonia or a salt thereof.

37. The method according to claim 36,
wherein the heterologous fusion protein is reacted with ammonia or a salt thereof, in the presence of a compound containing a nucleophilic thiol group.

FIG. 1

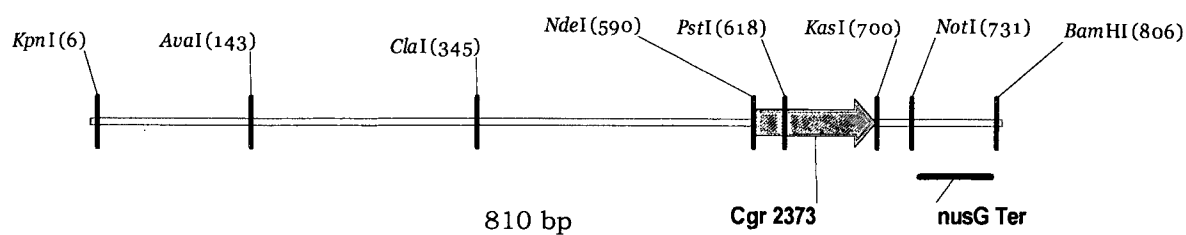


FIG. 2

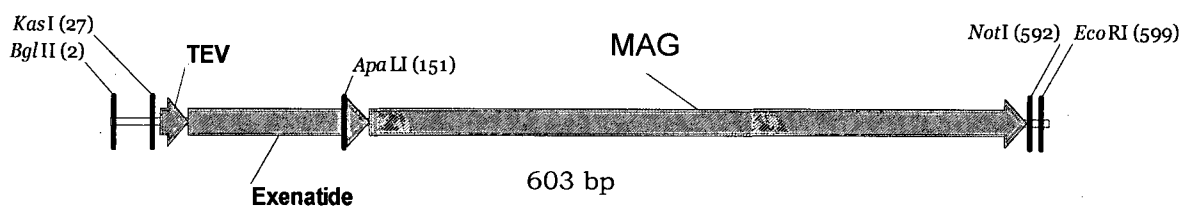


FIG. 3

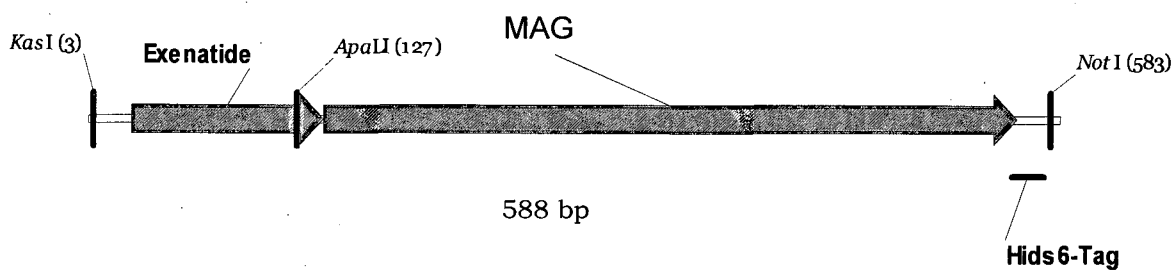


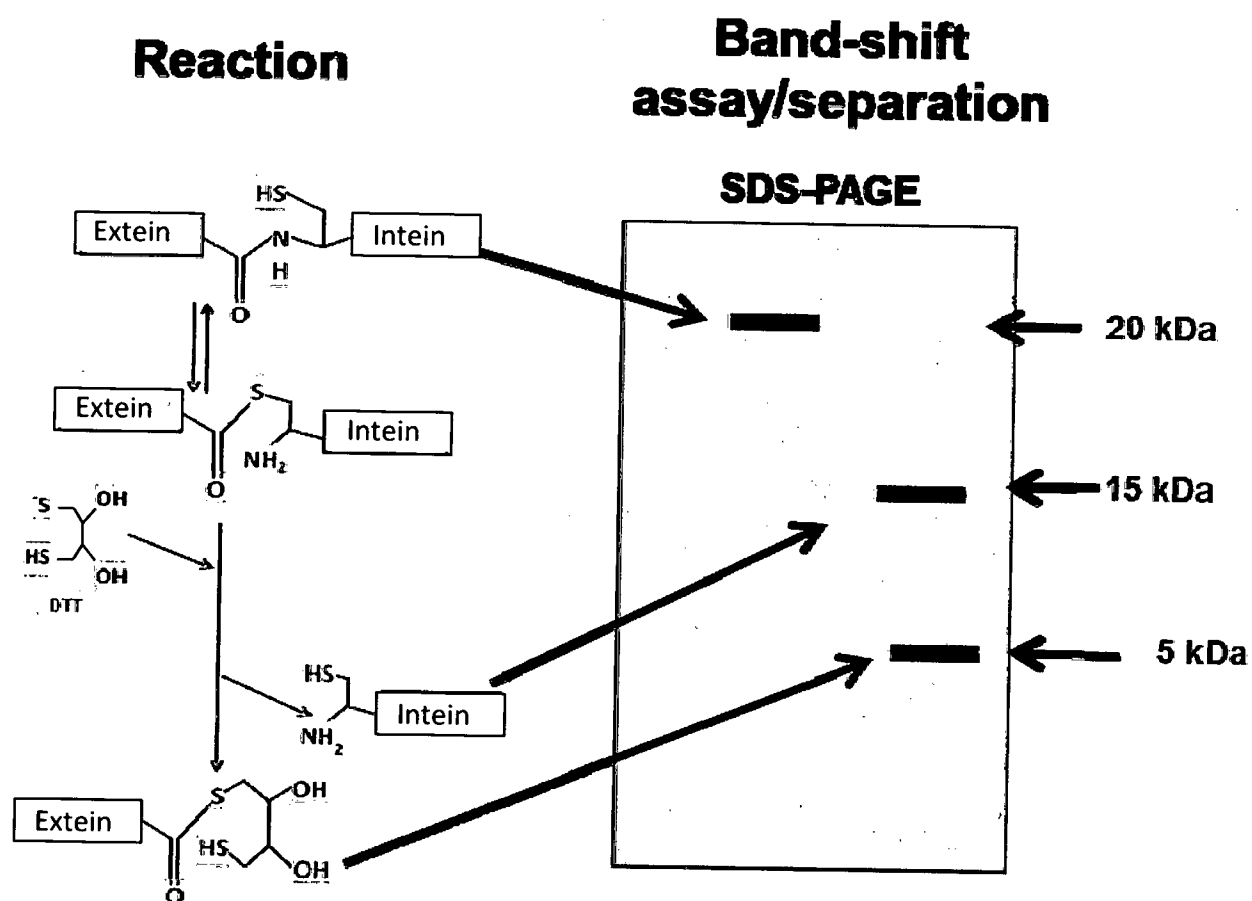
FIG. 4

FIG.5

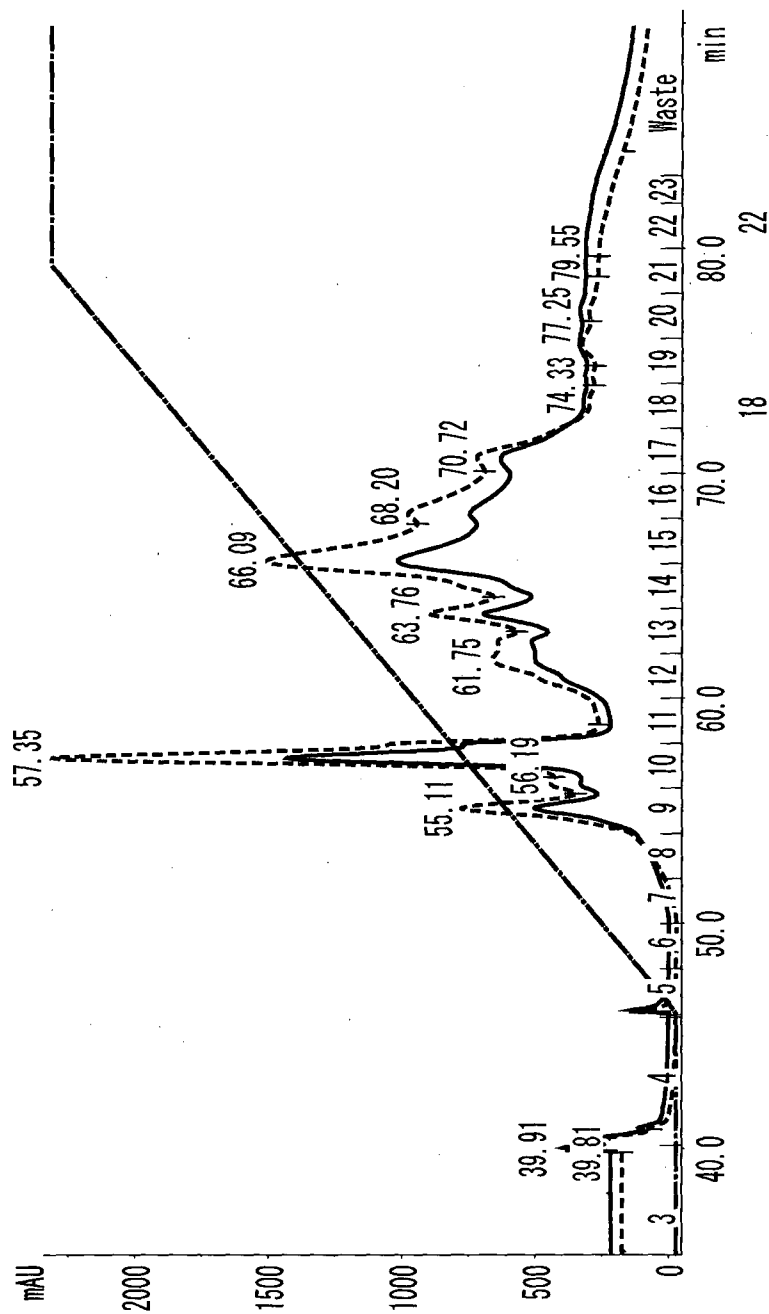
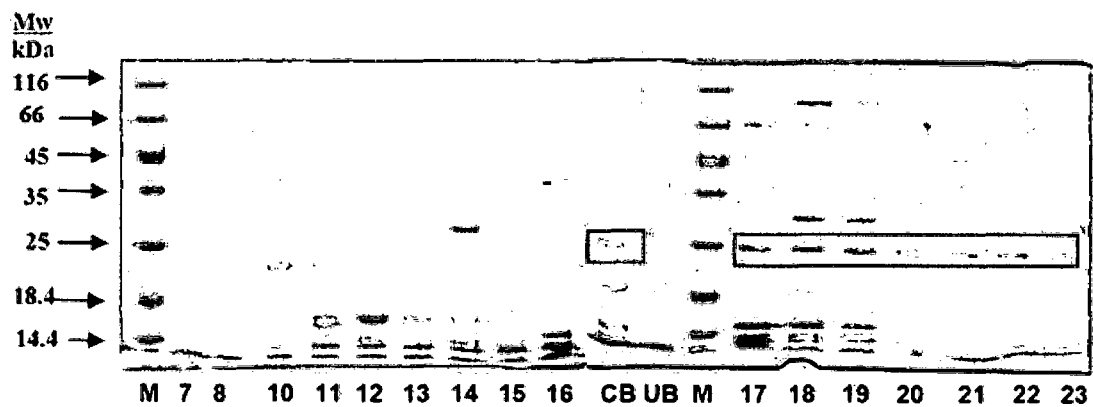


FIG. 6



Lanes:

M - Mw-marker of indicated molecular weights;

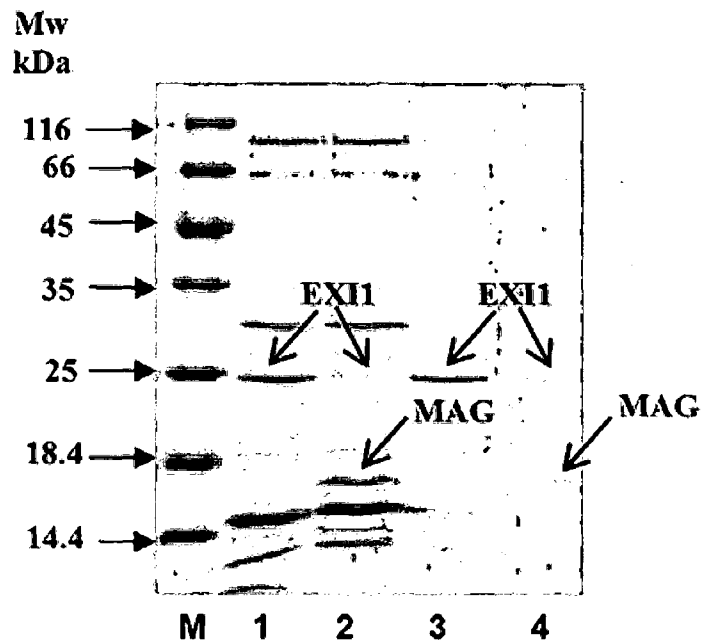
CB - culture broth;

UB - pooled fractions 1, 2, and 3 unbounded to a column;

7 - 23 - fractions eluted from the column.

The EXI1 fusion protein is marked by rectangles.

FIG. 7



Lanes:

M - Mw-marker of indicated molecular weights;

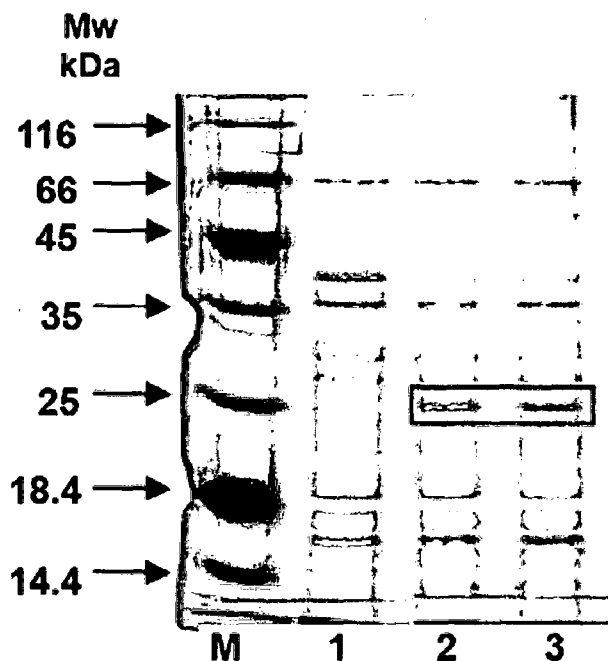
1 - EXI1 eluted in fraction 18 with 50 mM Tris-HCl pH 8.0, incubated at 0 °C for 16 hours;

2 - EXI1 eluted in fraction 18 with 50 mM Tris-HCl pH 8.0 and 100 mM DTT, incubated at 0 °C for 16 hours;

3 - EXI1 eluted in fraction 22 with 50 mM Tris-HCl pH 8.0, incubated at 0 °C for 16 hours;

4 - EXI1 eluted in fraction 22 with 50 mM Tris-HCl pH 8.0 and 100 mM DTT, incubated at 0 °C for 16 hours.

FIG. 8



Lanes:

M - Mw-marker of indicated molecular weights;

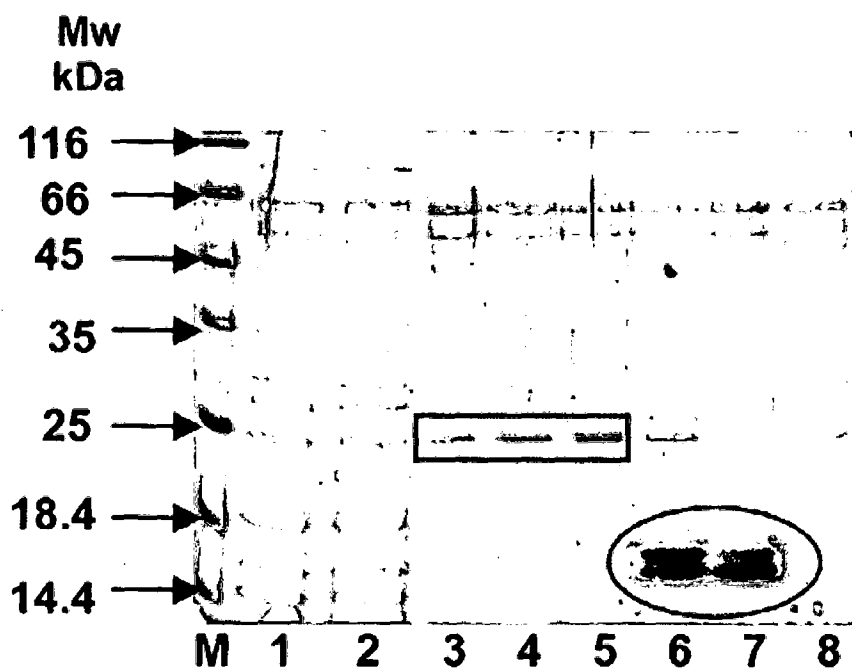
1 - culture broth of the YDK010[pEXC1] strain;

2 - culture broth of the YDK010[pEXC1-exe-MAG-HT#2] strain;

3 - the same as lane 2, but with addition of 100 mM DTT and incubation of the culture broth at 4 °C for about 16 hours.

The EXI1HT fusion protein is marked by rectangle.

FIG. 9



Lanes:

M - Mw-marker of indicated molecular weights;

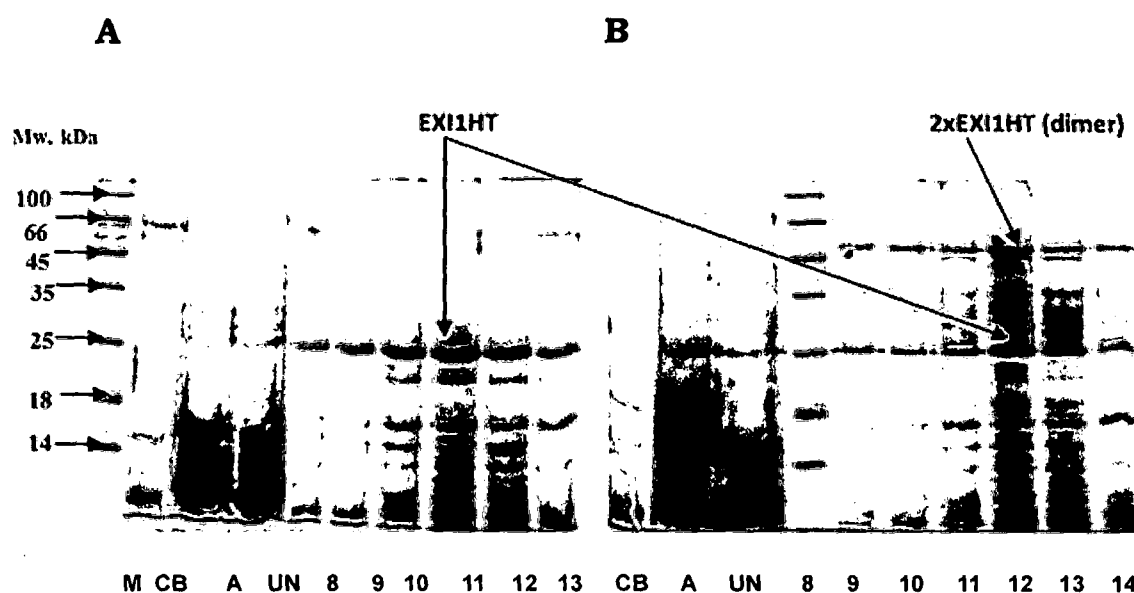
1 - culture broth;

2 - unbound fraction;

3 - 8 - eluted fractions.

The EXI1HT fusion protein is marked by rectangle.

FIG. 10



Reducing (A) and non-reducing (B) SDS-PAGE of fractions 8-13 eluted from HisTrap column (IMAC).

Lanes:

M - Mw-marker of indicated molecular weights;

CB - culture broth of YDK010 strain harboring pEXC1-exe-MAG-HT#2 plasmid;

A - protein preparation applied onto the HisTrap column;

UN - unbound fractions.

FIG. 11

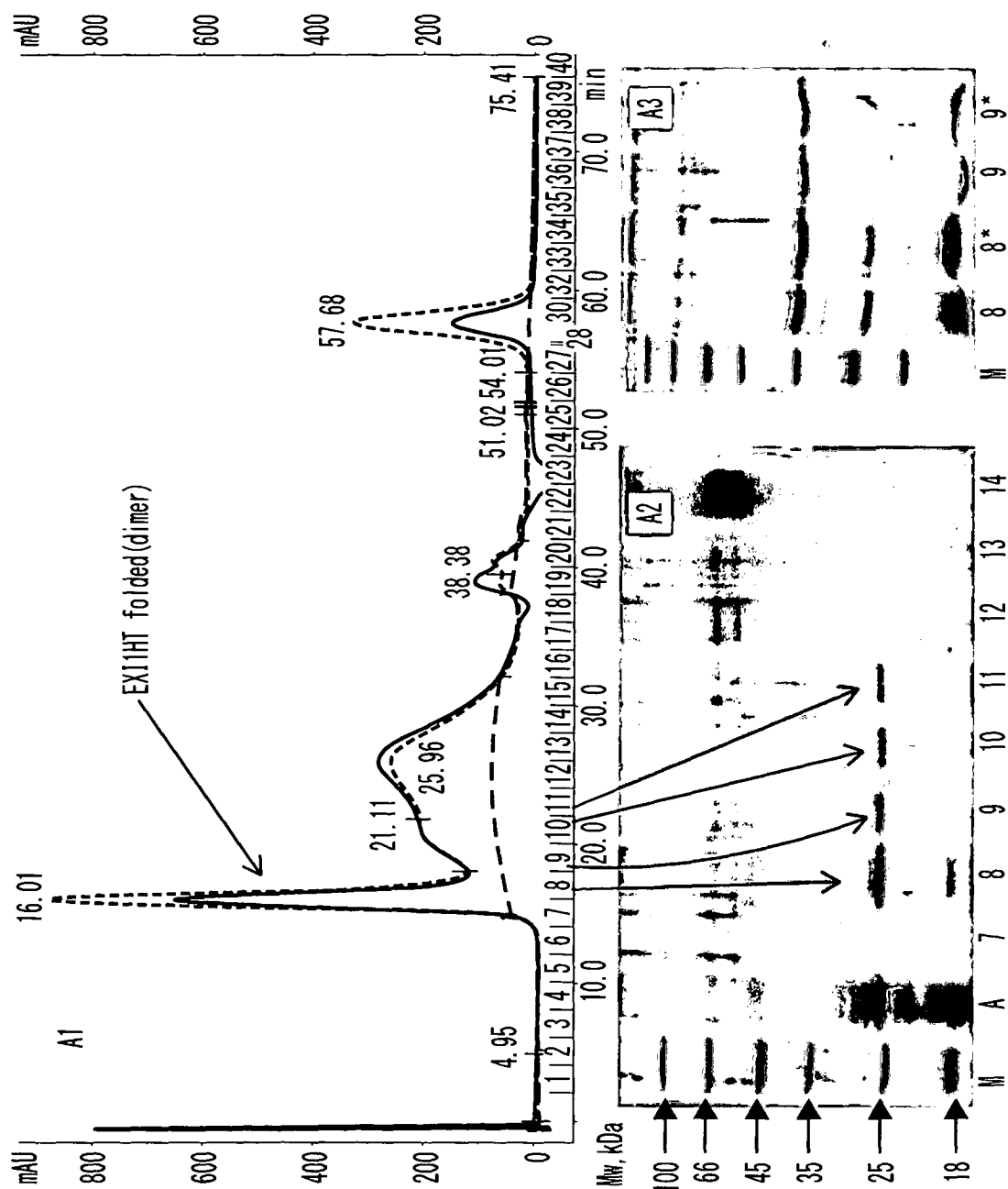


FIG.12

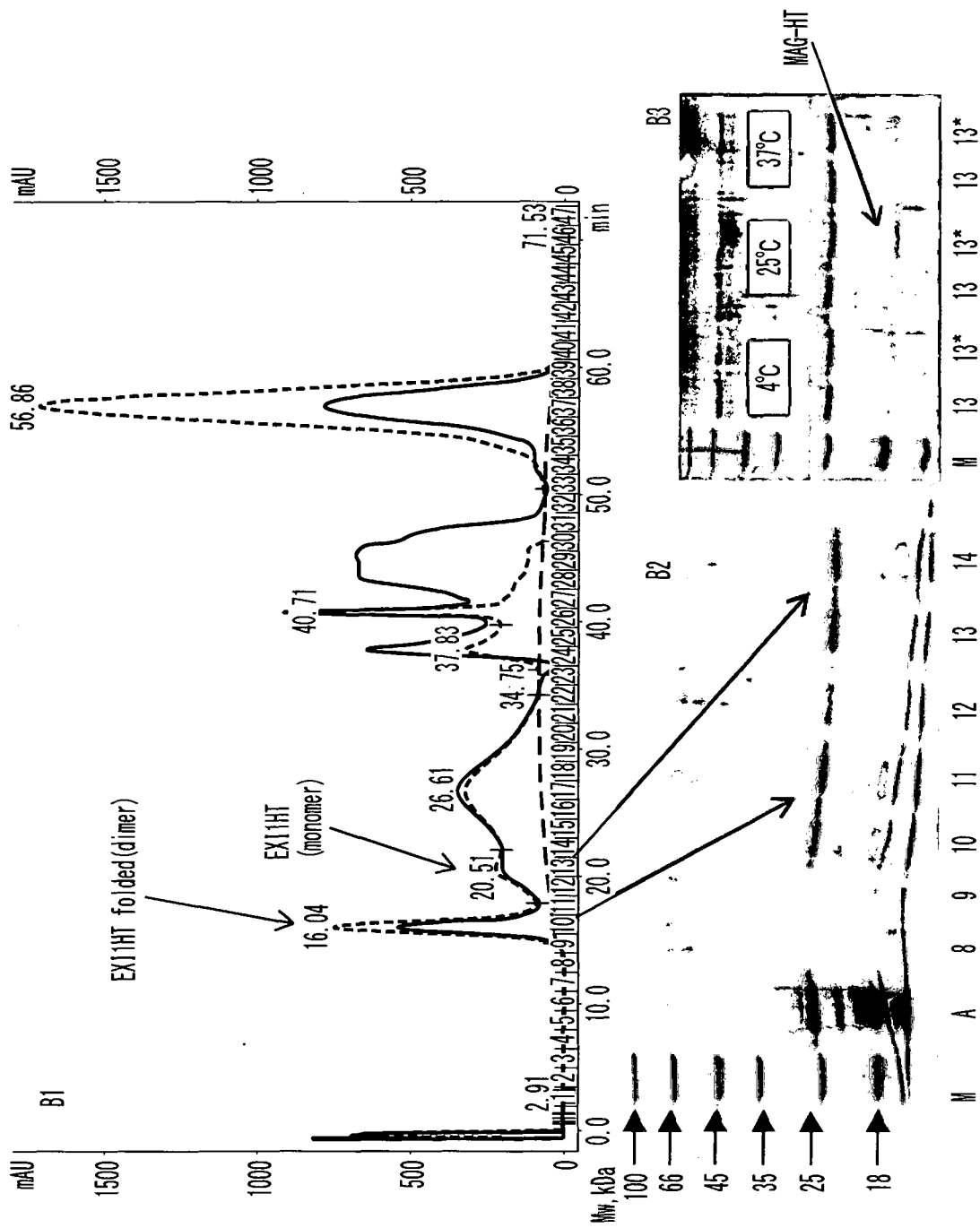


FIG. 13

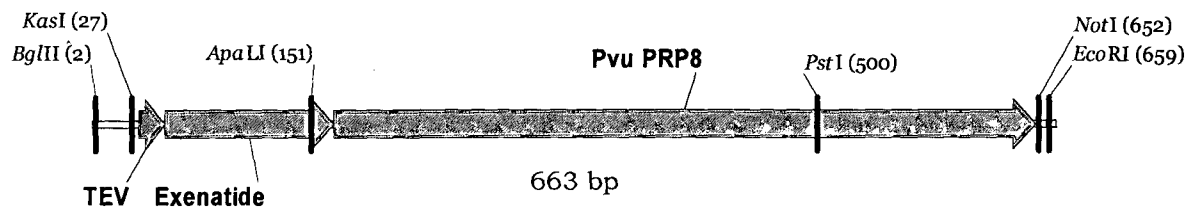


FIG. 14

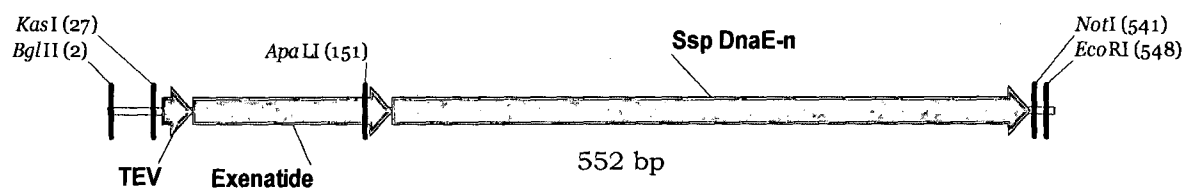


FIG. 15

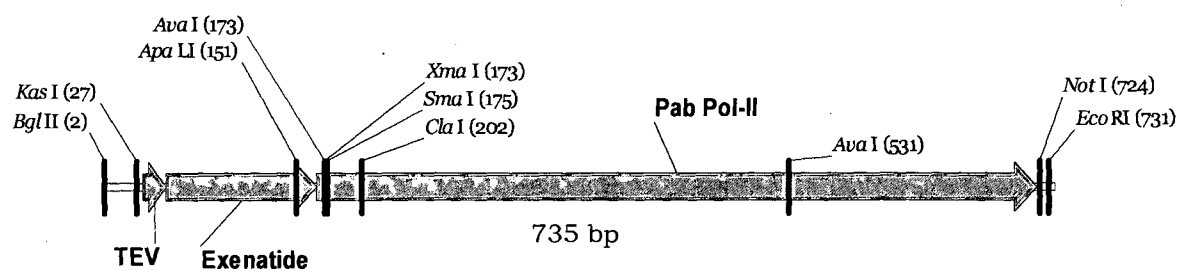


FIG. 16

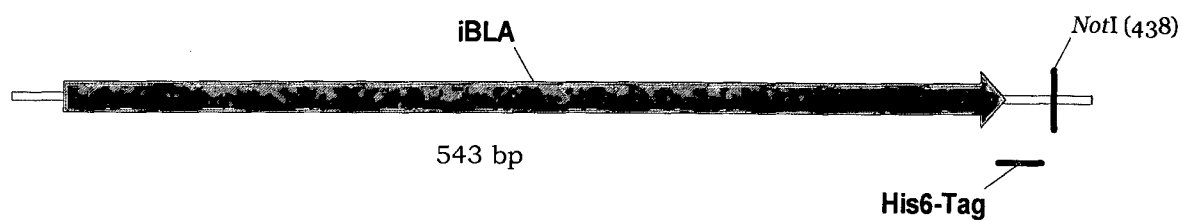


FIG. 17

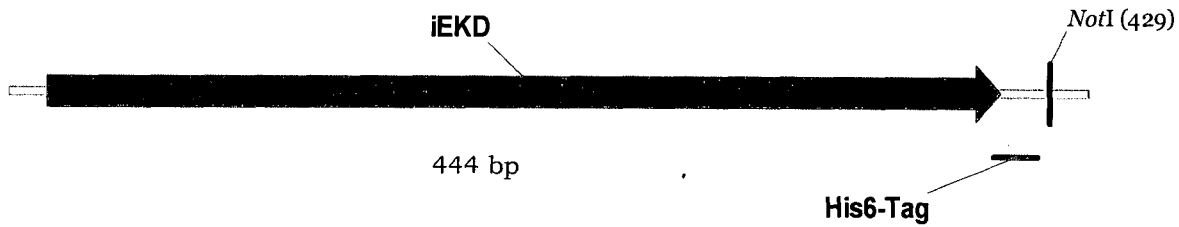
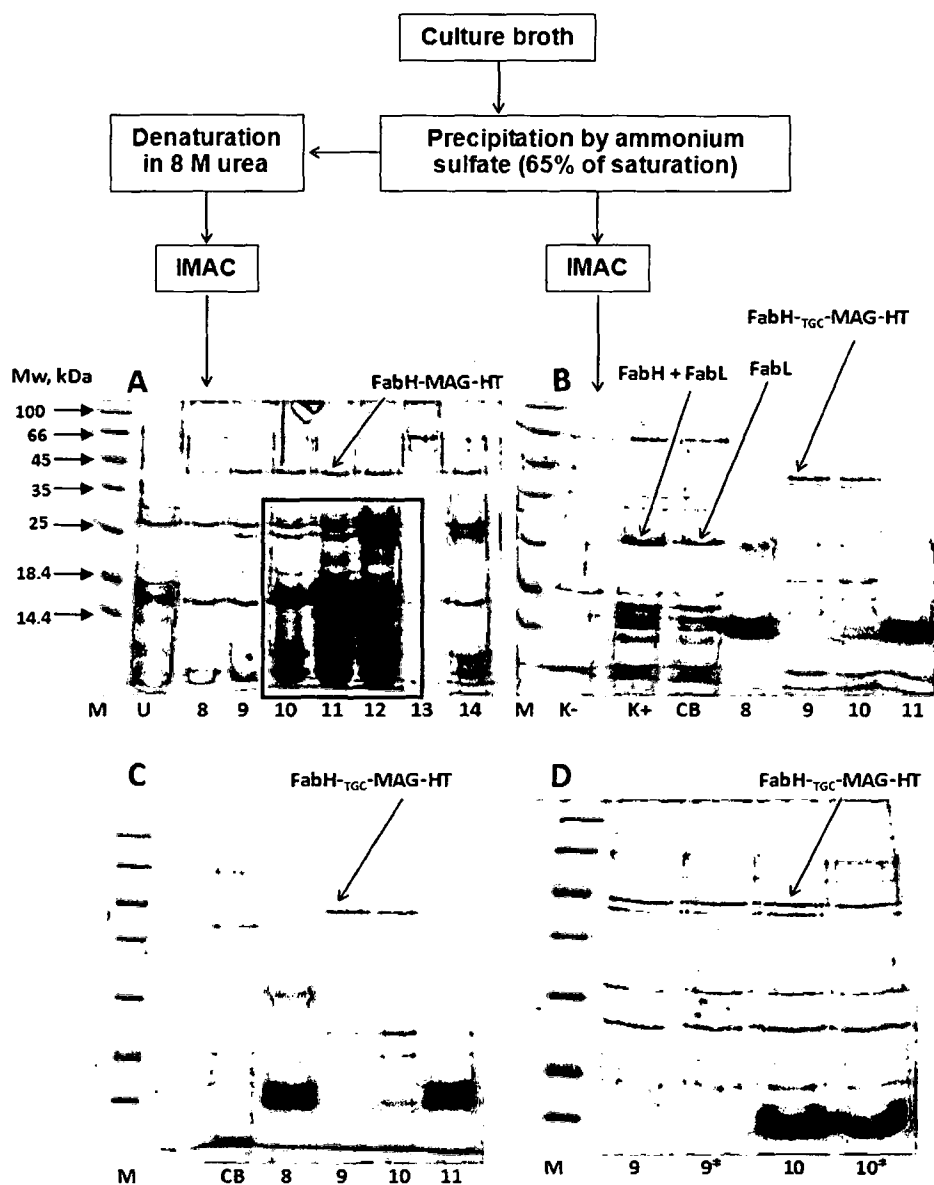


FIG. 18



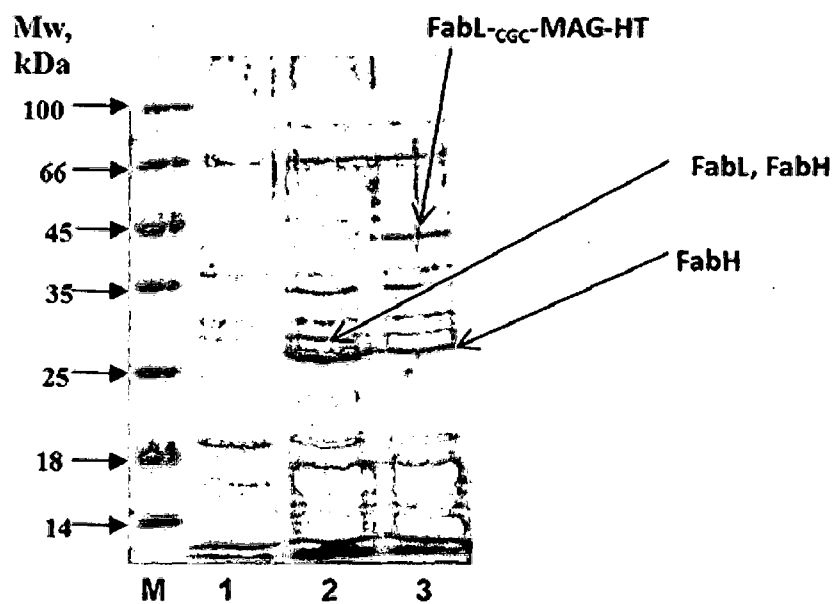
Panel A: lane U - crude protein preparation. Proteolytic fragments of FabH-TGC-MAG-HT are shown in square.

Panels B and C: lanes K-, K+, and CB - protein preparations from culture broths of YDK010ΔPBP1a strain harboring plasmid pEXC1, pPKSherFabHL, or pPKSherFabH-TGC-MAG-HT-FabL respectively.

Panel D: lanes 9, 10 - control reactions without DTT, lanes 9*, 10* - test reactions with addition of 100 mM DTT.

M - Mw-marker of indicated molecular weights.

FIG. 19



Lanes:

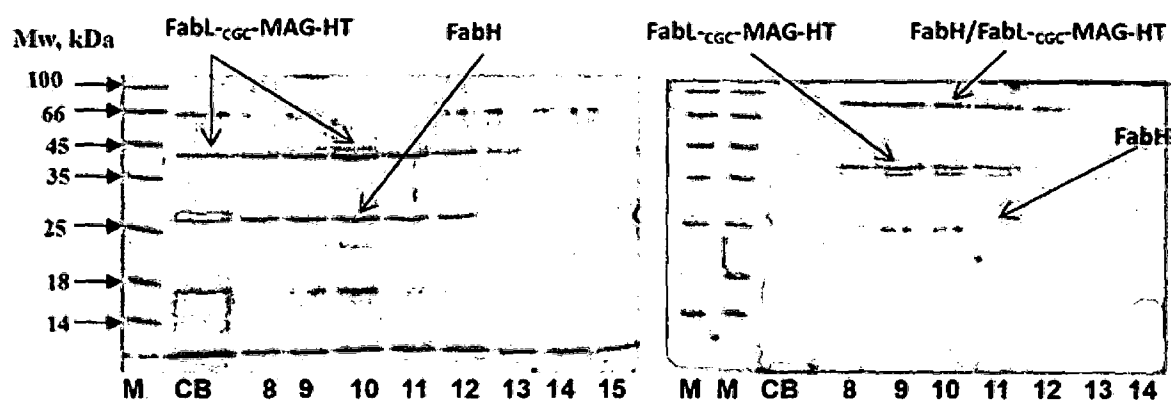
M - Mw-marker of indicated molecular weights;

1 - pEXC1 (negative control);

2 - pPKSherFabHL (positive control);

3 - pPKSherFabH-FabL-cgc-MAG-HT.

FIG. 20

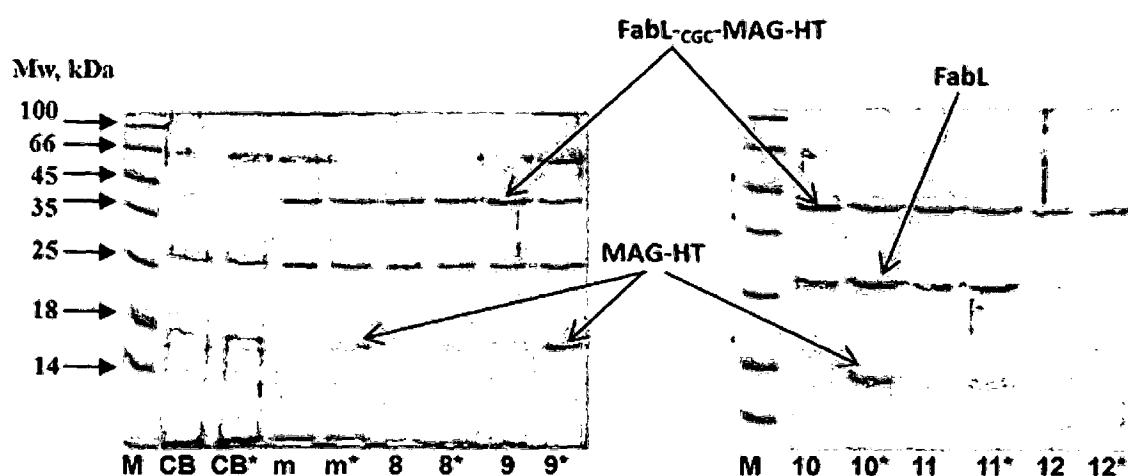


Lanes:

M - Mw-marker of indicated molecular weights;

CB - culture broth of YDK110ΔPBP1a harboring the pPKSherFabH-FabL-ccc-MAG-HT plasmid.

FIG. 21



Lanes:

M - Mw-marker of indicated molecular weights;

CB - culture broth without DTT;

8-12 - control reactions without DTT;

m - mixture of equal aliquots of fractions 8-12 without DTT;

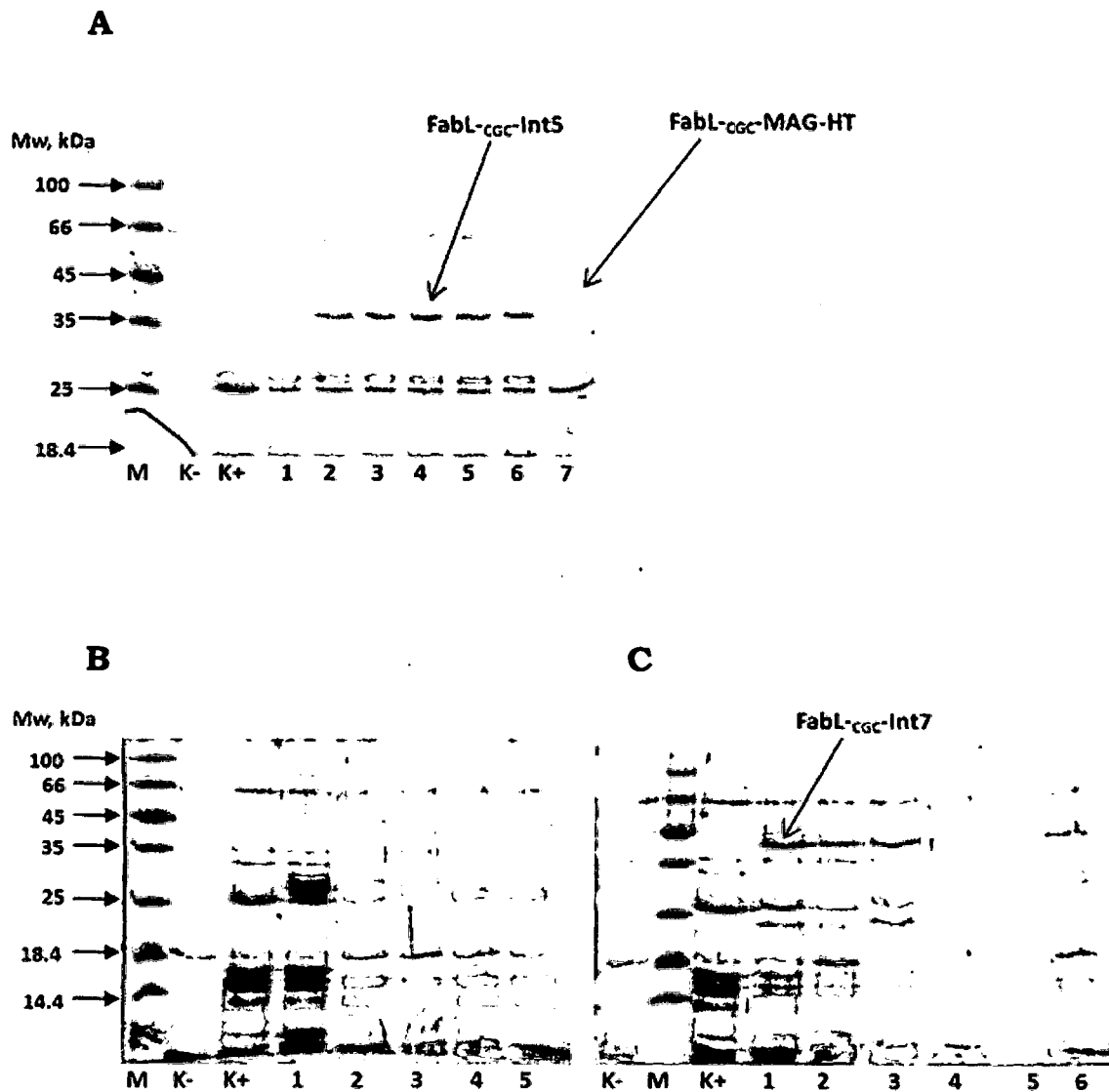
CB* - culture broth with 100 mM DTT;

8*-12* - test reactions with 100 mM DTT;

m* - mixture of equal aliquots of fractions 8*-12* with 100 mM DTT.

CB - culture broth of YDK110ΔPBP1a strain harboring the pPK-SherFabH-FabL-CGC-MAG-HT plasmid.

FIG. 22



Lanes:

M - Mw-marker of indicated molecular weights;

K- - pEXC1(negative control);

K+ - pPKSherFabHL (positive control);

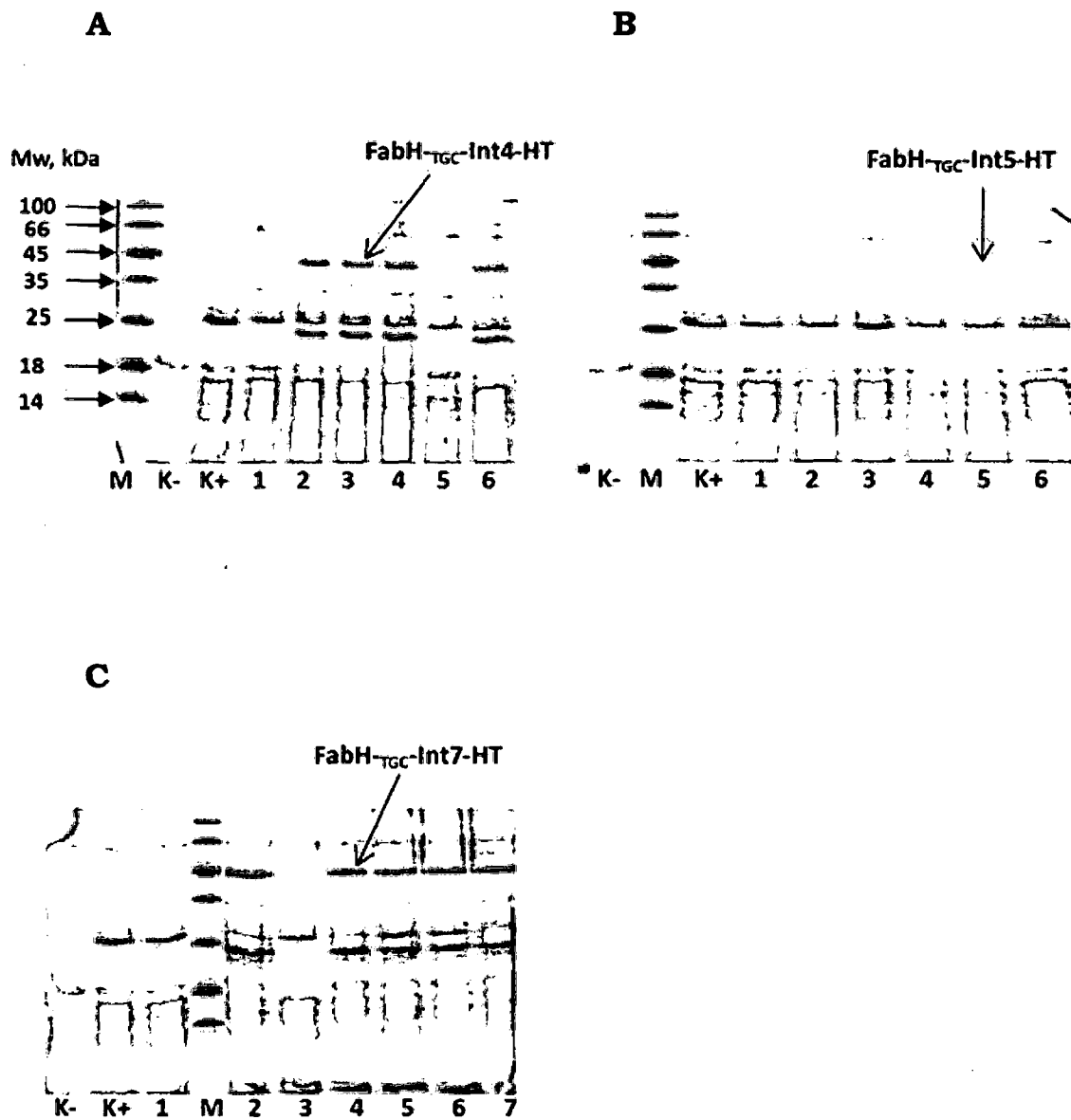
A7 - pPKSherFabH-FabL-cgc-MAG-HT (positive control);

A1-A6 - pPKSherFabH-FabL-cgc-Int5#(1-6);

B1-B5 - pPKSherFabH-FabL-cgc-Int4#(25-28,30);

C1-C5 - pPKSherFabH-FabL-cgc-Int7#(37-42).

FIG. 23



Lanes:

M - Mw-marker of indicated molecular weights;

K- - pEXC1(negative control);

K+ - pPKSherFabHL (positive control);

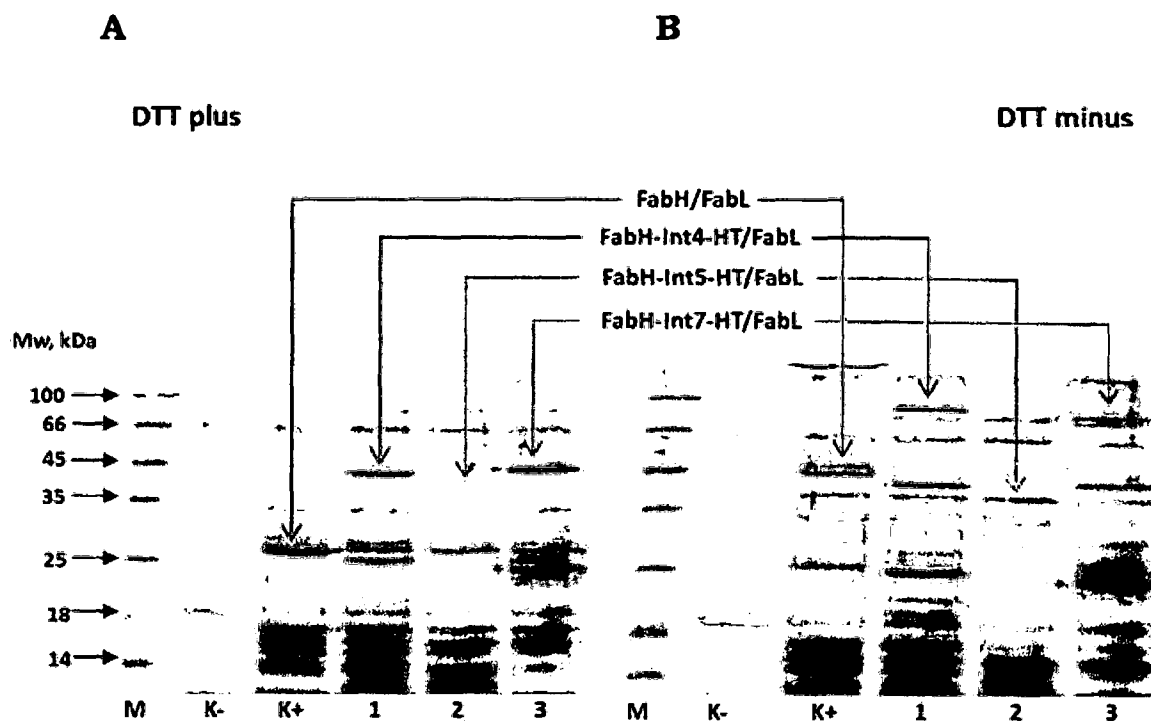
1 - pPKSherFabH_{TGC}-MAG-FabL (positive control);

A2-A6 - pPKSherFabH_{TGC}-Int4-HT-FabL#(41-43, 45, 46);

B2-B6 - pPKSherFabH_{TGC}-Int5-HT-FabL#(51-43, 55, 56);

C2-C7 - pPKSherFabH_{TGC}-Int7-HT-FabL#(71-76).

FIG. 24



Lanes:

M - Mw-marker of indicated molecular weights;

K- - pEXC1(negative control);

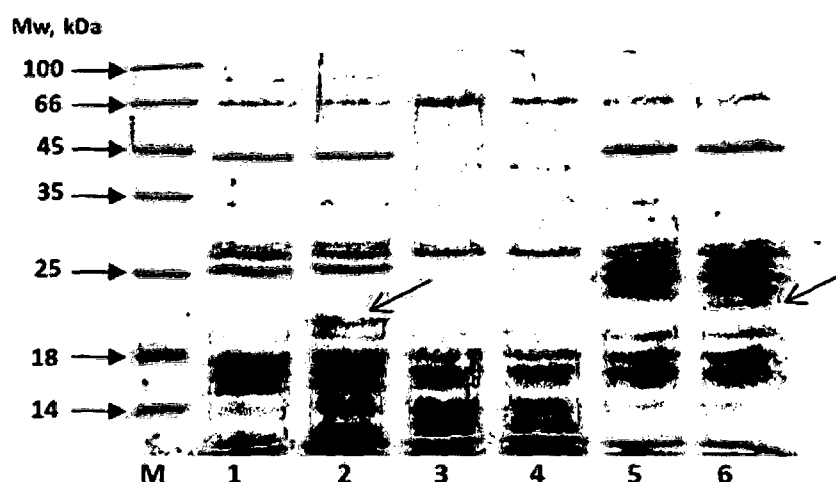
K+ - pPKSherFabHL (positive control);

1 - pPKSherFabH_{TGC}-Int4-HT-FabL;

2 - pPKSherFabH_{TGC}-Int5-HT-FabL;

3 - pPKSherFabH_{TGC}-Int7-HT-FabL.

FIG. 25



Lanes:

M - Mw-marker of indicated molecular weights;

1 - FabH-TGC-Int4-HT, without DTT;

2 - FabH-TGC-Int4-HT, with 100 mM DTT;

3 - FabH-TGC-Int5-HT, without DTT;

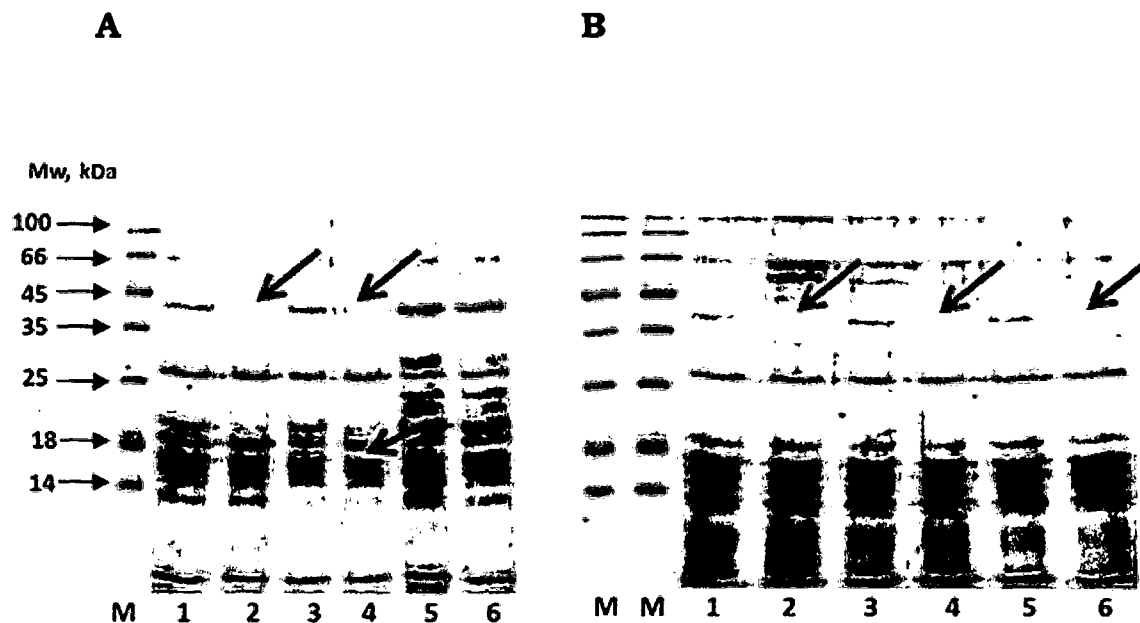
4 - FabH-TGC-Int5-HT, with 100 mM DTT;

5 - FabH-TGC-Int7-HT, without DTT;

6 - FabH-TGC-Int7-HT, with 100 mM DTT.

The protein bands corresponding to cleaved His6-tagged inteins are shown by arrows.

FIG. 26



Lanes:

M - Mw-marker of indicated molecular weights;

A1-A6 - FabH-TGC-Int18-HT#1-3;

B1-B6 - FabH-TGC-Int19-HT#3-5;

A1, A3, A5, B1, B3, B5 - without DTT;

A2, A4, A6, B2, B4, B6 - with 100 mM DTT.

The protein bands corresponding to cleaved His6-tagged inteins are shown by lower sloping arrow. Disappearance of bands corresponding to fused proteins FabH-TGC-IntX-HT, X = 18 or 19 is shown by upper sloping arrows.

FIG. 27

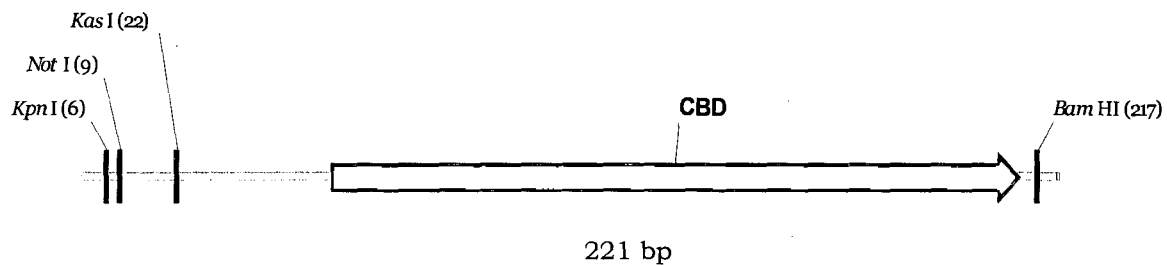
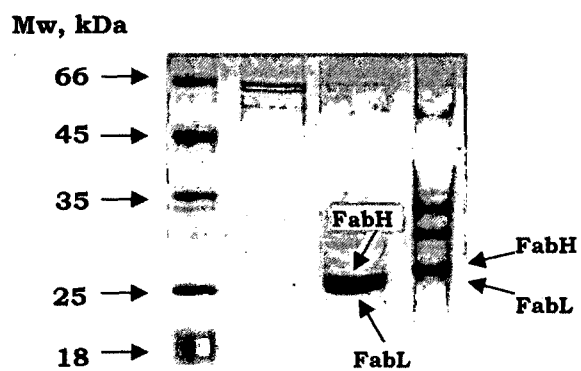
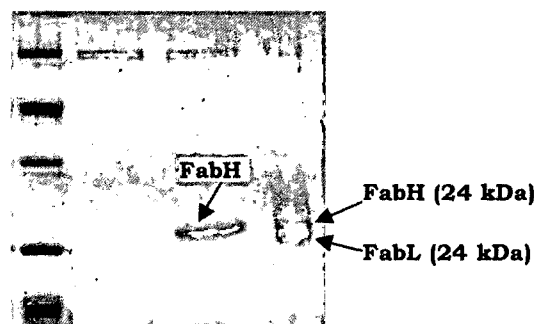


FIG. 28

A



B



Lanes:

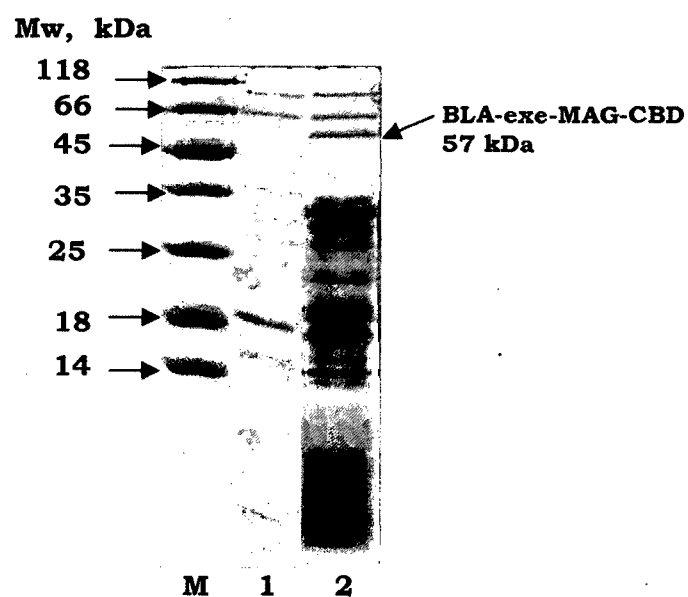
M - Mw-marker of indicated molecular weights;

1 - control reaction, without 50 mM DTT;

2 - test reaction, with 50 mM DTT;

3 - proteins bound to Chitin Resin but not eluted upon addition of DTT

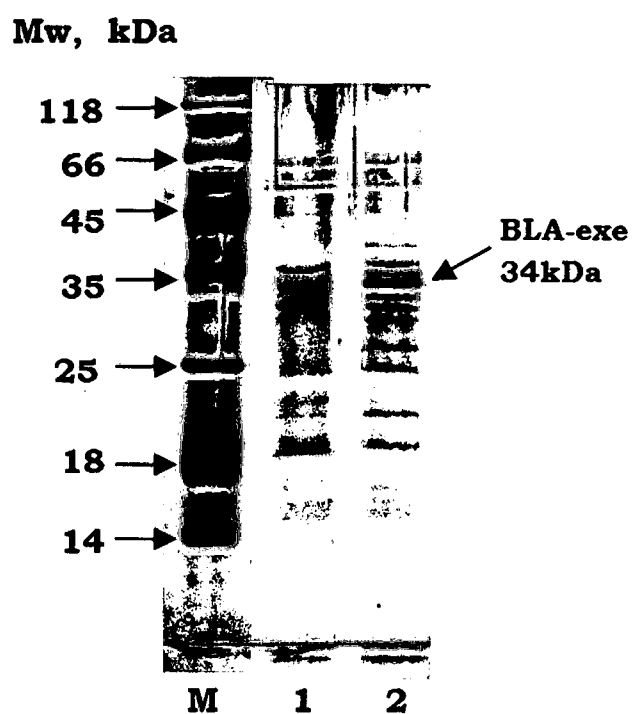
FIG. 29



Lanes:

- M - Mw-marker of indicated molecular weights;
- 1 - pEXC1 (negative control);
- 2 - pEXC1-BLA-exe-MAG-CBD (positive control).

FIG. 30



Lanes:

M - Mw-marker of indicated molecular weights;

1 - control reaction, without 50 mM DTT;

2 - test reaction, with 50 mM DTT.

FIG. 31

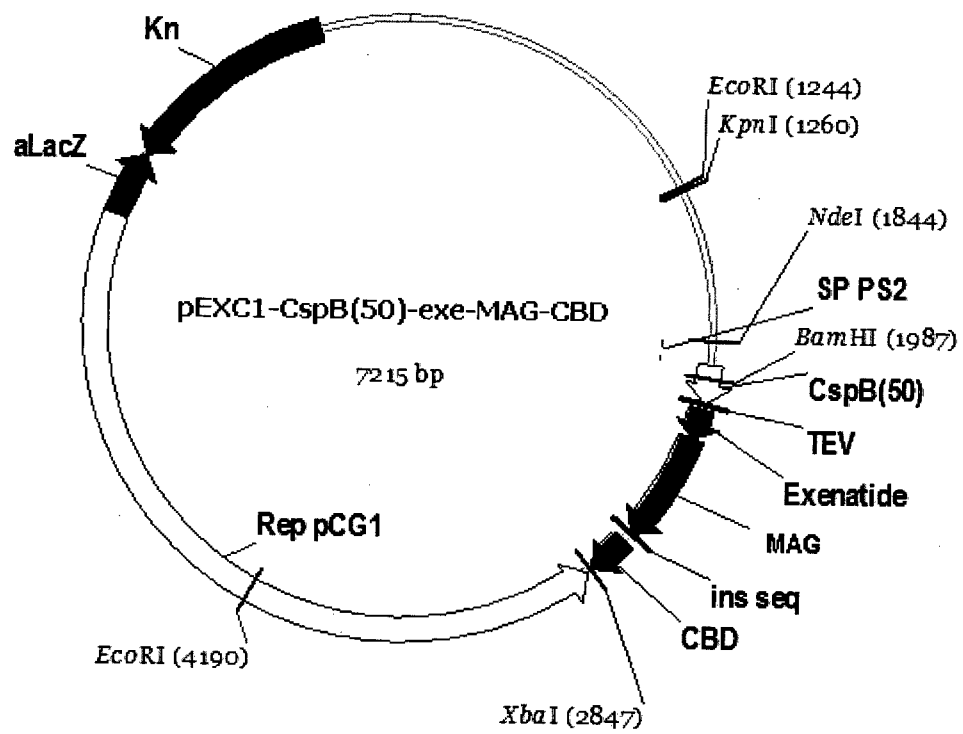


FIG. 32

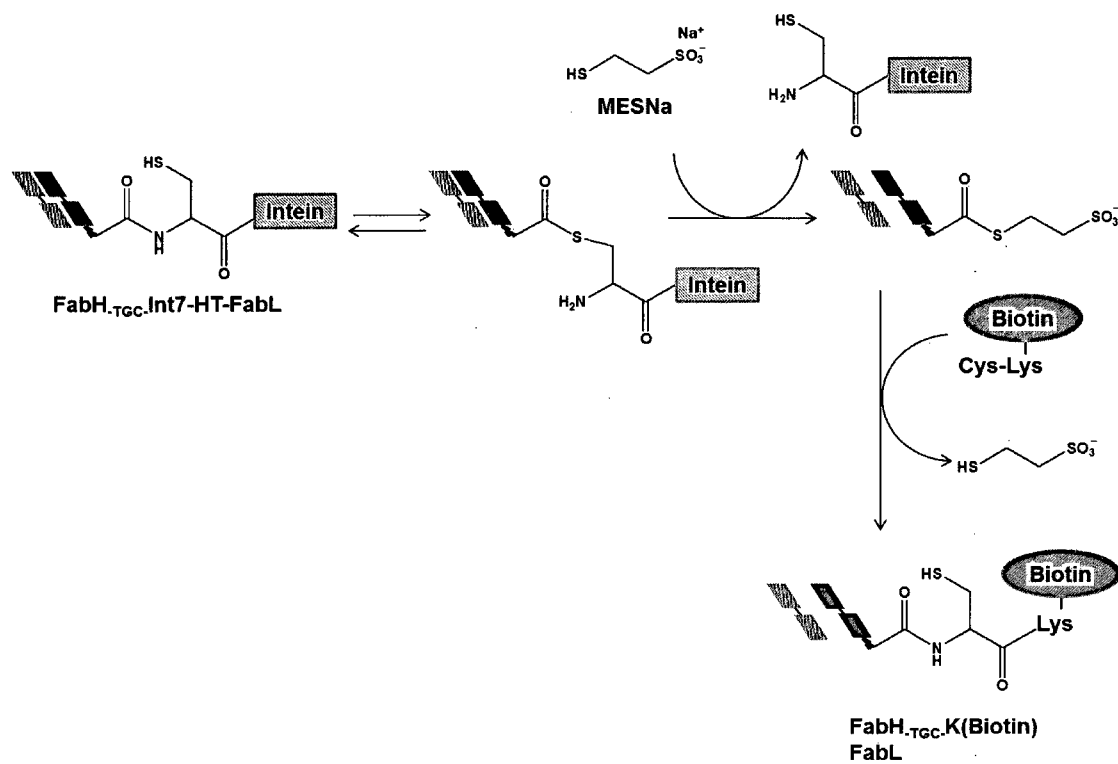
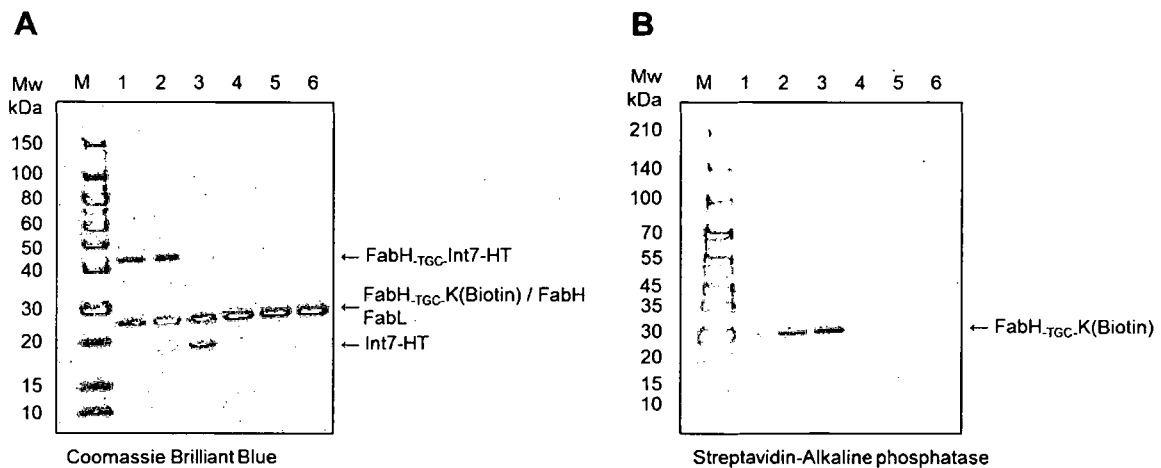


FIG. 33



Lanes:

- M - Mw-marker of indicated molecular weights;
- 1 - FabH_{TGC}-Int7-HT-FabL, without MESNa;
- 2 - FabH_{TGC}-Int7-HT-FabL, with 5 mM MESNa;
- 3 - FabH_{TGC}-Int7-HT-FabL, with 50 mM MESNa;
- 4 - FabHL, without MESNa;
- 5 - FabHL, with 5 mM MESNa;
- 6 - FabHL, with 50 mM MESNa.

FIG. 34

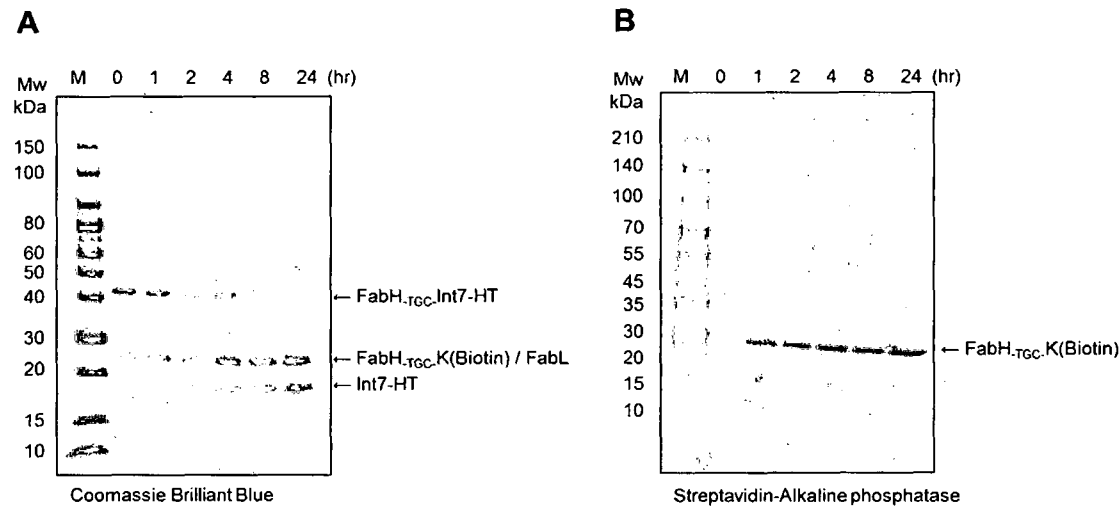


FIG. 35

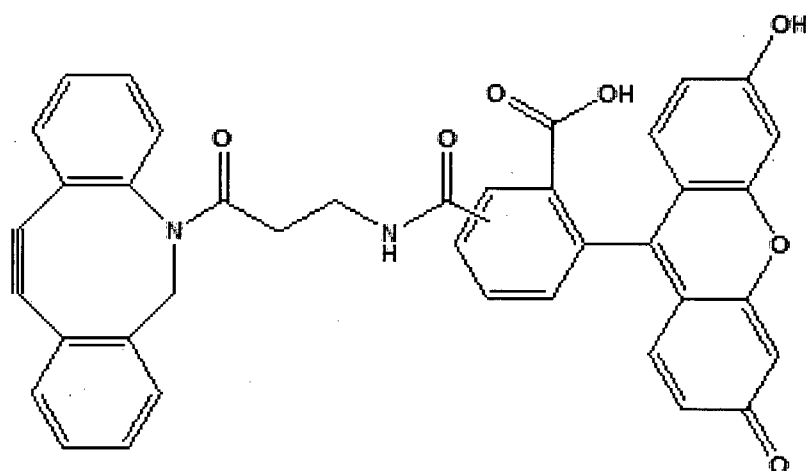


FIG. 36

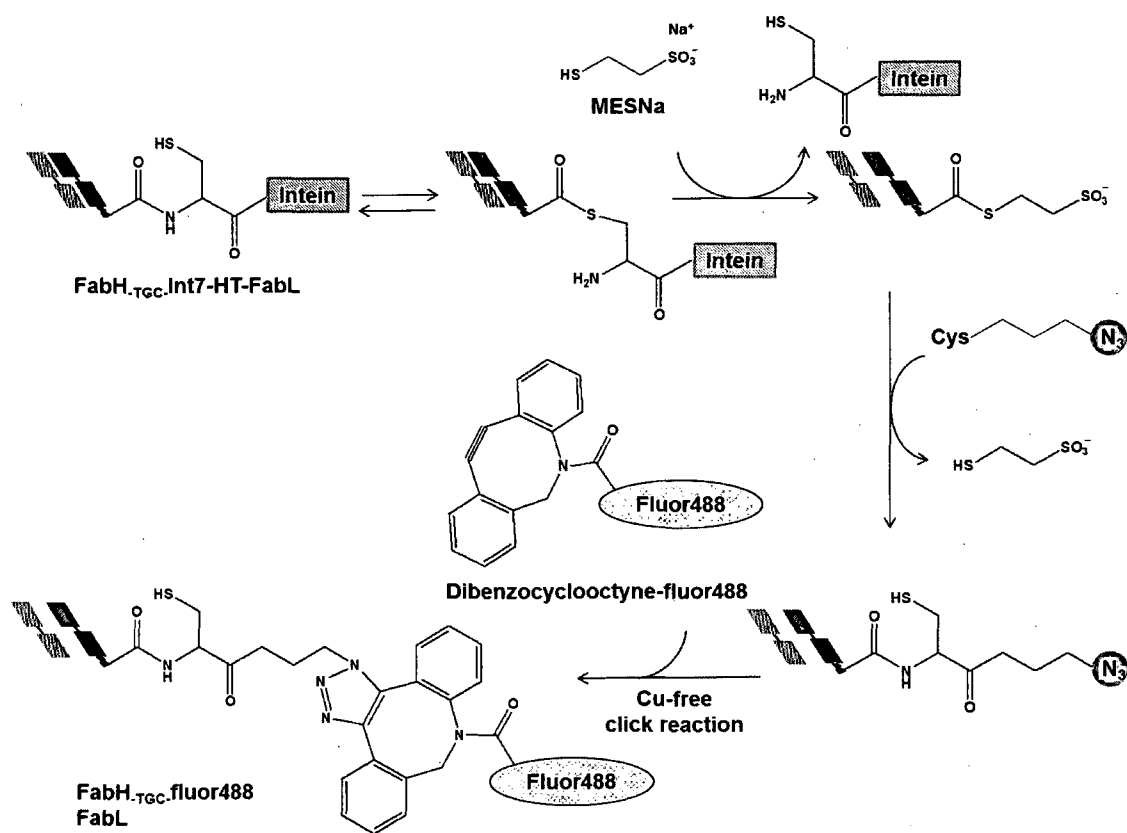
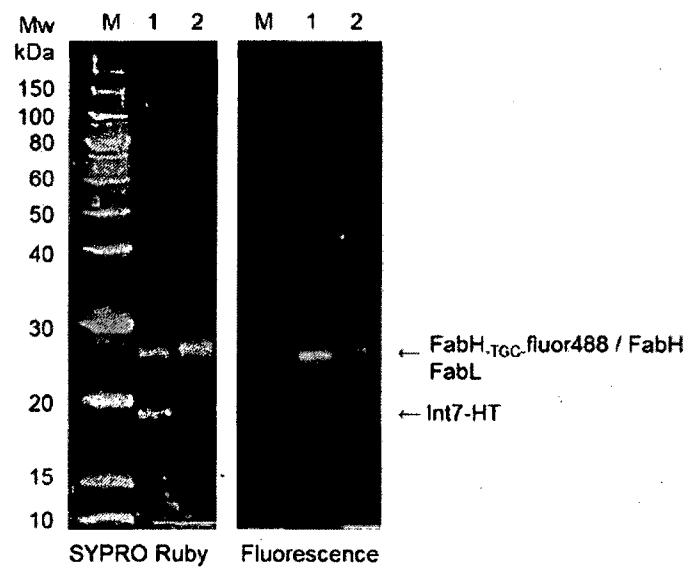


FIG. 37



Lanes:

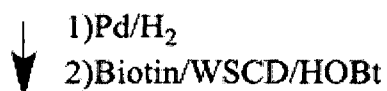
M - Mw-marker of indicated molecular weights;

1 - FabH_{TGC}-Int7-HT-FabL;

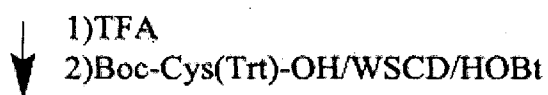
2 - FabHL.

FIG. 38

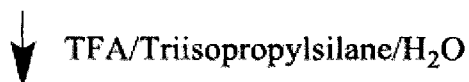
Boc-Lys(2-ClZ)-NH₂



Boc-Lys((Biot)-NH₂



Boc-Cys(Trt)-Lys(Biot)-NH₂



Cys-Lys(Biot)-NH₂

WSCD: Water-Soluble Carbodiimide,
1-Ethyl-3-(3-Dimethylaminopropyl)-Carbodiimide

HOBt: 1-Hydroxybenzotriazole

INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2014/062292

A. CLASSIFICATION OF SUBJECT MATTER		
INV.	C12P21/02 C07K14/34	C12N15/77 C07K1/107
	C07K14/605 C12N15/62	C07K16/32 C12P21/06
		C07K16/18 C12N9/90
ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) C12P C12N C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, Sequence Search, FSTA, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 1 219 713 A1 (AJINOMOTO KK [JP]) 3 July 2002 (2002-07-03) cited in the application the whole document	1,28,29, 36
A	EP 1 748 077 A1 (AJINOMOTO KK [JP]) 31 January 2007 (2007-01-31) cited in the application the whole document	1,28,29, 36
	----- -/--	
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 12 September 2014		Date of mailing of the international search report 22/09/2014
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer van de Kamp, Mart

INTERNATIONAL SEARCH REPORT

International application No

PCT/JP2014/062292

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MYSCOFSKI D M ET AL: "Cleavage and purification of intein fusion proteins using the Streptococcus gordonii spex system", PREPARATIVE BIOCHEMISTRY AND BIOTECHNOLOGY, vol. 31, no. 3, 1 August 2001 (2001-08-01), pages 275-290, XP009166411, ISSN: 1082-6068, DOI: 10.1081/PB-100104909 the whole document -----	1,28,29, 36
A	SINA MÖHLMANN ET AL: "Site-specific modification of ED-B-targeting antibody using intein-fusion technology", BMC BIOTECHNOLOGY, vol. 11, no. 1, 21 July 2011 (2011-07-21), page 76, XP021105286, ISSN: 1472-6750, DOI: 10.1186/1472-6750-11-76 cited in the application the whole document -----	1,28,29, 36
A	IAN R. COTTINGHAM ET AL: "A method for the amidation of recombinant peptides expressed as intein fusion proteins in Escherichia coli.", NATURE BIOTECHNOLOGY, vol. 19, no. 10, 1 October 2001 (2001-10-01), pages 974-977, XP055087005, ISSN: 1087-0156, DOI: 10.1038/nbt1001-974 cited in the application the whole document -----	1,28,29, 36
A	GERRIT VOLKMANN ET AL: "Recent progress in intein research: from mechanism to directed evolution and applications", CELLULAR AND MOLECULAR LIFE SCIENCES, vol. 70, no. 7, 28 August 2012 (2012-08-28), pages 1185-1206, XP055139858, ISSN: 1420-682X, DOI: 10.1007/s00018-012-1120-4 the whole document -----	1,28,29, 36
A	SKANDER ELLEUCHE ET AL: "Inteins, valuable genetic elements in molecular biology and biotechnology", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 87, no. 2, 7 May 2010 (2010-05-07), pages 479-489, XP019841626, ISSN: 1432-0614 the whole document ----- -/--	1,28,29, 36

INTERNATIONAL SEARCH REPORT

International application No

PCT/JP2014/062292

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MIQUEL VILA-PERELLÓ ET AL: "Biological Applications of Protein Splicing", CELL, vol. 143, no. 2, 1 October 2010 (2010-10-01), pages 191-200, XP055139595, ISSN: 0092-8674, DOI: 10.1016/j.cell.2010.09.031 cited in the application the whole document	1,28,29, 36
A,P	----- WO 2013/065869 A1 (AJINOMOTO KK [JP]) 10 May 2013 (2013-05-10) the whole document -----	1,16,20

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/JP2014/062292

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 1219713	A1	03-07-2002	AT 532870 T 15-11-2011
		AU 7449400 A 30-04-2001	
		BR 0014059 A 14-05-2002	
		CA 2391961 A1 05-04-2001	
		CN 1377413 A 30-10-2002	
		DK 1219713 T3 12-12-2011	
		EP 1219713 A1 03-07-2002	
		ES 2372806 T3 26-01-2012	
		JP 4320769 B2 26-08-2009	
		US 2003082746 A1 01-05-2003	
		US 2010297729 A1 25-11-2010	
		WO 0123591 A1 05-04-2001	
EP 1748077	A1	31-01-2007	DK 1748077 T3 08-04-2013
		EP 1748077 A1 31-01-2007	
		JP 4730302 B2 20-07-2011	
		US 2007184525 A1 09-08-2007	
		WO 2005103278 A1 03-11-2005	
WO 2013065869	A1	10-05-2013	AU 2012333451 A1 03-04-2014
		CN 103946371 A 23-07-2014	
		EP 2748340 A1 02-07-2014	
		US 2014255996 A1 11-09-2014	
		WO 2013065869 A1 10-05-2013	