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(54) **MUTANT TRNA FOR INTRODUCING UNNATURAL AMINO ACID INTO PROTEIN**

(75) Inventor: **Takahiro Hohsaka**, Ishikawa (JP)

(73) Assignee: **Japan Science and Technology Agency**, Kawaguchi-shi (JP)

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(30) **Foreign Application Priority Data**

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(51) **Int. Cl.**
    C07K 1/107   (2006.01)
    C07H 21/02   (2006.01)
(52) **U.S. Cl.** .... **536/23.1**; 514/44 A; 530/409
(58) **Field of Classification Search** .... None
    See application file for complete search history.

(56) **References Cited**

PUBLICATIONS

Turacatti et al Probing the Structure and Function of the Tachykinin Neurokinin-2 Receptor through Biosynthetic Incorporation of Fluorescent Amino Acids at Specific Sites.*

Probing the Structure and Function of the Tachykinin Neurokinin-2 receptor through Biosynthetic Incorporation of Fluorescent Amino Acids at Specific Sites JBC 1996, vol. 271, No. 33, pp. 19991-19998.*
Yamao, F. et al., "UGA is read as tryptophan in *Mycoplasma capricolum*", Proc. Natl. Acad. Sci. USA, vol. 82, pp. 2306-2309, (1985).
Anderson, J.C. et al., "An expanded genetic code with a functional quadruplet codon", Proc. Natl. Acad. Sci. USA, vol. 101 No. 20, pp. 7566-7571, (2004).
Santoro, S.W. et al., "An archaebacteria-derived glutamyl-Trna synthetase and tRNA pair for unnatural amino acid mutagenesis of proteins in *Escherichia coli*", Nucleic Acids Research, vol. 31, No. 23, pp. 6700-6709, (2003).
Hohsaka, T. et al. "Efficient Incorporation of Nonnatural Amino Acids with Large Aromatic Groups into Streptavidin in In Vitro Protein Synthesizing Systems", Journal, American Chemical Society, vol. 121 pp. 34-40, (1999).
Kajihara, D. et al., "2 Shurui no Keiko Kyoshiki Aminosan o Bui Tokuiteki ni Donyu shita Tanpaushitsu no Sakusei to FRET Bunsekl", The Chemical Society of Japan Koen Yokoshu, vol. 85, p. 1213, (2005) (with partial English translation).
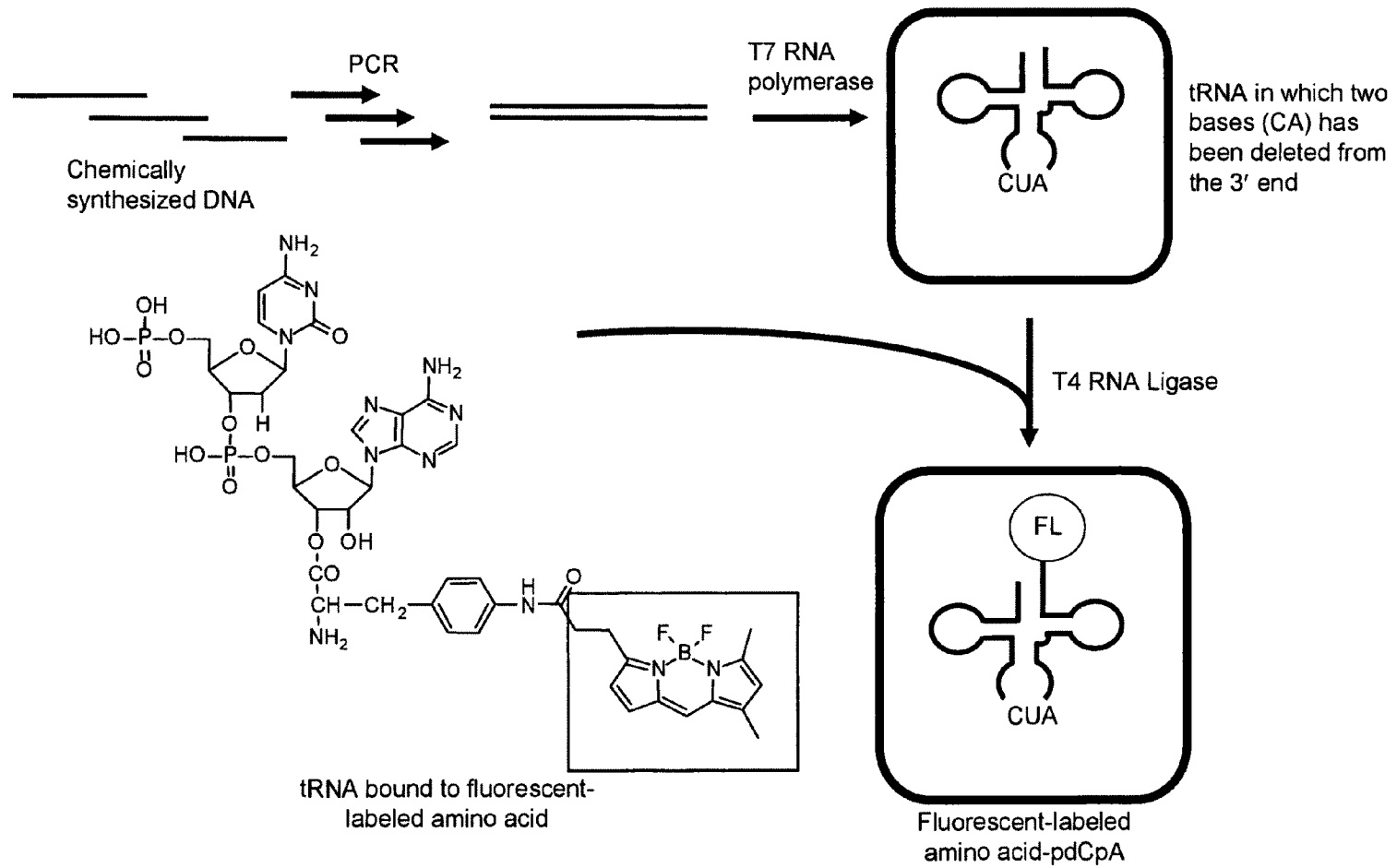
* cited by examiner

Primary Examiner — Kagnew H Gebreyesus
(74) *Attorney, Agent, or Firm* — Oblon, Spivak, McClelland, Maier & Neustadt, L.L.P.

(57) **ABSTRACT**

It is an objective of the present invention to provide tRNA that has CUA or CCCG as an anticodon and is aminoacylated with an unnatural amino acid, such tRNA being capable of efficiently introducing an unnatural amino acid into a protein without competing with a termination factor. Such tRNA is a mutant tRNA for tryptophan which has G at the 5' end, C as a base pairing with the G at the 5' end, and A as a base next to the C on the 3' side, such tRNA being a mutant tRNA which pairs with a stop codon and has CUA as an anticodon or a mutant tRNA which pairs with a stop codon or a 4-base codon has CUA or CCCG as an anticodon, into which a single base has been inserted just before the CCA sequence at the 3' end.
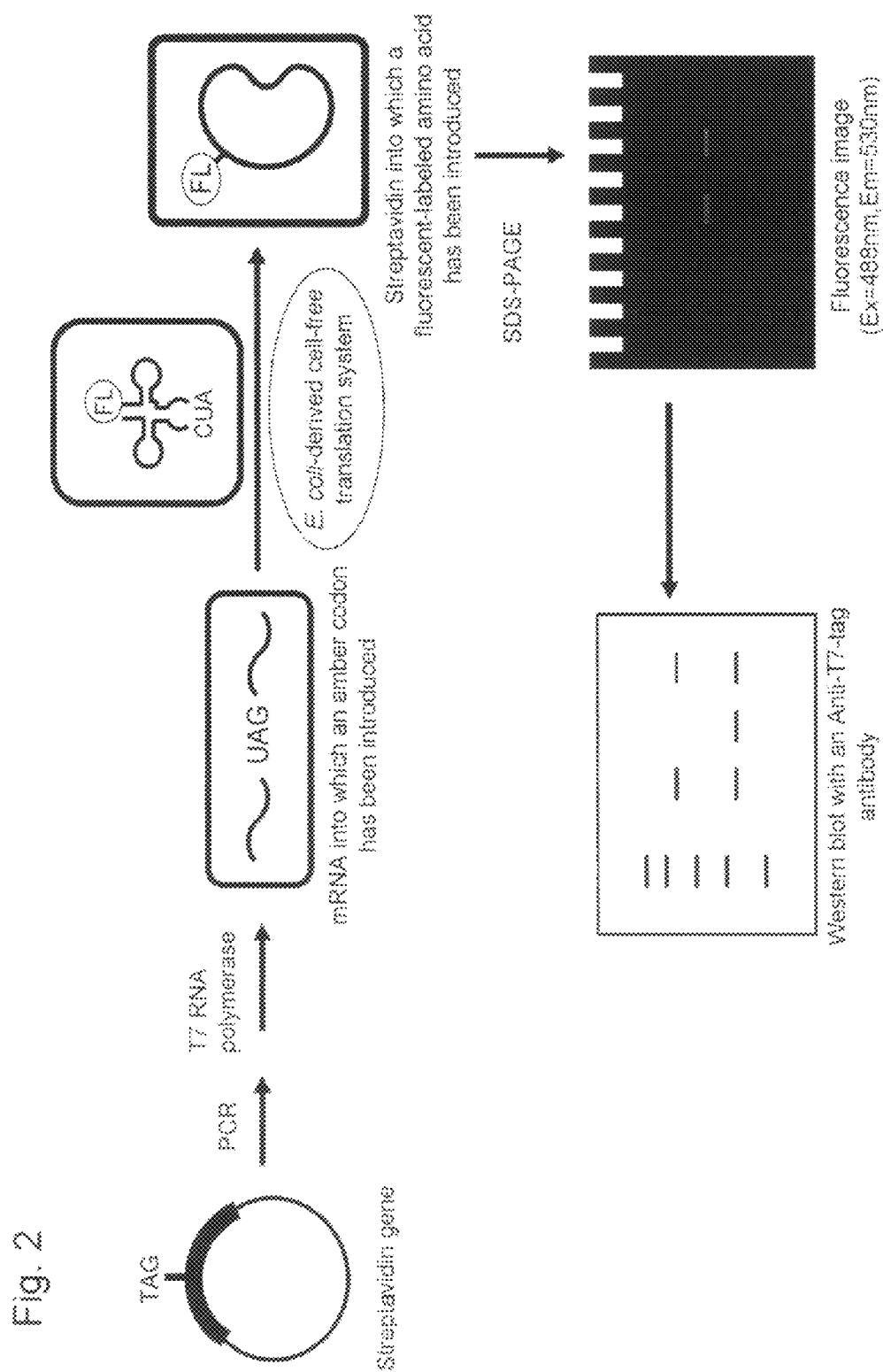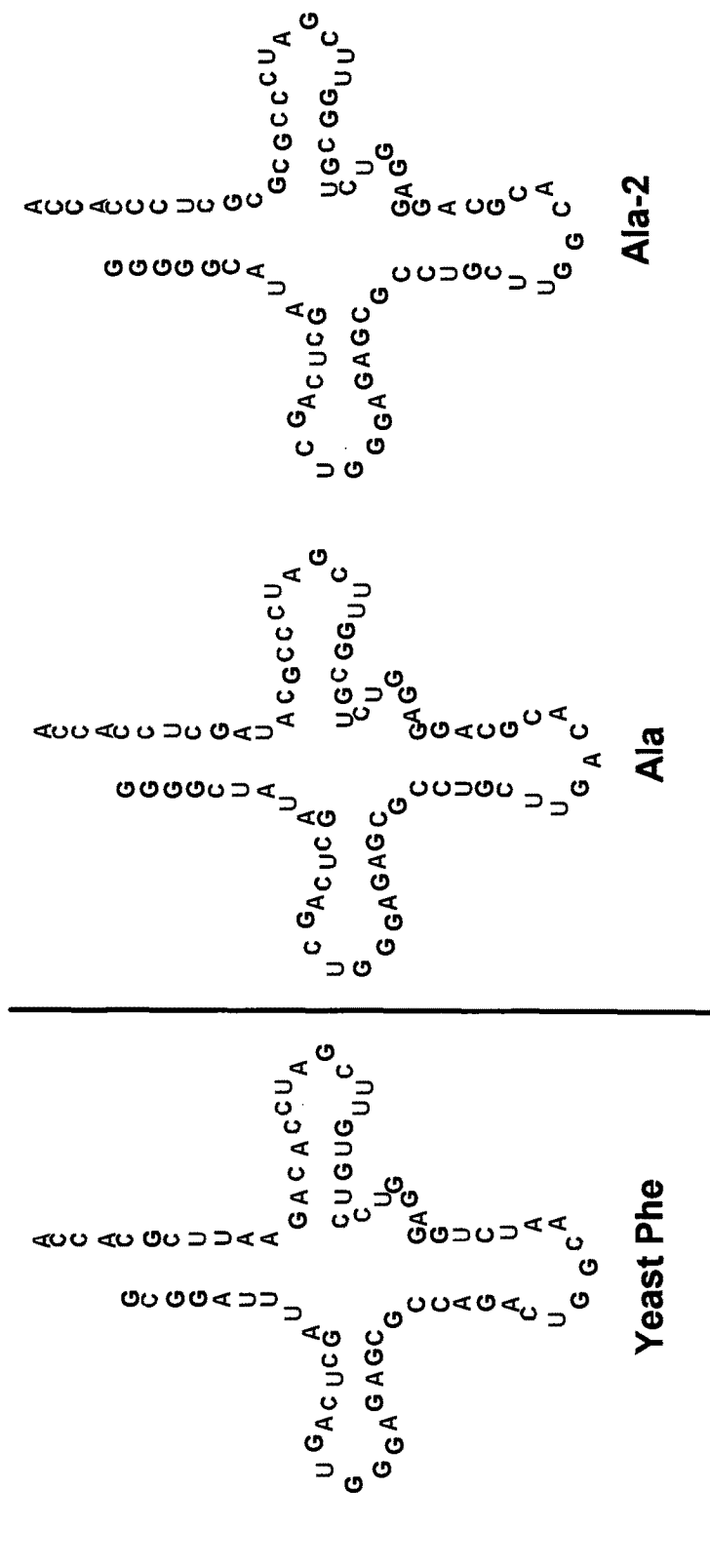
11 Claims, 52 Drawing Sheets

Fig. 1

PCR

Chemically
synthesized DNA

T7 RNA
polymerase

CUA

tRNA in which two
bases (CA) has
been deleted from
the 3' end

T4 RNA Ligase

FL

CUA

Fluorescent-labeled
amino acid-pdCpA

tRNA bound to fluorescent-
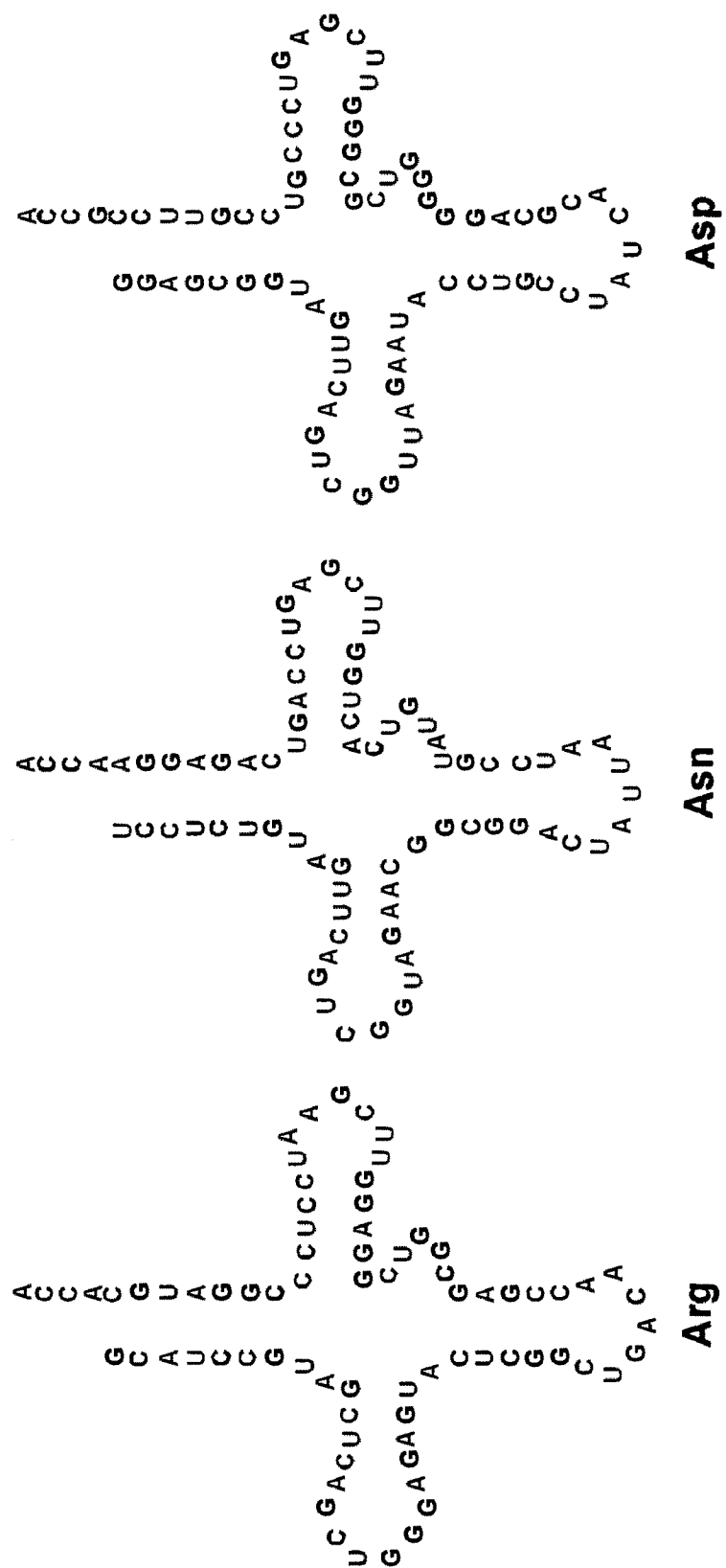labeled amino acid

Fig. 2

Fig. 3A

Yeast Phe

Ala

Ala-2

Fig. 3B

Fig. 3C

Fig. 3D
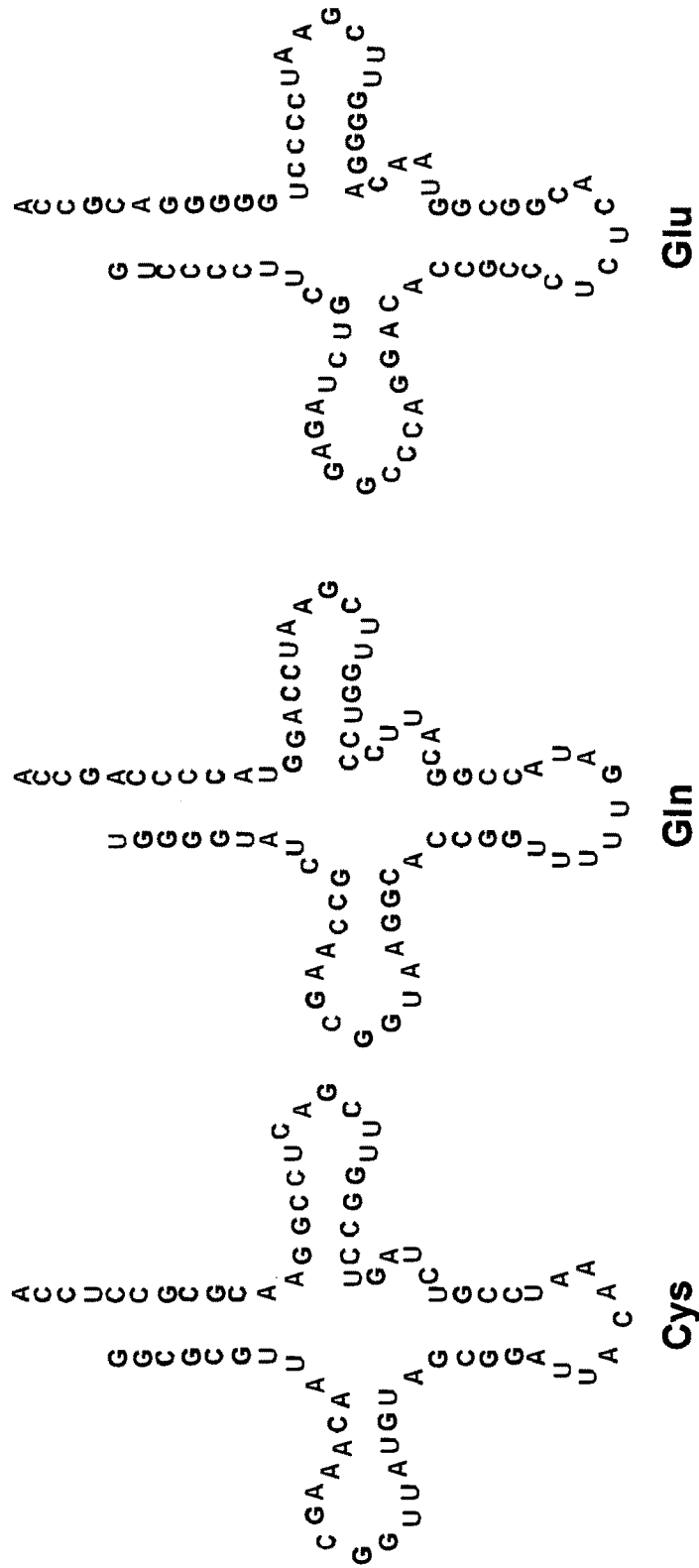
Fig. 3E
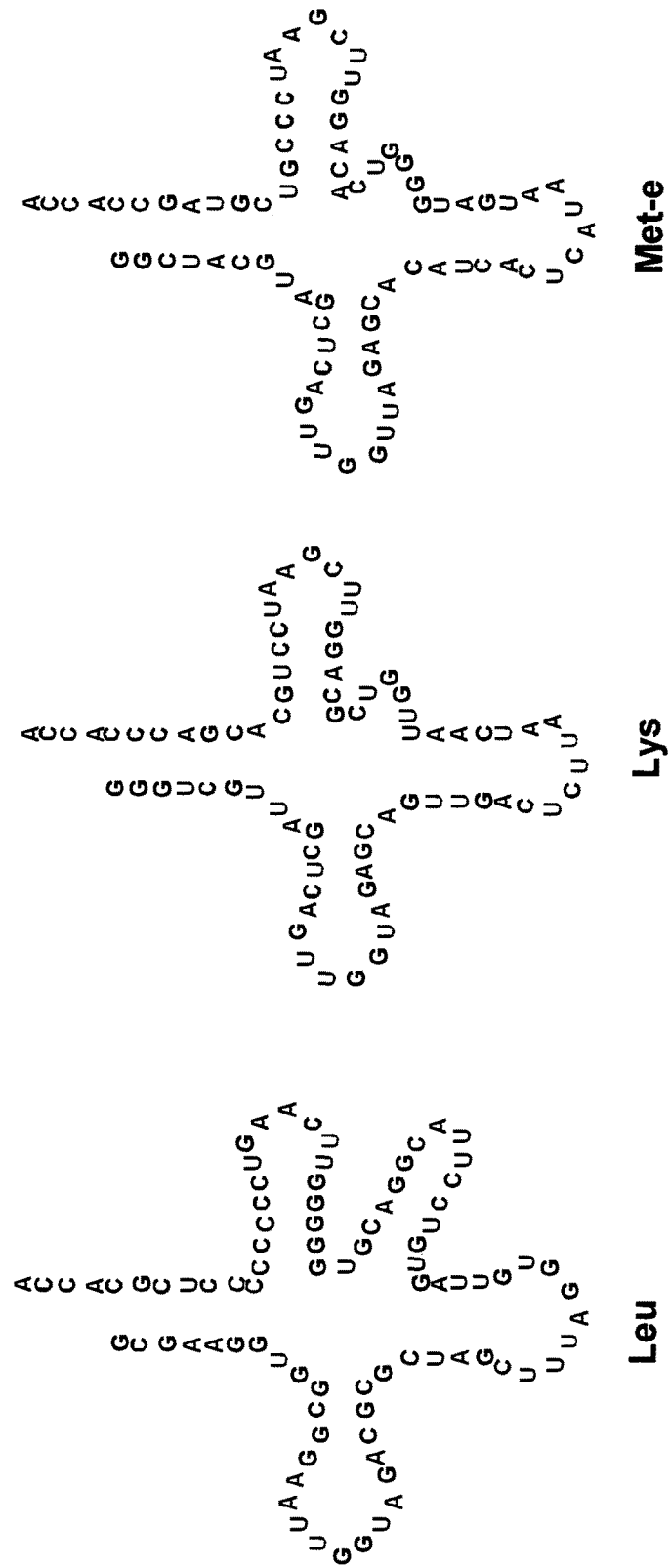
Fig. 3F

Fig. 3G

Fig. 3H

Fig. 4

Fig. 5
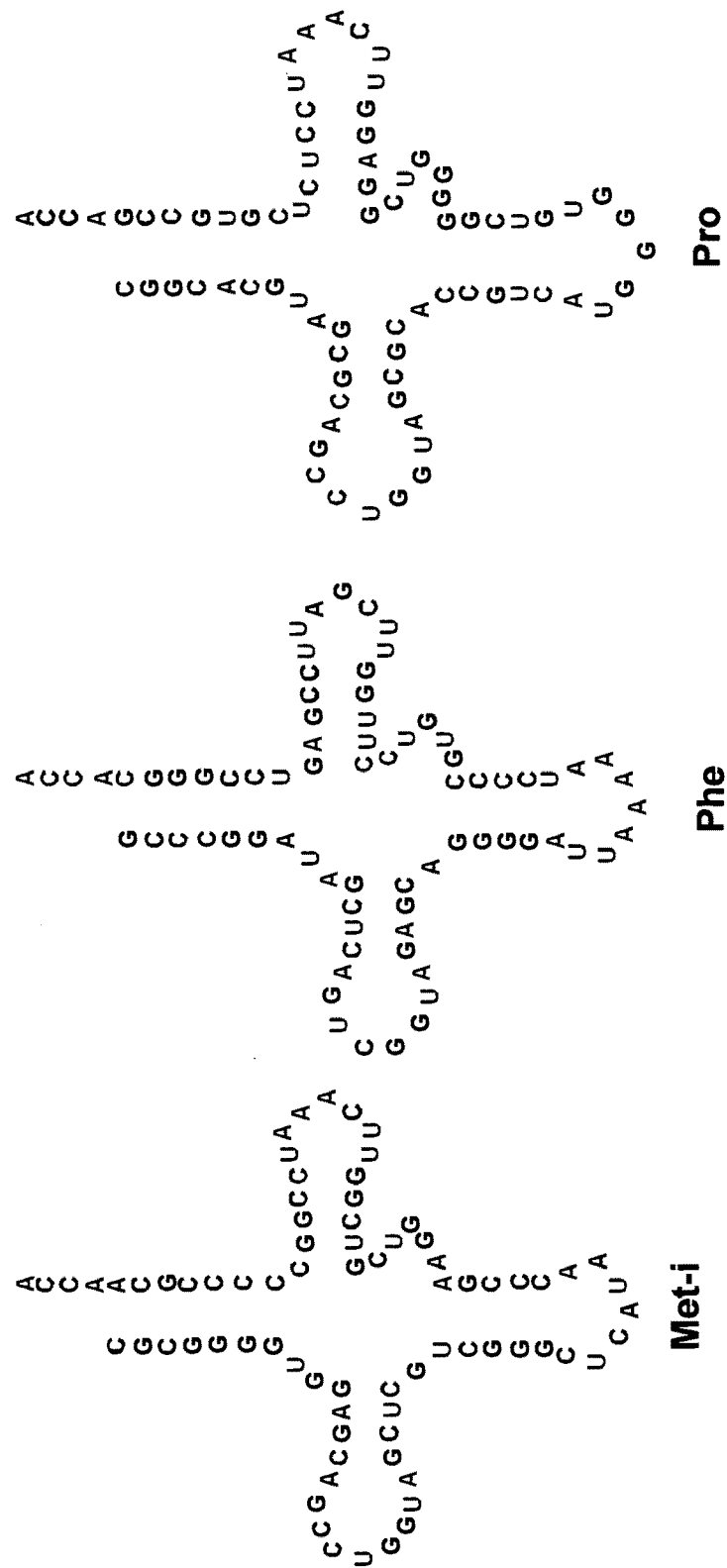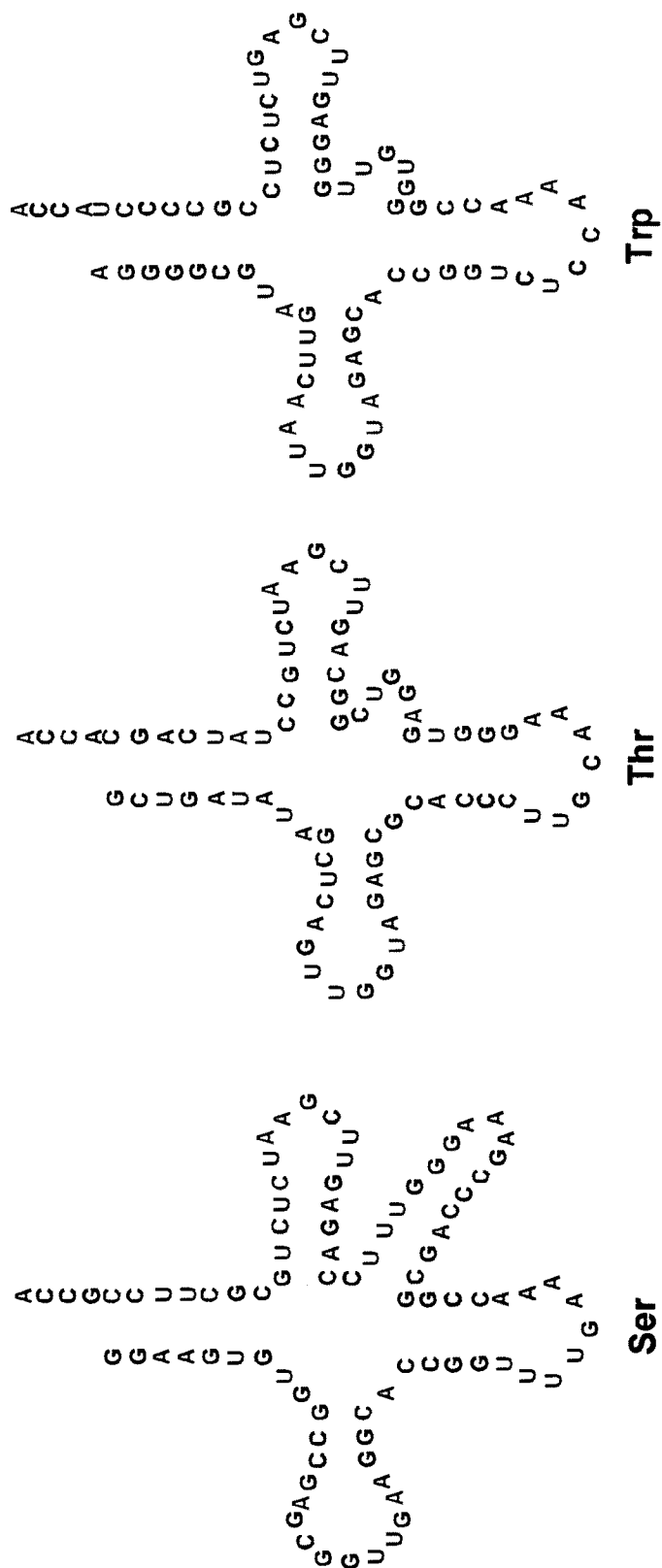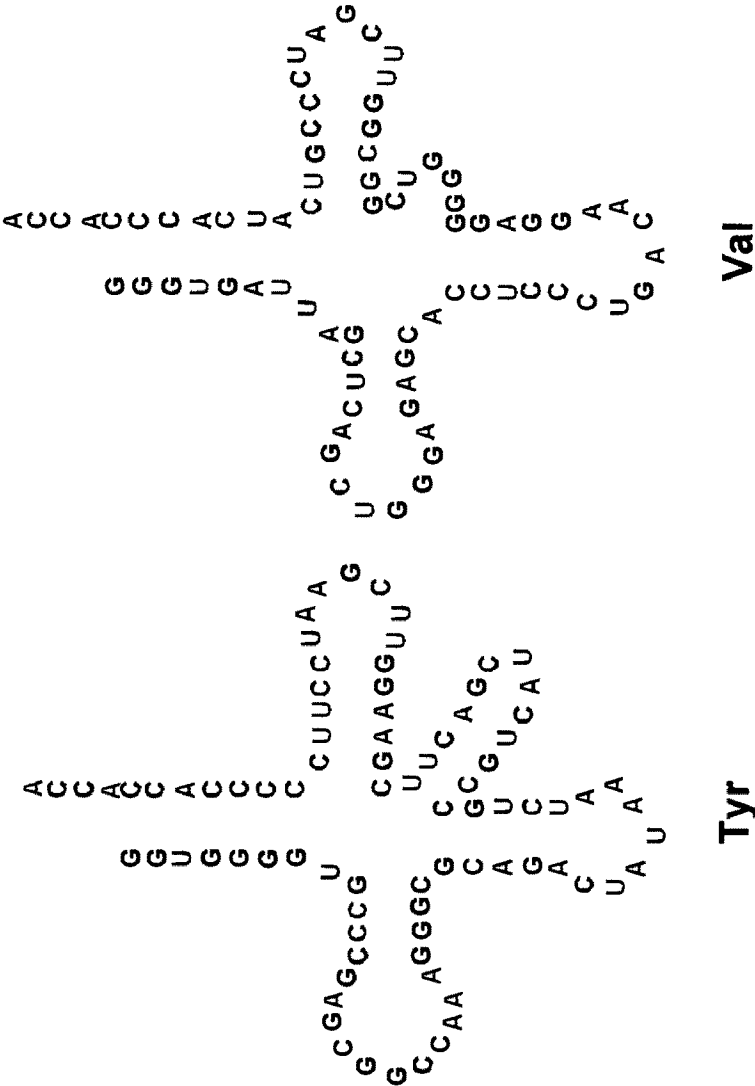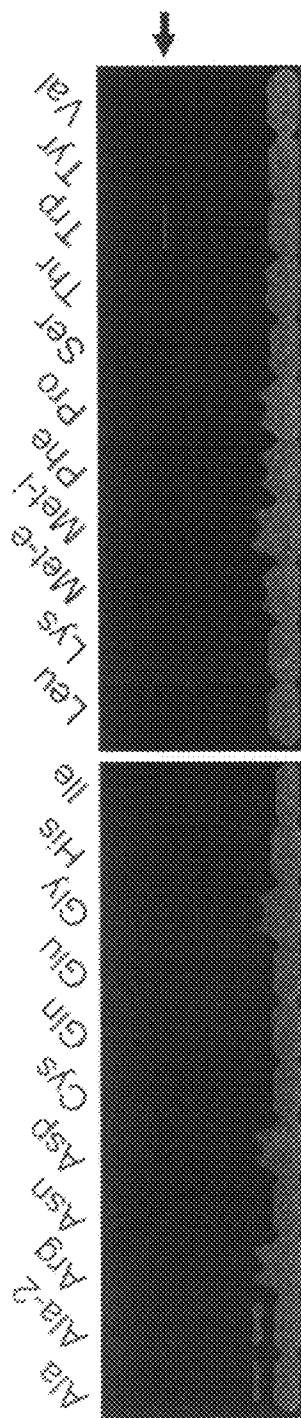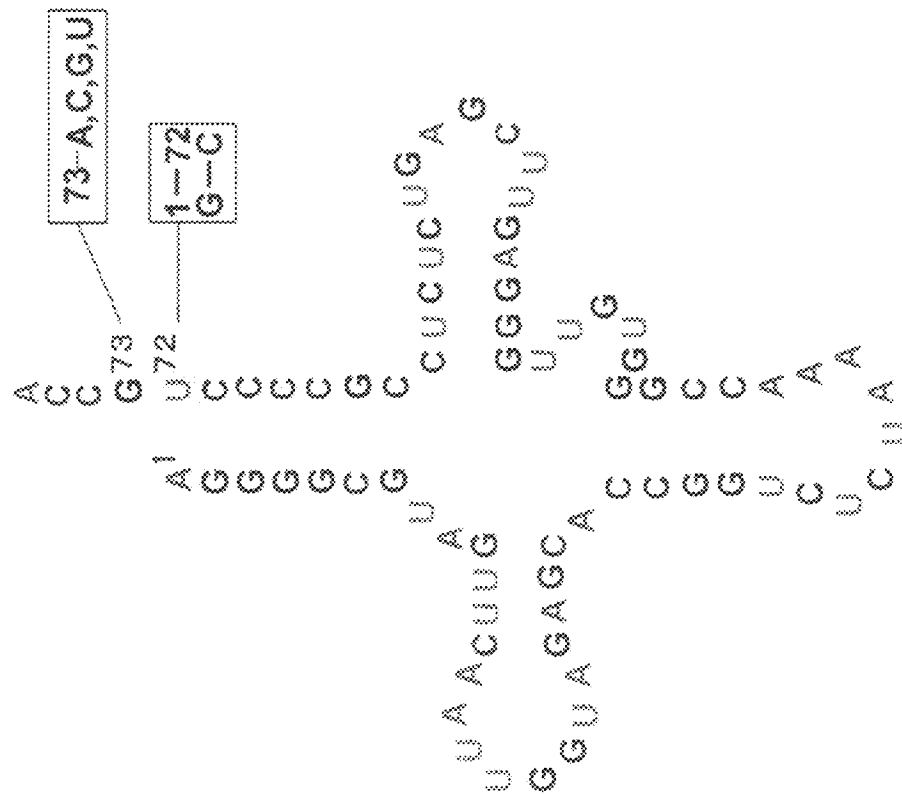
Fig. 6A
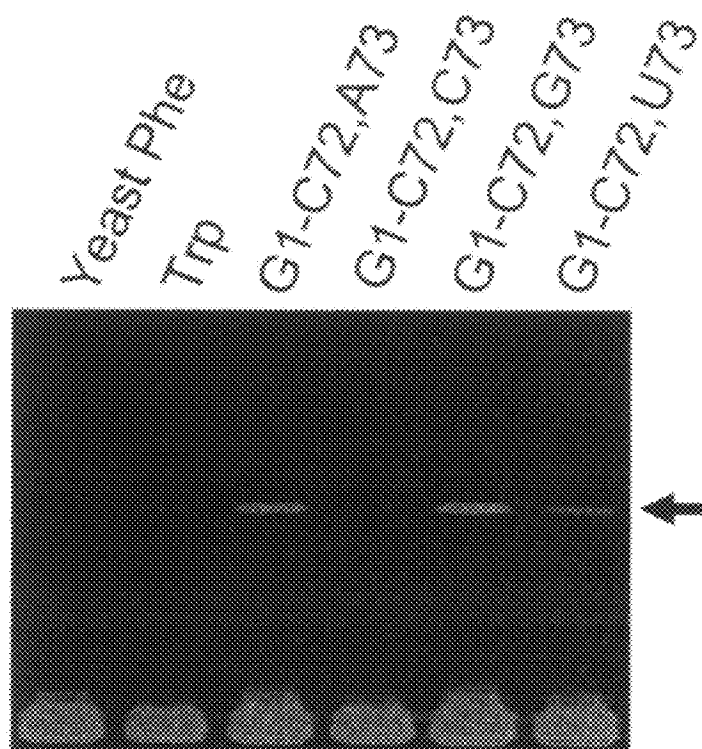
Fig. 6B

Fig. 6C

Fig. 7A
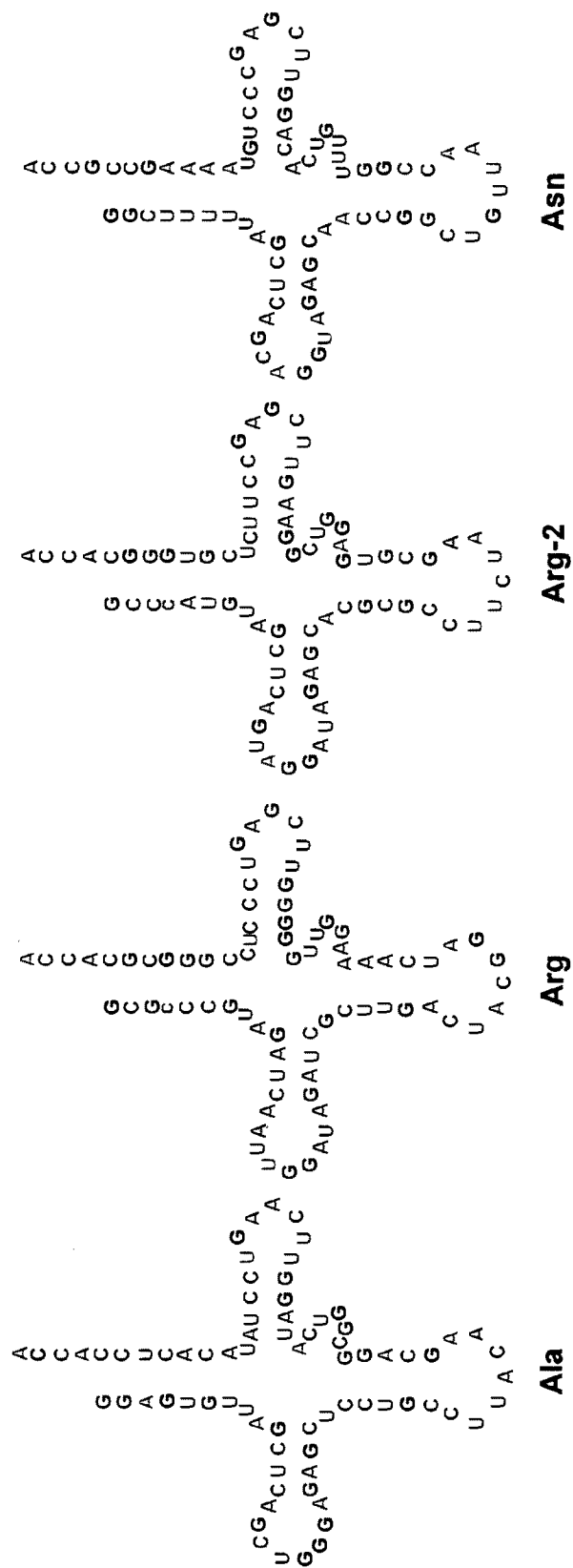
Fig. 7B
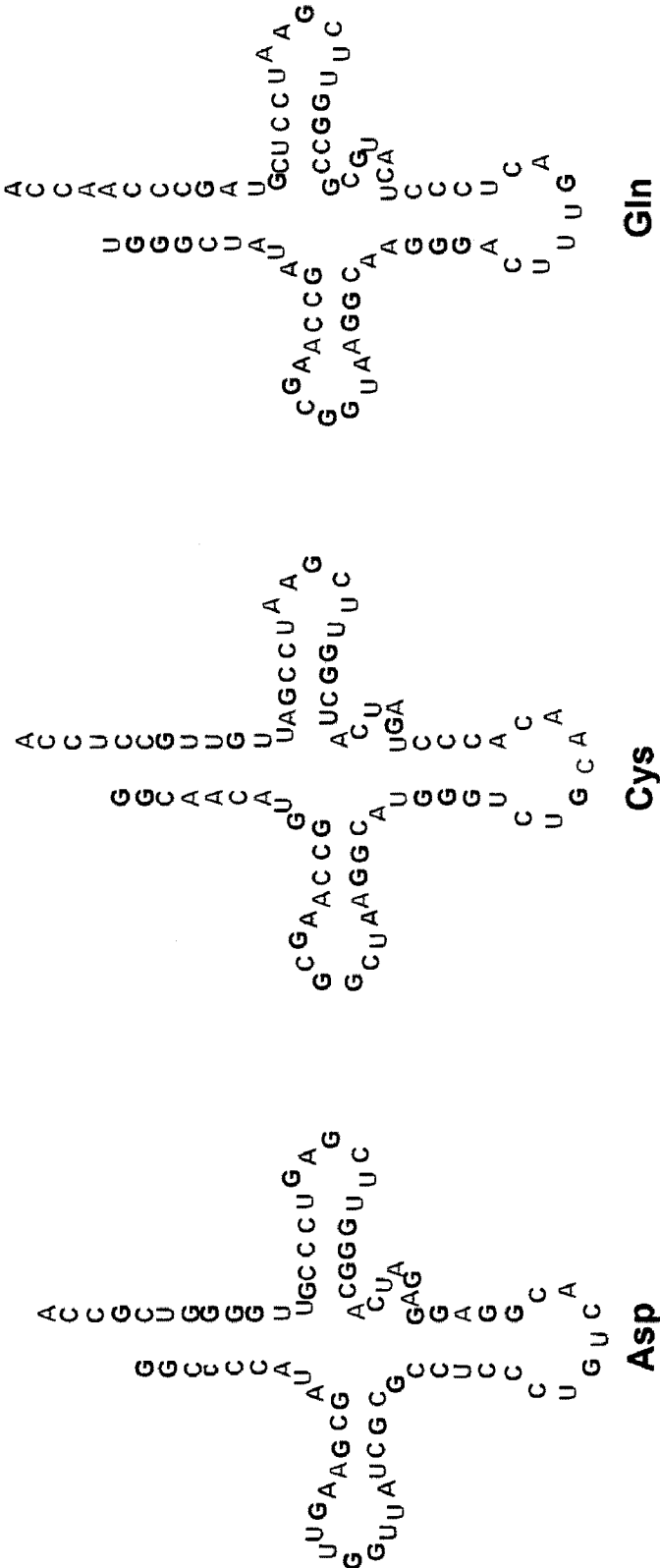
Gln

Cys

Asp

Fig. 7C

Fig. 7D

Fig. 7E

Met-e

Lys-2

Lys

Leu-3

Fig. 7F

Pro

Phe

Met-i

Fig. 7G

Fig. 7H

Fig. 8

Fig. 9
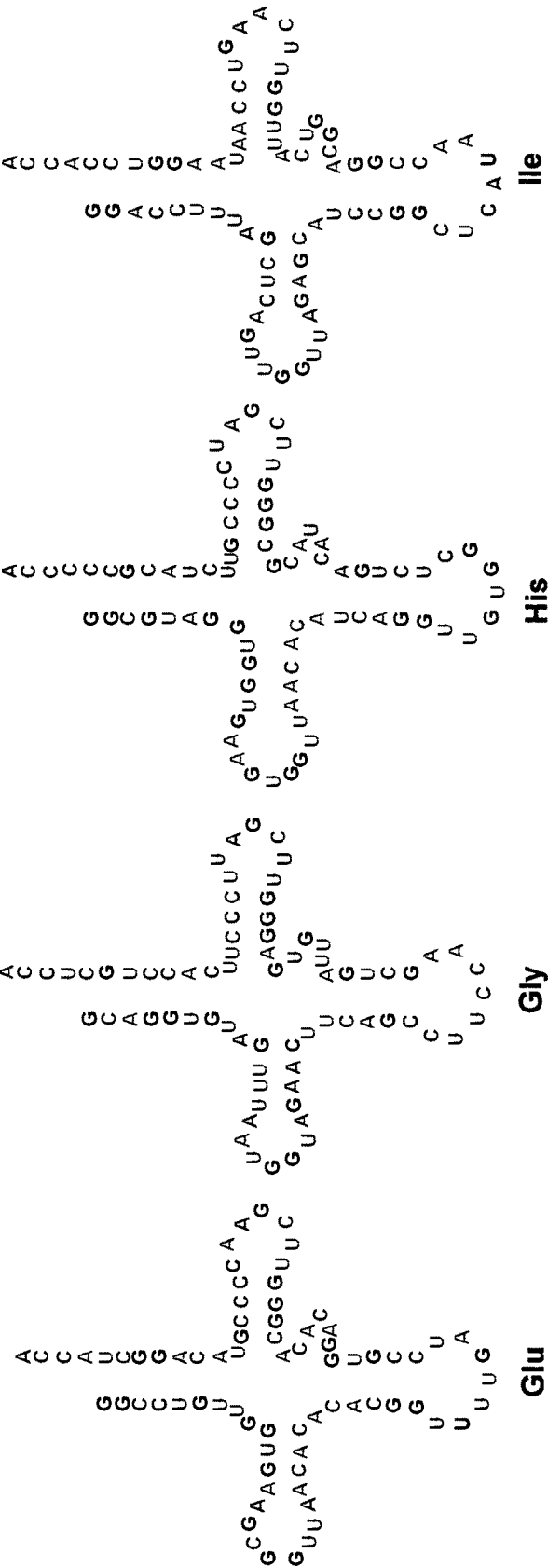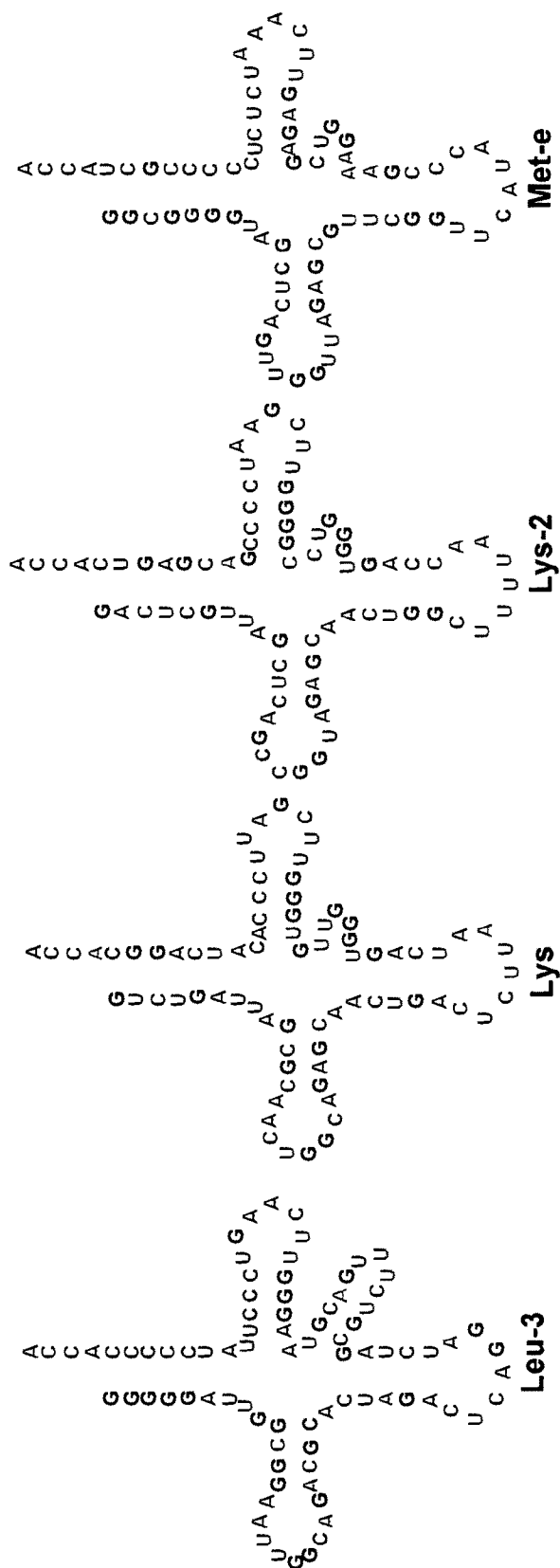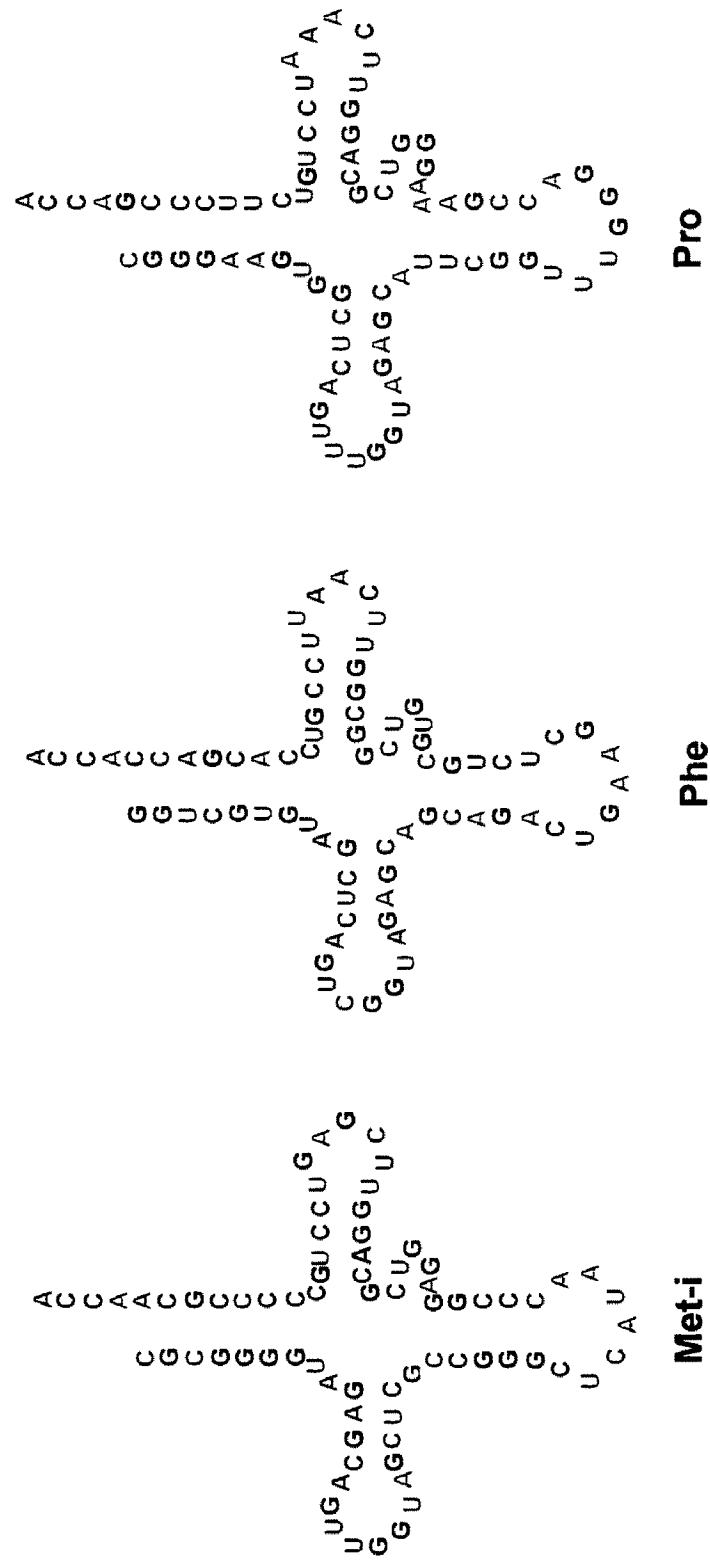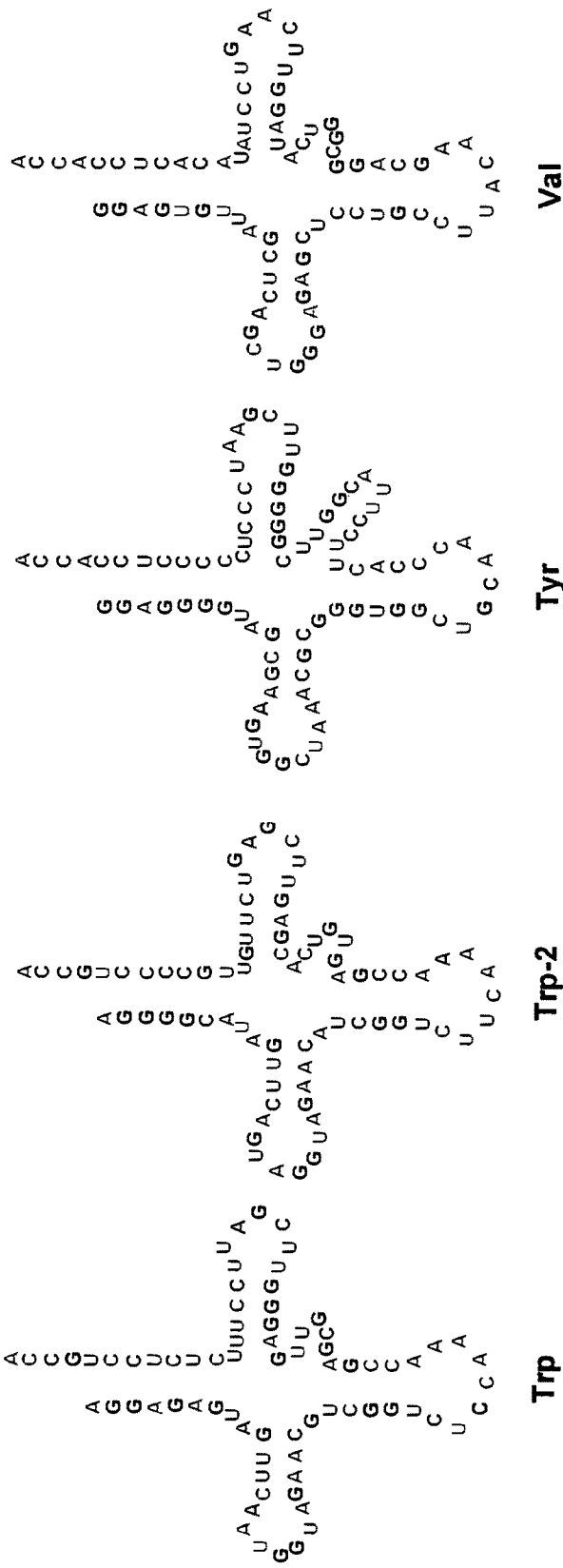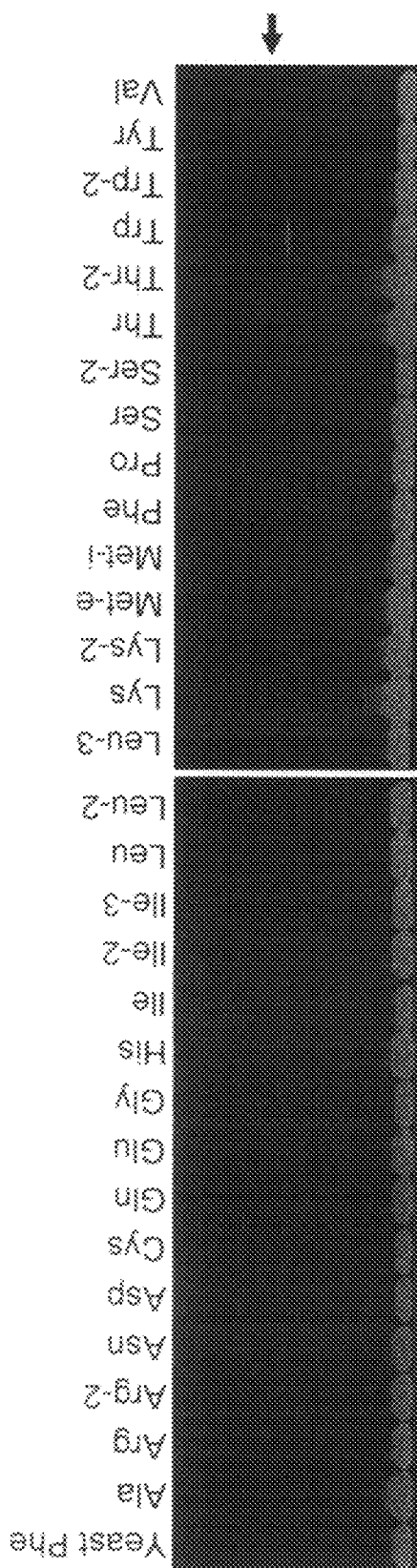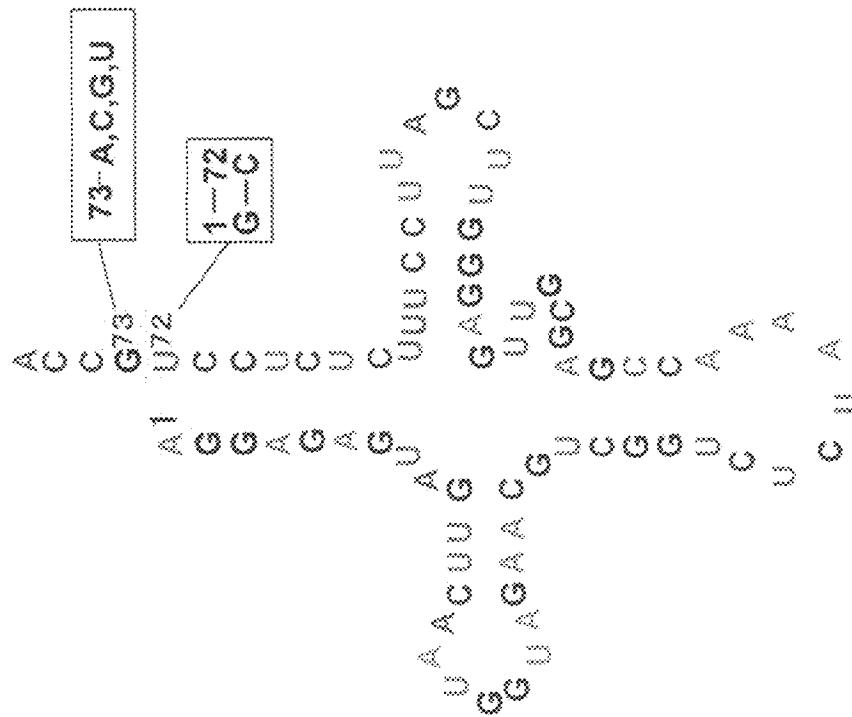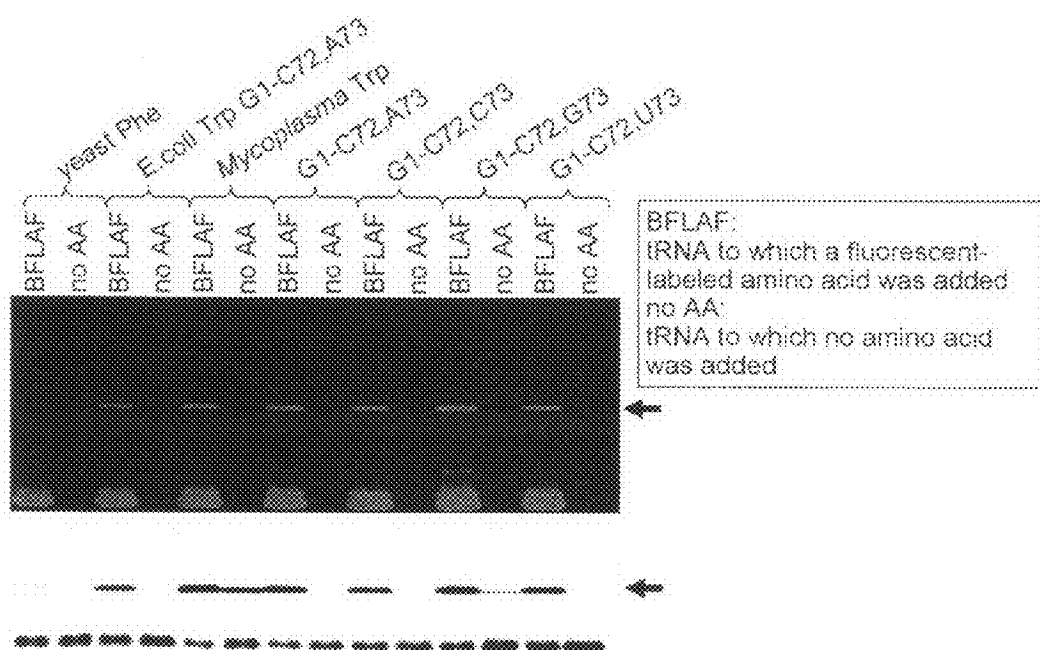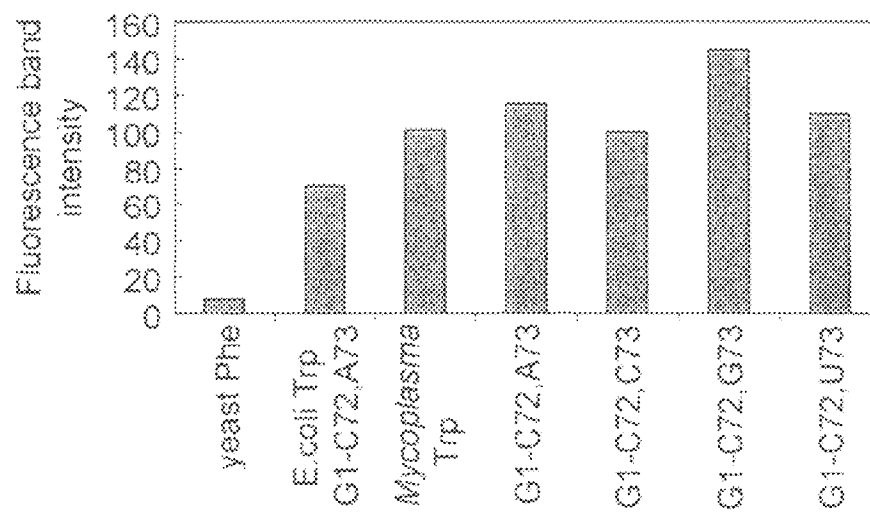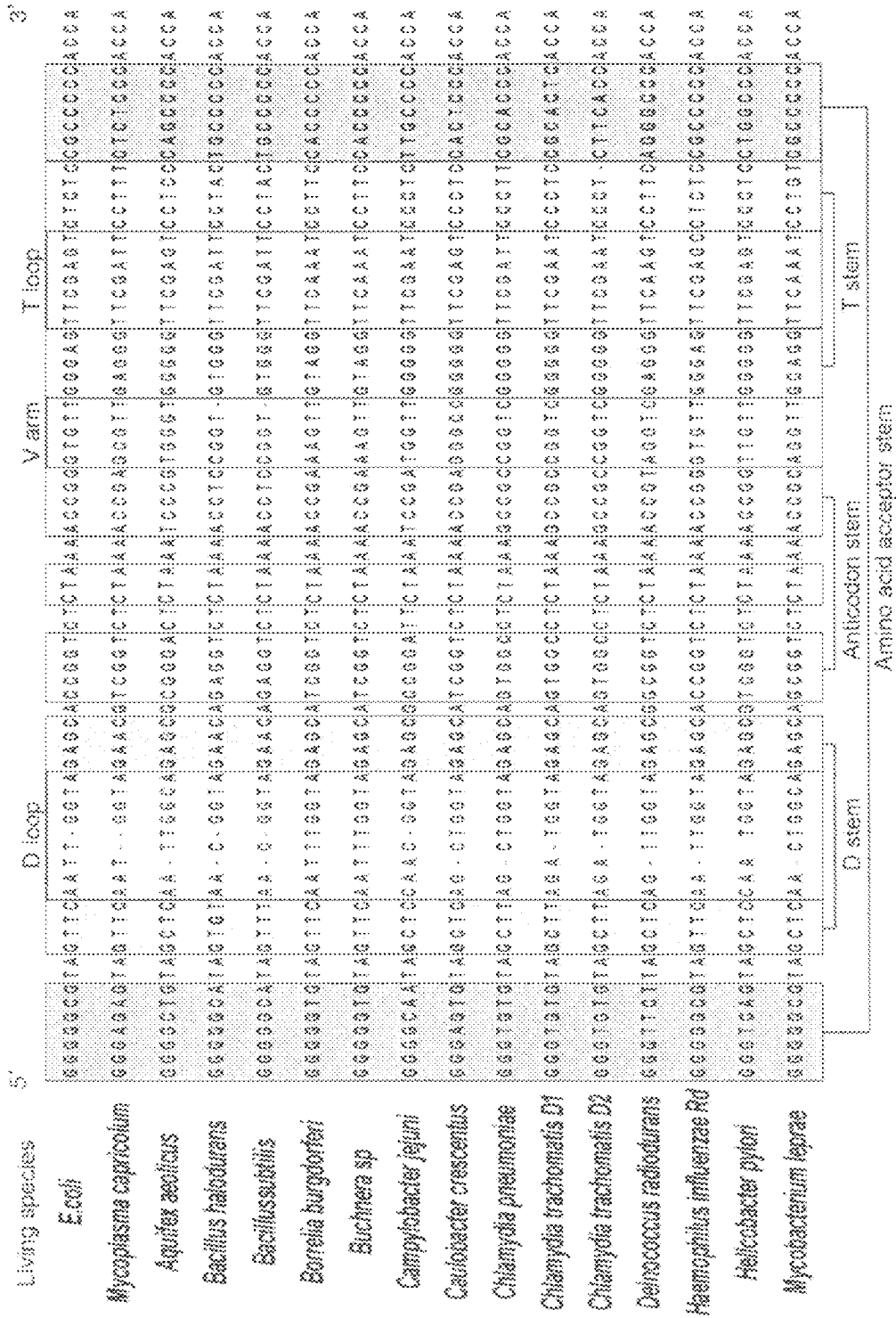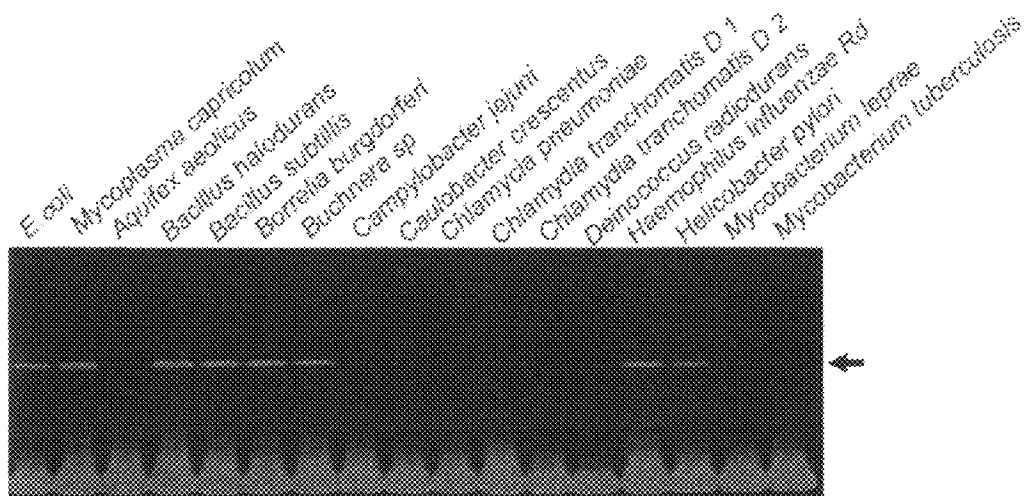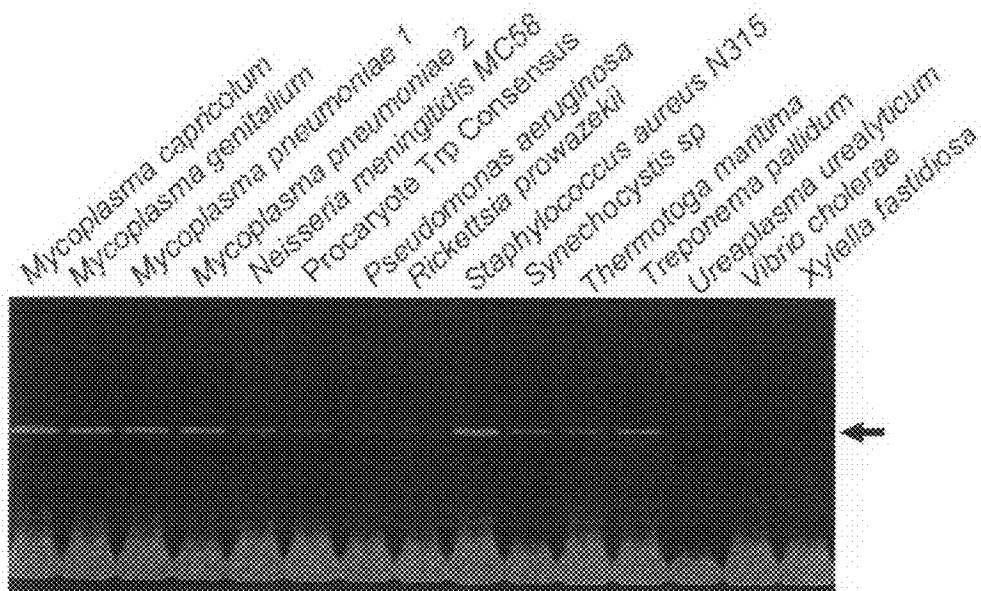
Fig. 10A
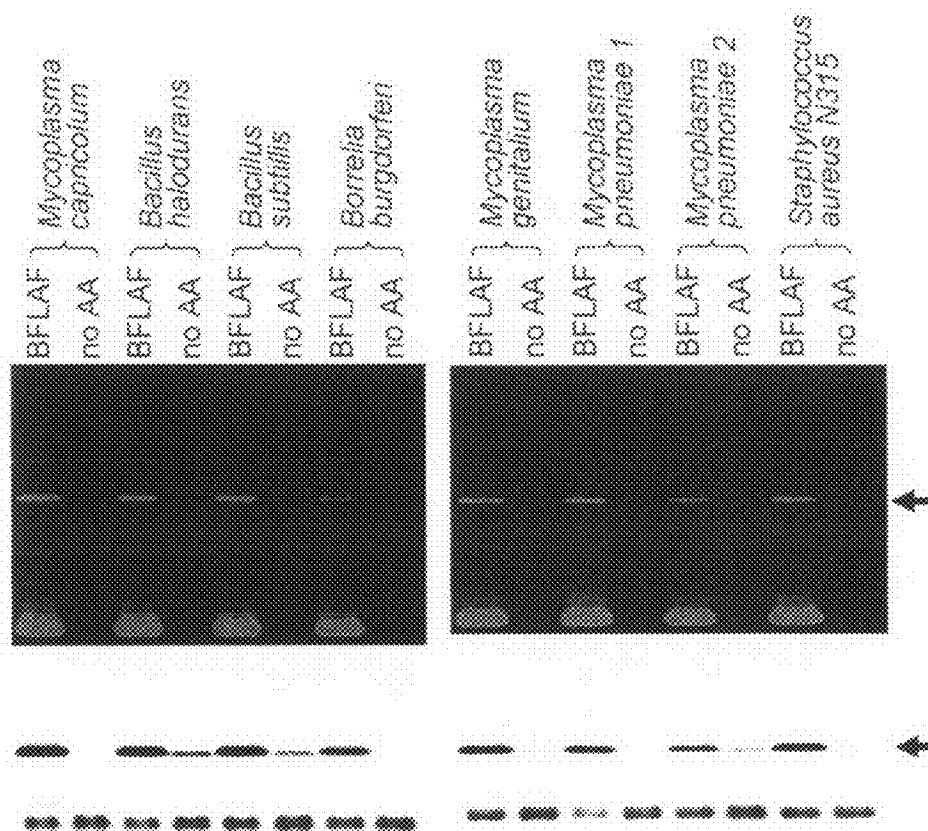
BFLAF:
tRNA to which a fluorescent-
labeled amino acid was added
no AA:
tRNA to which no amino acid
was added

Fig. 10B

Fig. 11A

Fig. 11B

Living species	5'	D loop			V arm	T loop	3'

Mycobacterium tuberculosis

Mycoplasma genitalium

Mycoplasma pneumoniae 1

Mycoplasma pneumoniae 2

Neisseria meningitides MC58

Procaryote Trp consensus

Pseudomonas aeruginosa

Rickettsia prowazekii

Staphylococcus aureus N315

Synechocystis sp

Thermotoga maritime

Treponema pallidum

Ureaplasma urealyticum

Vibrio choleraes

Xylella fastidiosa

D stem   Anticodon stem   T stem

Amino acid acceptor stem

Fig. 12A

Fig. 12B

Fig. 13A

Fig. 13B

Fig. 14A

Fig. 14B

Fig. 15A
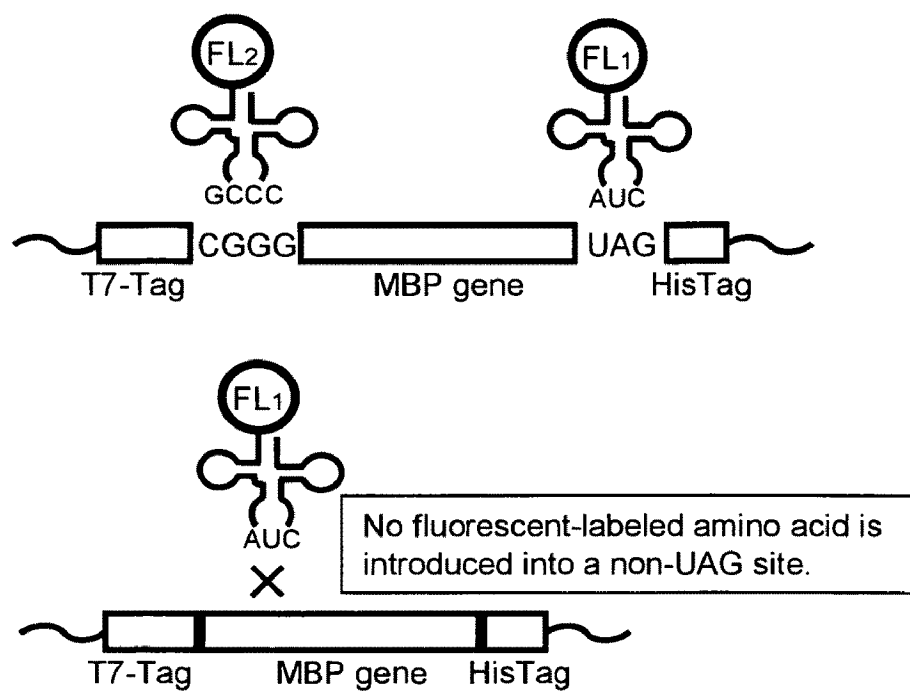
Fig. 15B
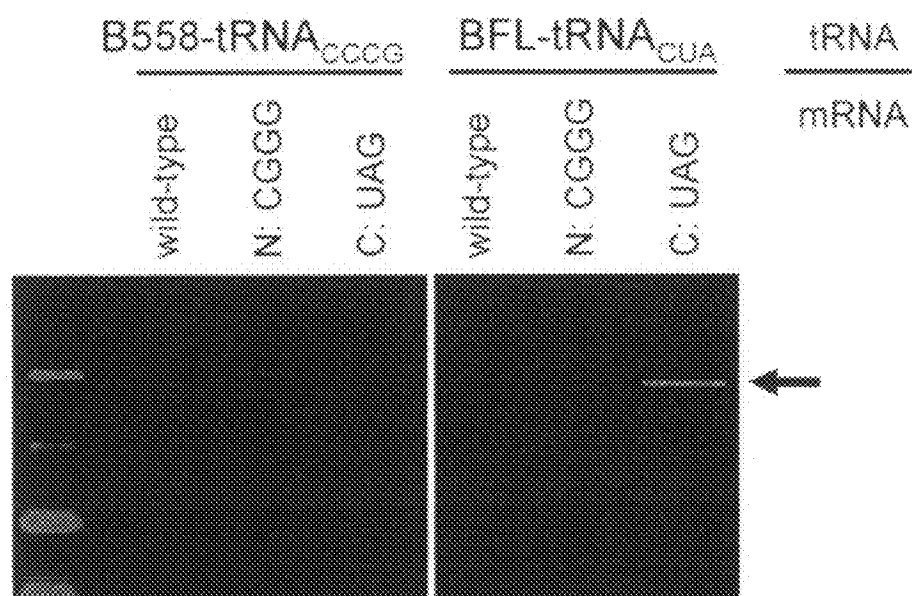
Fig. 16A
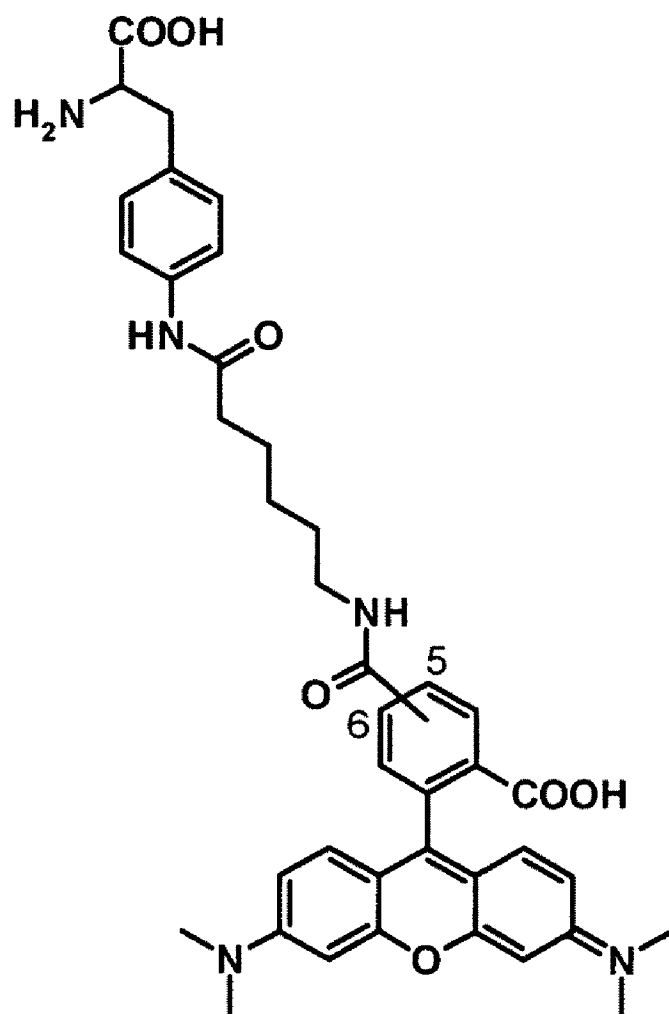
Fig. 16B

Type of codon incorporated into the
2nd base of streptavidin

CGGG   UAG

Fig. 16C

Fig. 17

Mutant tRNA for
Mycoplasma capricolum **tryptophan**
(normal)

73.1A
73.1C
73.1G
73.1U

Fig. 18A
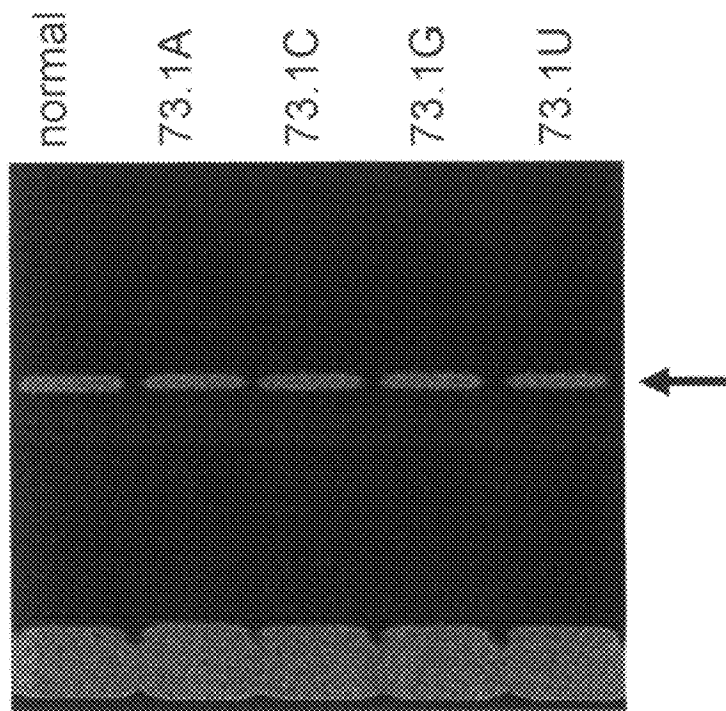
Fig. 18B
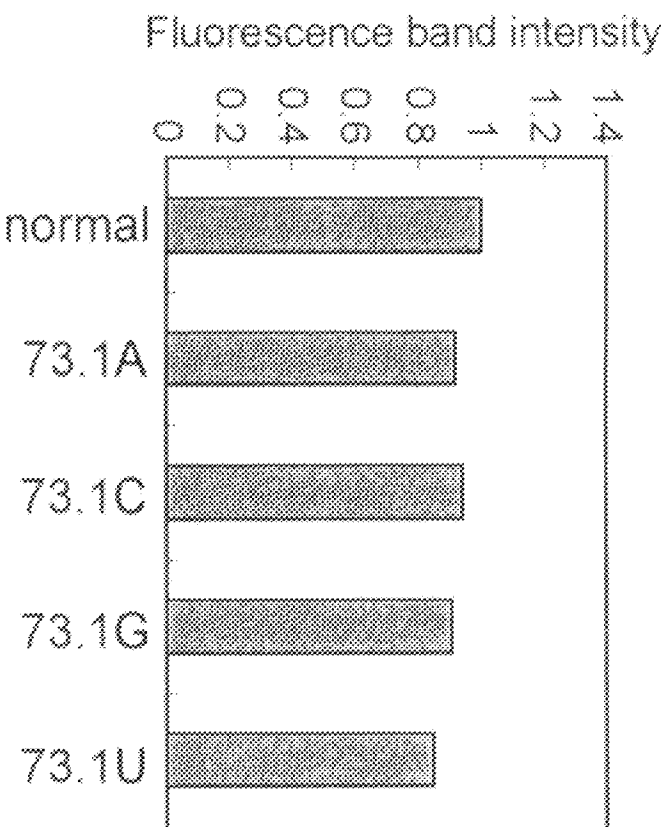
Fig. 19A
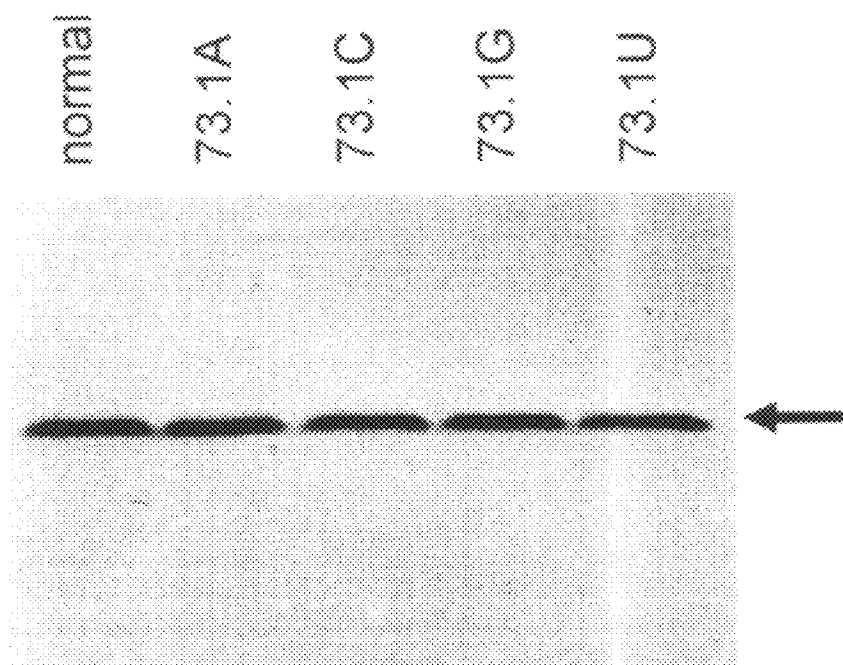
Fig. 19B

Fig. 20A

Fig. 20B

Fig. 21

Mutant tRNA for yeast phenylalanine
(normal)

73.1A
73.1C
73.1G
73.1U
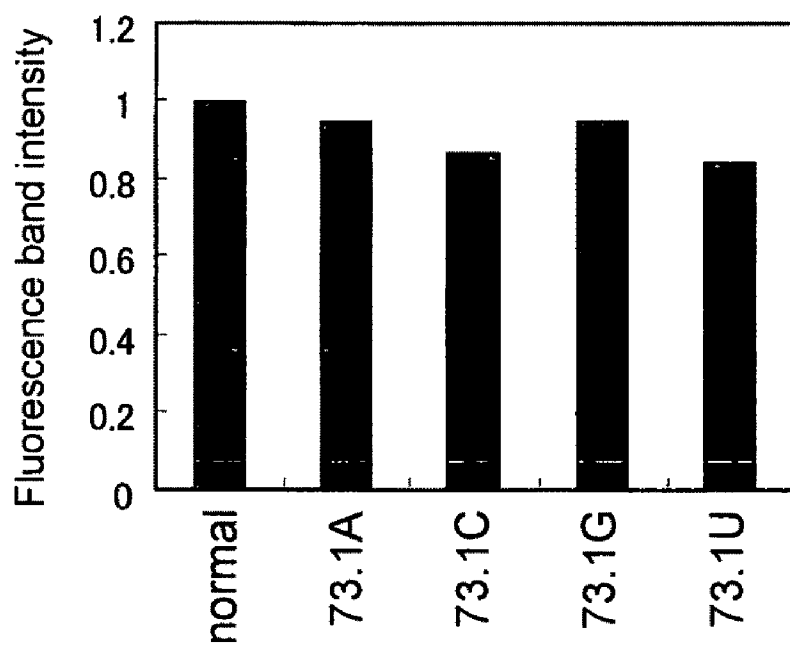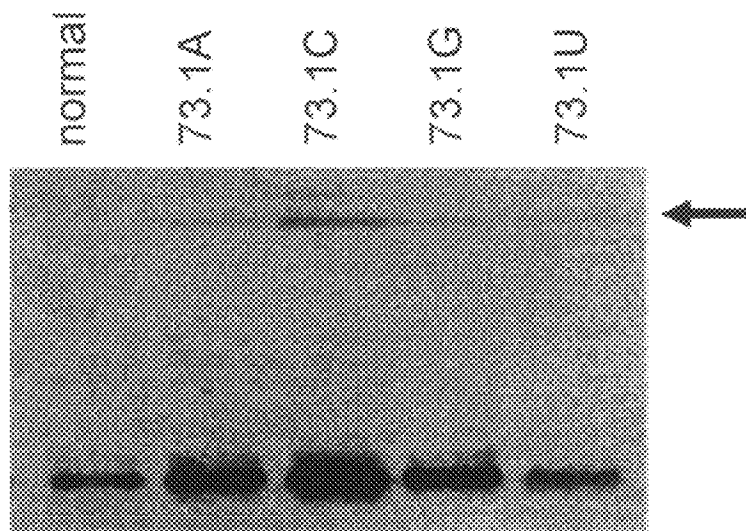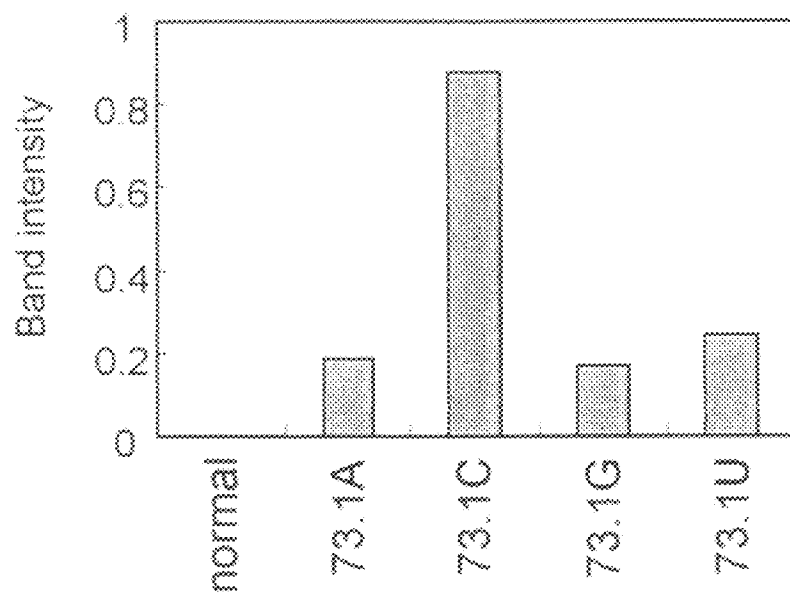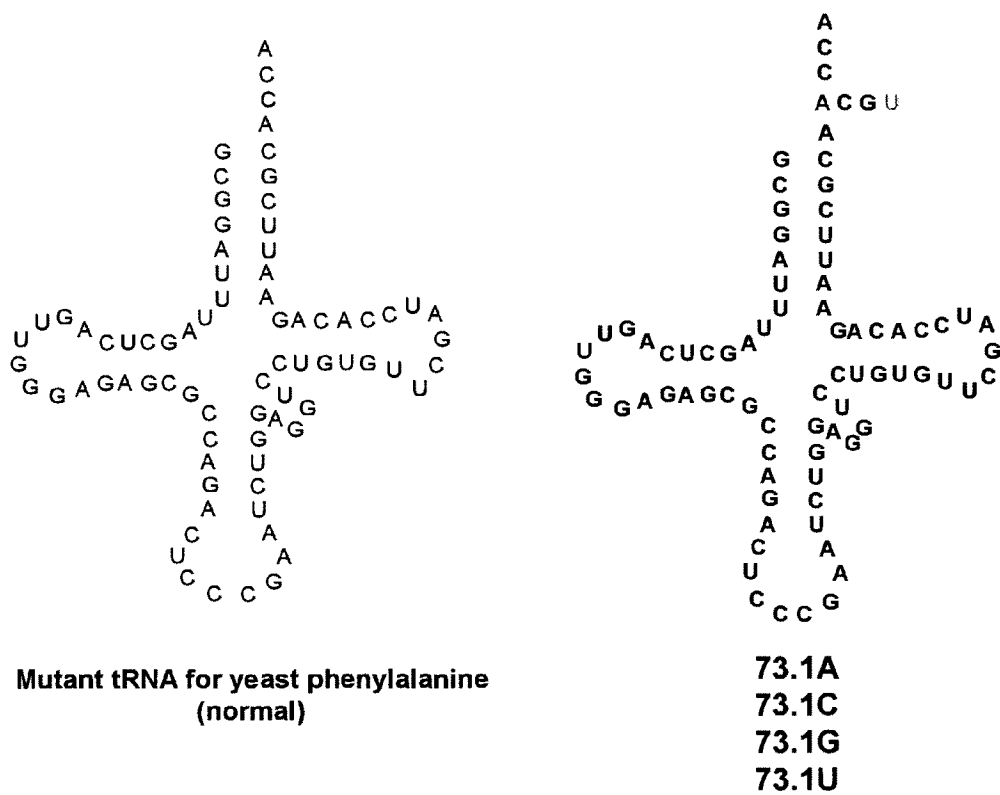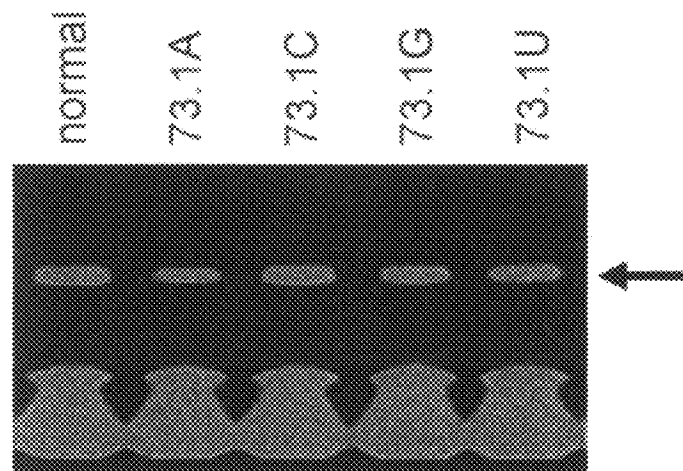
Fig. 22A

Fig. 22B
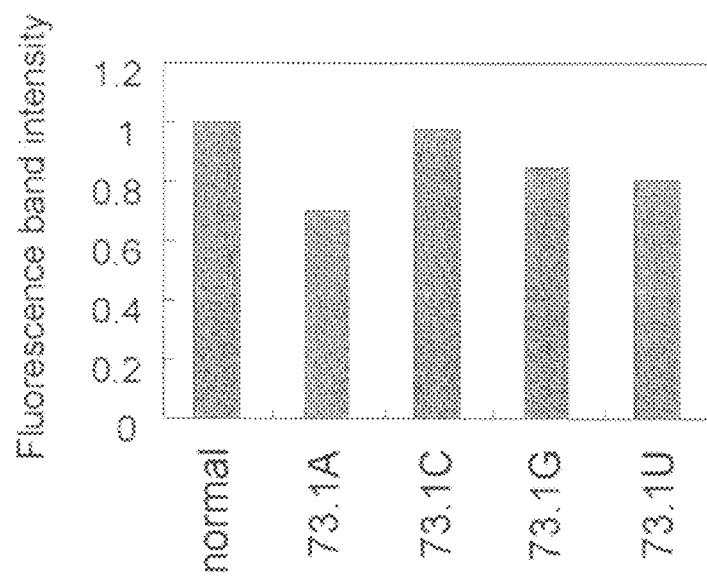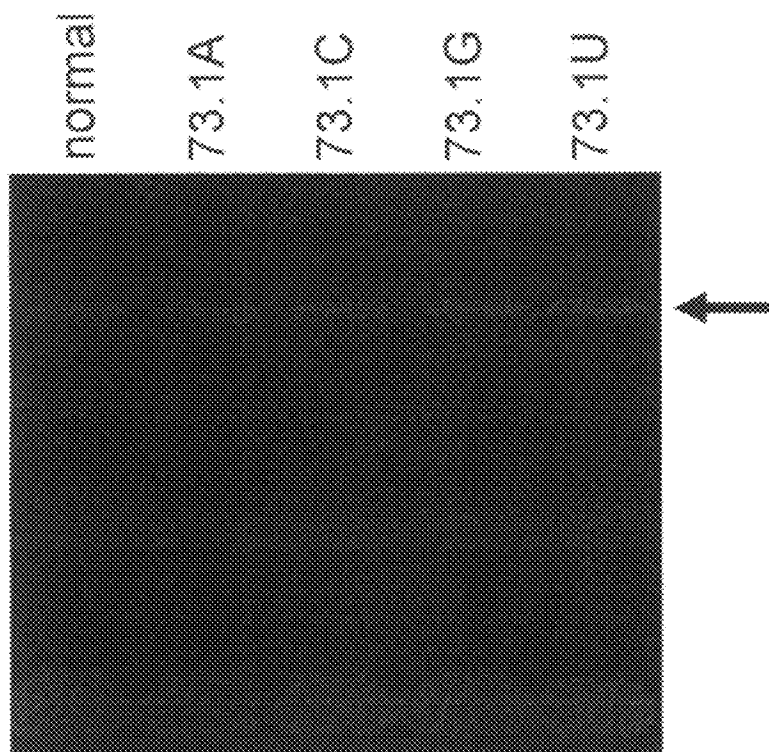
Fig. 23A

Fig. 23B
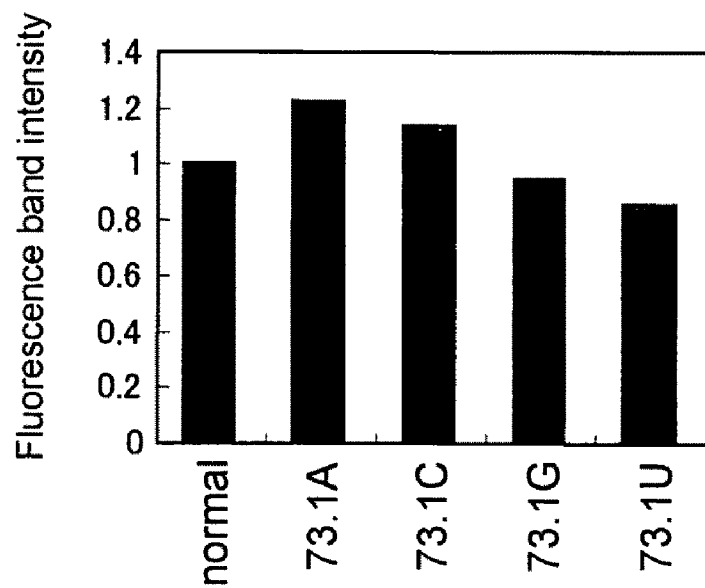
MUTANT TRNA FOR INTRODUCING UNNATURAL AMINO ACID INTO PROTEIN

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is a 35 U.S.C. §371 National Stage patent application of International patent application PCT/JP2006/323064, filed on Nov. 14, 2006, which claims priority to Japanese patent application JP 2005-329115, filed on Nov. 14, 2005.

TECHNICAL FIELD

The present invention relates to tRNA for introducing an unnatural amino acid, such as a labeled amino acid, into a protein.

BACKGROUND ART

In general, chemical modification of a specific protein residue is carried out to introduce a functional group onto the surface of a protein. Such chemical modification can be readily carried out and many specific residues can be modified at once, which is advantageous. On the other hand, excellent results are unlikely to be obtained in terms of reproducibility of the control of modified sites and/or the number of modified sites, which is problematic. Along with the recent developments in genetic engineering, it has become possible to substitute amino acid residues in proteins. Thus, it has become possible to introduce a desired unnatural amino acid having an amino skeleton into a protein by modifying a protein synthesis system, resulting in the realization of synthesis of a protein carrying functional groups with good reproducibility.

During protein synthesis, an amino acid first binds to the 3' end of tRNA and then is transferred to a ribosome, where protein synthesis takes place. In a ribosome, translation from codons to amino acids takes place. With the use of tRNA bound to an unnatural amino acid, an unnatural amino acid can be incorporated into a protein.

The following method can be used for introducing an unnatural amino acid into a protein: a method wherein a codon at a target site for introduction is first substituted with a stop codon UAG and then translation is carried out in the presence of tRNA that has CUA as an anticodon and is aminoacylated with an unnatural amino acid (see Non-Patent Documents 1 to 4). In such a method, examples of tRNA used include tRNA for yeast phenylalanine (see Non-Patent Documents 1 and 2), tRNA for *E. coli* asparagine, tRNA for tetrahymena glutamine (see Non-Patent Document 3), and tRNA for *E. coli* glycine (see Non-Patent Document 4).

However, since tRNA that has CUA as an anticodon and is aminoacylated with an unnatural amino acid competes with a termination factor upon translation of UAG, the efficiency of introduction of an unnatural amino acid is not high in such case.

Non-Patent Document 1: Science, 244, p. 182, 1989
Non-Patent Document 2: Nucleic Acids Res., 18, 83-88, 1989
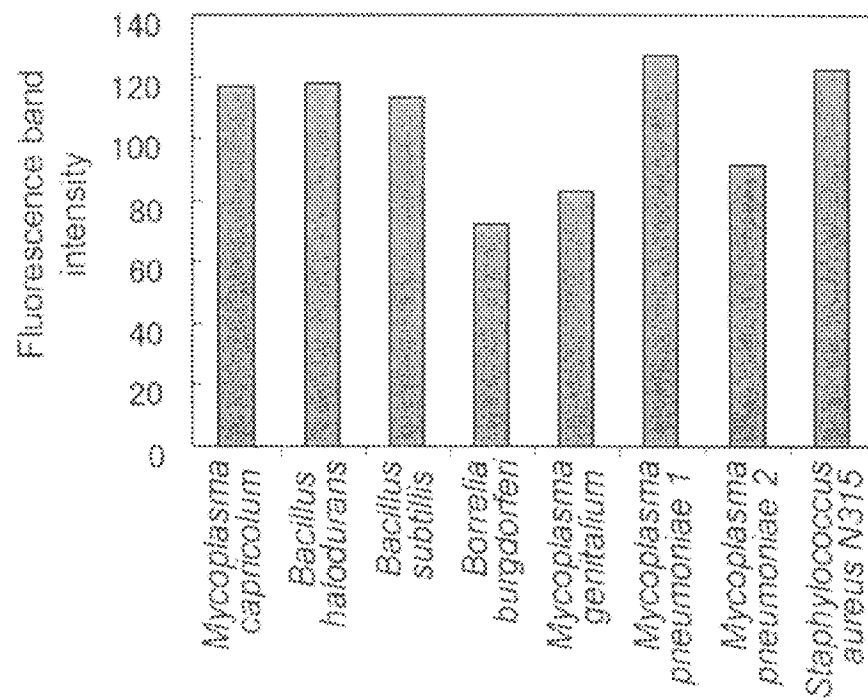Non-Patent Document 3: Chem. Biol., 3, 1033-1038, 1996
Non-Patent Document 4: J. Am. Chem. Soc., 111, p. 8013, 1989

DISCLOSURE OF THE INVENTION

It is an objective of the present invention to provide tRNA that has CUA as an anticodon and is aminoacylated with an

unnatural amino acid, such tRNA being capable of efficiently introducing an unnatural amino acid into a protein without competing with a termination factor.

The present inventors searched for tRNA that can efficiently introduce an unnatural amino acid into a protein with the use of a stop codon UAG among tRNAs derived from a variety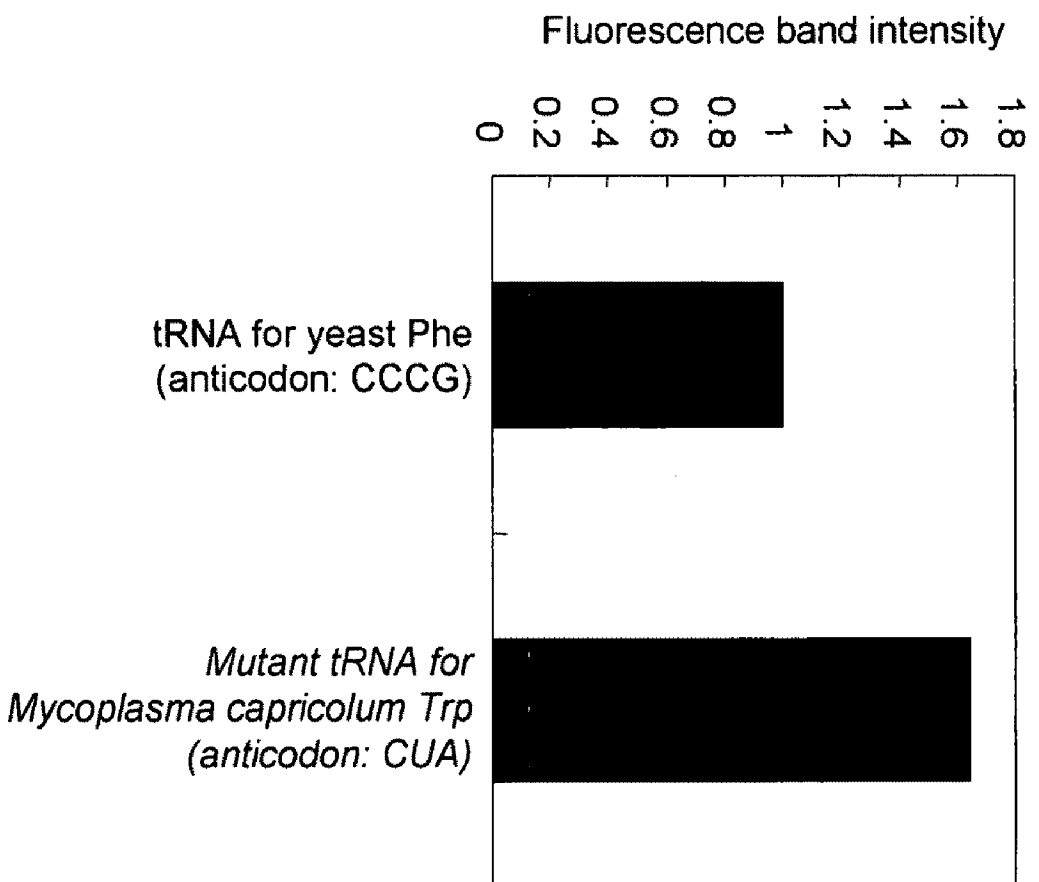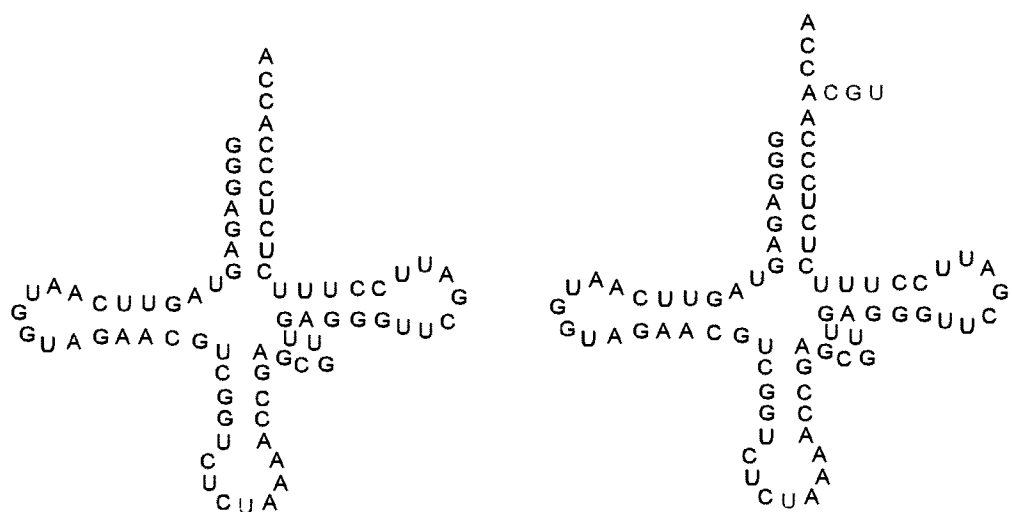 of the living species. Accordingly, they have found that a tRNA for Trp having CUA as an anticodon can introduce an unnatural amino acid with good efficiency. In addition, they have found that tRNA for Trp obtained by modifying the above tRNA can introduce an unnatural amino acid with better efficiency while the addition of a natural amino acid caused by aminoacyl tRNA synthase does not take place, such tRNA for TRp having an anticodon CUA, G at the 5' end, C as a base paring with the G at the 5' end (generally designated as the 72nd base), and A as a base next to the C. Further, they have found that the most preferable tRNA among the above examples is *Mycoplasma capricolumn* Trp tRNA (a G1-C72, A73 mutant) having the following sequence: GGGAGAGUAG UUCAAUGGUA GAACGUCGGU CUCUAAAACC GAGCGUUGAG GGUUCGAUUC CUUUCUCUCC CACCA (SEQ ID NO: 1). Furthermore, they have found that the efficiency of introduction of an unnatural amino acid can be improved by inserting a single base just before the CCA sequence at the 3' end of tRNA. According to the present invention, the above objective has been achieved in a manner such that efficient introduction of an unnatural amino acid can be realized with the use of the above tRNAs even when such tRNA competes with a termination factor.

Specifically, the present invention is described as follows.

[1] A mutant tRNA for tryptophan which pairs with a stop codon and has CUA an anticodon.

[2] The mutant tRNA which pairs with a stop codon according to [1], which has G at the 5' end, C as a base pairing with the G at the 5' end, and A as a base next to the C on the 3' side.

[3] The mutant tRNA which pairs with a stop codon according to [2], in which C pairing with the G at the 5' end is the 72nd base from the 5' end and A next to the C on the 3' side is the 73rd base from the 5' end.

[4] The mutant tRNA which pairs with a stop codon according to any one of [1] to [3], which is a prokaryotic-cell-derived tRNA.

[5] The mutant tRNA which pairs with a stop codon according to [4], which is an *E. coli* or *Mycoplasma capricolum*-derived tRNA.

[6] A mutant tRNA which pairs with a stop codon, which is a *Mycoplasma capricolum*-derived mutant tRNA comprising the base sequence represented by SEQ ID NO: 1.

[7] The mutant tRNA which pairs with a stop codon according to any one of [1] to [6], into which a single base A, C, G, or U has been inserted just before the CCA sequence at the 3' end.

[8] The mutant tRNA which pairs with a stop codon according to [7], in which C pairing with the G at the 5' end is the 72nd base from the 5' end, A next to the C on the 3' side is the 73rd base from the 5' end, and a single base A, C, G, or U has been further inserted so as to serve as the 74th base from the 5' end.

[9] The mutant tRNA which pairs with a stop codon according to any one of [1] to [8], which is aminoacylated with an amino acid.

[10] The mutant tRNA which pairs with a stop codon according to [9], wherein the amino acid is an unnatural amino acid or a derivative thereof.

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[11] The mutant tRNA which pairs with a stop codon according to [10], wherein the amino acid derivative is selected from the group consisting of hydroxy acid, mercapto acid, and carboxylic acid.

[12] The mutant tRNA which pairs with a stop codon according to any one of [9] to [11], wherein the amino acid is fluorescent-labeled.

[13] A method for introducing a desired amino acid into a protein, wherein an amino acid is introduced into a protein in which mRNA of the protein into which an amino acid is introduced, the mRNA having a stop codon that is corresponding to a site at which the amino acid is introduced, and the mutant tRNA which pairs with a stop codon according to any one of [1] to [12] are used and the mutant tRNA is allowed to pair with the stop codon.

[14] A mutant tRNA which pairs with a 4-base codon and has a sequence consisting of 4 bases as an anticodon, in which a single base A, G, C, or U has been inserted just before the CCA sequence at the 3' end.

[15] The mutant tRNA which pairs with a 4-base codon according to [14], which has CCCG as an anticodon and pairs with a 4-base codon CGCG.

[16] The mutant tRNA which pairs with a 4-base codon according to [14] or [15], which is a mutant tRNA for phenylalanine.

[17] The mutant tRNA which pairs with a 4-base codon according to any one of [14] to [16], which is a yeast-derived tRNA.

[18] A mutant tRNA which pairs with a 4-base codon, which is a yeast-derived mutant tRNA comprising the base sequence represented by SEQ ID NO: 40.

[19] The mutant tRNA which pairs with a 4-base codon according to any one of [14] to [18], which is aminoacylated with an amino acid.

[20] The mutant tRNA which pairs with a 4-base codon according to [19], wherein the amino acid is an unnatural amino acid, a modified amino acid, or a derivative of either thereof.

[21] The mutant tRNA which pairs with a 4-base codon according to [20], wherein the amino acid derivative is selected from the group consisting of hydroxy acid, mercapto acid, and carboxylic acid.

[22] The mutant tRNA which pairs with a 4-base codon according to any one of [19] to [21], wherein the amino acid is fluorescent-labeled.

[23] A method for introducing a desired amino acid into a protein, wherein an amino acid is introduced into a protein in which mRNA of the protein into which an amino acid is introduced, the mRNA having a 4-base codon that is corresponding to a site at which the amino acid is introduced) and the mutant tRNA which pairs with a stop codon according to any one of [14] to [22] are used and the mutant tRNA is allowed to pair with the 4-base codon.

[24] A method for producing a protein comprising a fluorescent-labeled amino acid labeled with a fluorescent substance that serves as an energy donor for fluorescence resonance energy transfer and a fluorescent-labeled amino acid labeled with a fluorescent substance that serves as an energy acceptor for fluorescence resonance energy transfer, such protein comprising the two different fluorescent-labeled amino acids located at positions on the protein at which the distance between the fluorescent substance that serves as an energy donor and the fluorescent substance that serves as an energy acceptor and their orientation relative to each other are changed due to changes in the protein conformation caused by the binding between the protein and a molecule capable of binding to a protein,

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resulting in changes in the efficiency of fluorescence resonance energy transfer, such method comprising the steps of:

preparing mRNA of the protein into which a single 4-base codon and a single stop codon have been inserted, in which the positions on the mRNA at which a 4-base codon and the stop codon are inserted correspond to the positions on the protein such that the distance and the orientation of the fluorescent substances are changed due to interaction between the protein and other molecules, resulting in changes in the efficiency of fluorescence resonance energy transfer;

preparing two different amino acids separately labeled with two types of fluorescent substances that can cause fluorescence resonance energy transfer, which are an amino acid bound to the tRNA having an anticodon that pairs with a 4-base codon and an amino acid bound to the tRNA according to any one of [1] to [12], respectively; and

synthesizing the protein with the use of the mRNA and the amino-acid-binding tRNA.

[25] The method for producing a protein according to [24], wherein the tRNA comprising an anticodon corresponding to a 4-base codon is the mutant tRNA which pairs with a 4-base codon according to any one of [14] to [22].

[26] The method according to [13], wherein protein synthesis is carried out in a cell-free translation system.

[27] The method according to [23], wherein protein synthesis is carried out in a cell-free translation system.

[28] The method according to [24], wherein protein synthesis is carried out in a cell-free translation system.

[29] The method according to [25], wherein protein synthesis is carried out in a cell-free translation system.

This description includes part or all of the contents as disclosed in the description of Japanese Patent Application No. 2005-329115, which is a priority document of the present application.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the outline of a method for producing fluorescent-labeled amino acid-tRNA.

FIG. 2 shows the outline of a method for evaluating the introduction of fluorescent-labeled amino acid.

FIG. 3A shows the structures of E. coli-derived tRNAs.

FIG. 3B shows the structures of E. coli-derived tRNAs.

FIG. 3C shows the structures of E. coli-derived tRNAs.

FIG. 3D shows the structures of E. coli-derived tRNAs.

FIG. 3E shows the structures of E. coli-derived tRNAs.

FIG. 3F shows the structures of E. coli-derived tRNAs.

FIG. 3G shows the structures of E. coli-derived tRNAs.

FIG. 3H shows the structures of E. coli-derived tRNAs.

FIG. 4 shows a fluorescence image of SDS-PAGE in the case involving the addition of tRNA having a fluorescent-labeled amino acid. The image indicates the results of introduction of fluorescent-labeled amino acid into UAG with the use of an E. coli-derived mutant tRNA.

FIG. 5 shows the structure of a mutant tRNA for E. coli-derived tryptophan.

FIG. 6A is an image showing the results of fluorescence imaging of SDS-PAGE in the case involving the use of tRNA to which no fluorescent-labeled amino acid was added. The image indicates the results of introduction of fluorescent-labeled amino acid into UAG with the use of a mutant tRNA for E. coli-derived tryptophan.

FIG. 6B is an image showing the results of Western blotting in the case involving the use of tRNA having a fluorescent-labeled amino acid. The image indicates the results of intro-

duction of fluorescent-labeled amino acid into UAG with the use of a mutant tRNA for *E. coli*-derived tryptophan.

FIG. **6C** is a graph showing fluorescence band intensities of proteins subjected to introduction with the use of the individual mutants. The graph indicates the results of introduction of fluorescent-labeled amino acid into UAG with the use of a mutant tRNA for *E. coli*-derived tryptophan.

FIG. **7A** shows the structures of *Mycoplasma capricolum*-derived tRNAs.

FIG. **7B** shows the structures of *Mycoplasma capricolum*-derived tRNAs.

FIG. **7C** shows the structures of *Mycoplasma capricolum*-derived tRNAs.

FIG. **7D** shows the structures of *Mycoplasma capricolum*-derived tRNAs.

FIG. **7E** shows the structures of *Mycoplasma capricolum*-derived tRNAs.

FIG. **7F** shows the structures of *Mycoplasma capricolum*-derived tRNAs.

FIG. **7G** shows the structures of *Mycoplasma capricolum*-derived tRNAs.

FIG. **7H** shows the structures of *Mycoplasma capricolum*-derived tRNAs.

FIG. **8** is an image showing the results of introduction of fluorescent-labeled amino acid into UAG with the use of a *Mycoplasma capricolum*-derived mutant tRNA.

FIG. **9** shows the structure of a mutant tRNA for *Mycoplasma capricolum*-derived tryptophan.

FIG. **10A** is an image showing the results of fluorescence imaging in the case involving the use of tRNA to which no amino acid was added and tRNA to which a fluorescent-labeled amino acid was added. The image indicates the results of introduction of fluorescent-labeled amino acid into UAG with the use of a mutant tRNA for *Mycoplasma capricolum*-derived tryptophan. In FIG. **10A**, "BFLAF" denotes tRNA to which a fluorescent-labeled amino acid was added and "no AA" denotes tRNA to which no amino acid was added.

FIG. **10B** is a graph showing fluorescence band intensities of proteins subjected to introduction with the use of the individual mutants. The graph indicates the results of introduction of fluorescent-labeled amino acid into UAG with the use of a mutant tRNA for *Mycoplasma capricolum*-derived tryptophan.

FIG. **11A** shows the structures of mutant tRNAs (G1-C72, A73) for tryptophan derived from a different organism.

FIG. **11B** shows the structures of mutant tRNAs (G1-C72, A73) for tryptophan derived from a different organism.

FIG. **12A** is an image showing the results of fluorescent-labeled amino acid introduction into UAG with the use of mutant tRNAs (G1-C72, A73) for tryptophan derived from a different organism.

FIG. **12B** is an image showing the results of fluorescent-labeled amino acid introduction into UAG with the use of mutant tRNAs (G1-C72, A73) for tryptophan derived from a different organism.

FIG. **13A** is an image showing the results of fluorescence imaging in the case involving the addition of tRNA to which no amino acid was added and tRNA to which a fluorescent-labeled amino acid was added. The image indicates the results of fluorescent-labeled amino acid introduction into UAG with the use of mutant tRNAs (G1-C72, A73) for tryptophan derived from a different organism. In FIG. **13A**, "BFLAF" denotes tRNA to which a fluorescent-labeled amino acid was added and "no AA" denotes tRNA to which no amino acid was added.

FIG. **13B** is a graph showing fluorescence band intensities of proteins subjected to introduction with the use of the indi-

vidual mutants. The graph indicates the results of fluorescent-labeled amino acid introduction into UAG with the use of mutant tRNAs (G1-C72, A73) for tryptophan derived from a different organism.

FIG. **14A** shows the outline of a method for double introduction of fluorescent-labeled amino acids into a maltose binding protein (MBP) based on the 4-base codon method.

FIG. **14B** is an image showing the results of double introduction of fluorescent-labeled amino acids into a maltose binding protein (MBP) based on the 4-base codon method.

FIG. **15A** shows the outline of a method for double introduction of fluorescent-labeled amino acids into a maltose binding protein (MBP) based on the 4-base codon method and the stop codon method with the use of the mutant tRNA of the present invention. In the method, no fluorescent-labeled amino acid is introduced into a non-UAG site.

FIG. **15B** is an image showing the results of double introduction of fluorescent-labeled amino acids into a maltose binding protein (MBP) based on the 4-base codon method and the stop codon method with the use of the mutant tRNA of the present invention.

FIG. **16A** shows a chemical structure of a fluorescent-labeled amino acid having TAMRA.

FIG. **16B** is an image showing the results of fluorescence imaging in the case involving the use of tRNA having a fluorescent-labeled amino acid. The image shows the results of introduction of a fluorescent-labeled amino acid having TAMRA into UAG with the use of a mutant tRNA for *Mycoplasma capricolum*-derived tryptophan and introduction of a fluorescent-labeled amino acid having TAMRA into CGGG with the use of a mutant tRNA for yeast-derived phenylalanine.

FIG. **16C** is a graph showing fluorescence band intensities of proteins subjected to introduction. The graph indicates the results of introduction of a fluorescent-labeled amino acid having TAMRA into UAG with the use of a mutant tRNA for *Mycoplasma capricolum*-derived tryptophan and introduction of a fluorescent-labeled amino acid having TAMRA into CGGG with the use of a mutant tRNA for yeast-derived phenylalanine.

FIG. **17** shows the structure of a mutant tRNA for *Mycoplasma capricolum*-derived tryptophan into which a single base has been inserted just before the CCA sequence at the 3' end.

FIG. **18A** is an image showing the results of fluorescence imaging in the case involving the use of tRNA having a fluorescent-labeled amino acid. The image indicates the results of fluorescent-labeled amino acid introduction into UAG with the use of a mutant tRNA for *Mycoplasma capricolum*-derived tryptophan into which a single base was inserted just before the CCA sequence at the 3' end.

FIG. **18B** is a graph showing fluorescence band intensities of proteins subjected to introduction with the use of the individual mutants. The graph indicates the results of fluorescent-labeled amino acid introduction into UAG with the use of a mutant tRNA for *Mycoplasma capricolum*-derived tryptophan into which a single base was inserted just before the CCA sequence at the 3' end.

FIG. **19A** is an image showing the results of Western blot with an anti-fluorescent substanceylated tyrosine antibody in the case involving the use of tRNA having fluorescent substanceylated tyrosine. The image indicates the results of introduction of fluorescent substanceylated tyrosine with the use of a mutant tRNA for *Mycoplasma capricolum*-derived tryptophan into which a single base was inserted just before the CCA sequence at the 3' end and streptavidin mRNA in which the 2nd site was substituted with UAG.

FIG. **19**B is a graph showing Western blot band intensities of proteins subjected to introduction with the use of the individual mutants. The graph indicates the results of introduction of fluorescent substanceylated tyrosine with the use of a mutant tRNA for *Mycoplasma capricolum*-derived tryptophan into which a single base was inserted just before the CCA sequence at the 3' end and streptavidin mRNA in which the 2nd site was substituted with UAG.

FIG. **20**A is an image showing the results of Western blot of tRNA having fluorescent substanceylated tyrosine with the use of an anti-T7tag antibody. The image indicates the results of introduction of fluorescent substanceylated tyrosine with the use of a mutant tRNA for *Mycoplasma capricolum*-derived tryptophan into which a single base was inserted just before the CCA sequence at the 3' end and streptavidin mRNA having T7tag at the N terminal, in which the 83rd site was substituted with UAG.

FIG. **20**B is a graph showing Western blot band intensities of proteins subjected to introduction with the use of the individual mutants. The graph indicates the results of introduction of fluorescent substanceylated tyrosine with the use of a mutant tRNA for *Mycoplasma capricolum*-derived tryptophan into which a single base was inserted just before the CCA sequence at the 3' end and streptavidin mRNA having T7tag at the N terminal, in which the 83rd site was substituted with UAG.

FIG. **21** shows the structure of a mutant tRNA for yeast-derived phenylalanine having an anticodon comprising 4 bases (CCCG) into which a single base has been added just before the CCA sequence at the 3' end (73.1A, 73.1C, 73.1G, 73.1U).

FIG. **22**A is an image showing the results of fluorescence imaging in the case involving the use of tRNA having a fluorescent-labeled amino acid. The image indicates the results of introduction of a fluorescent-labeled amino acid into a 4-base codon CGGG (introduced at the 83rd site of streptavidin) with the use of a mutant tRNA for yeast-derived phenylalanine into which a single base was inserted just before the CCA sequence at the 3' end.

FIG. **22**B is a graph showing fluorescence band intensities of proteins subjected to introduction with the use of the individual mutants. The graph indicates the results of introduction of fluorescent-labeled amino acid for a 4-base codon CGGG (introduced at the 83rd site of streptavidin) with the use of a mutant tRNA for yeast-derived phenylalanine into which a single base was inserted just before the CCA sequence at the 3' end.

FIG. **23**A is an image showing the results of fluorescence imaging in the case involving the use of tRNA having a fluorescent-labeled amino acid. The image indicates the results of introduction of a fluorescent-labeled amino acid having TAMRA with the use of a mutant tRNA for yeast-derived phenylalanine into which a single base was inserted just before the CCA sequence at the 3' end and streptavidin mRNA having a T7tag sequence at the N terminal, into which CGGG was introduced just after the T7tag sequence.

FIG. **23**B is a graph showing fluorescence band intensities of proteins subjected to introduction with the use of the individual mutants. The graph indicates the results of introduction of fluorescent-labeled amino acid having TAMRA with the use of a mutant tRNA for yeast-derived phenylalanine into which a single base was inserted just before the CCA sequence at the 3' end and streptavidin mRNA having a T7tag sequence at the N terminal, into which CGGG was introduced just after the T7tag sequence.

BEST MODE FOR CARRYING OUT THE INVENTION

The tRNA of the present invention is tRNA for tryptophan, which is a mutant tRNA that has CUA as an anticodon and pairs with a stop codon. Such mutant tRNA can be obtained by modifying tRNA for microorganism-derived tryptophan such that it has CUA as an anticodon. Preferred examples of a relevant microorganism include *E. coli*, *Mycoplasma capricolum*, *Bacillus halodouranns*, *Bacillus subtillis*, *Borrelia burgdorferi*, *Mycoplasma genitalium*, *Mycoplasma pneumoniae*, and *Staphylococcus aureus* N315. FIGS. **3**A to **3**H and **7**A to **7**H show the structures of naturally occurring tRNAs for *E. coli* or *Mycoplasma capricolumn* tryptophan. Among the above examples, an *E. coli* or *Mycoplasma capricolum*-derived mutant tRNA is preferable. In particular, a *Mycoplasma capricolum*-derived mutant tRNA is preferable. When a known tRNA having CUA as an anticodon is used, such tRNA competes with a termination factor, resulting in poor introduction efficiency. Meanwhile, when an unnatural amino acid such as a labeled amino acid is allowed to bind to the above tRNA of the present invention so as to be introduced into a protein, the tRNA does not compete with a termination factor, and thus the unnatural amino acid can be efficiently introduced into the protein.

Further, the tRNA of the present invention is the above tRNA for tryptophan, including a mutant obtained by inducing a further mutation in a mutant tRNA that has CUA as an anticodon and pairs with a stop codon. When a base at the 5' end of the tRNA base sequence is substituted with G so that the resulting tRNA has a cloverleaf model structure, such mutant is a mutant in which a base pairing with the G at the 5' end has been substituted with C and a base next to the C on the 3' side has been substituted with A. In general, the base pairing with the G at the 5' end is the 72nd base from the 5' end. That is, in a typical example of the mutant tRNA of the present invention, the 72nd base is substituted with C and the 73rd base is substituted with A or G. In such a case, a mutant tRNA in which the 73rd base has been substituted with A is particularly preferable. According to the present invention, a mutant tRNA obtained by substitution of a base at the 5' end with G, the 72nd base with C, and the 73rd base with A or G is referred to as "G1-C72, A73" or "G1-C72, G73." When an unnatural amino acid such as a labeled amino acid is allowed to bind to such mutant tRNA so as to be introduced into a protein, the efficiency of introduction of such unnatural amino acid is further increased. Moreover, in the case of G1-C72, A73, no natural amino acid is added to tRNA, and thus the efficiency of introduction of such unnatural amino acid is further increased.

Examples of the tRNA of the present invention include tRNAs for tryptophan derived from *Mycoplasma capricolum*, *E. coli*, *Bacillus halodouranns*, *Bacillus subtillis*, *Borrelia burgdorferi*, *Mycoplasma genitalium*, *Mycoplasma pneumoniae* 1, *Mycoplasma pneumoniae* 2, and *Staphylococcus aureus* N315, such tRNA being a mutant tRNA that pairs with a stop codon and has CUA as an anticodon, in which G is located at the 5' end, C (pairing with the G at the 5' end) is the 72nd base from the 5' end, and A (being next to the C on the 3' side) is the 73rd base from the 5' end. The individual sequences are represented by SEQ ID NOS: 1, 2, 4, 5, 6, 18, 19, 20, and 25. In addition, FIG. **5** shows the structure of tRNA for *E. coli*-derived tryptophan. FIG. **9** shows the structure of tRNA for *Mycoplasma capricolum*-derived tryptophan. FIGS. **5** and **9** show that a base at the 5' end is substituted with G, the 72nd base that pairs with the base at the

5' end is substituted with C, and the base on the 3' side of the base substituted with C is substituted with a different base.

In addition to the above, the tRNA of the present invention includes a mutant into which a single base (A, G, C, or U) has been inserted just before the CCA sequence at the 3' end. The above tRNA of the present invention has a single base positioned just before the CCA sequence at the 3' end (i.e., the 73rd base in general), which does not pair with a base at the 5' end. A mutant tRNA into which a single base (A, G, C, or U) has been inserted just before the CCA sequence at the 3' end includes tRNA in which the 72nd base, in general, is substituted with C, the 73rd base, in general, is substituted with A or G, and A, G, C, or U is inserted as the 74th base. The number of bases at a loop portion or a step portion differs depending on types of tRNA. In some cases, a base pairing with the base at the 5' end is not the 72nd base. Specifically, the base numbers of the above 72nd, 73rd, and 74th bases (at the substituted sites) from the 5' end may differ by 1, 2, 3, 4, or 5 bases, for example. The mutant tRNA of the present invention includes any tRNA having a conformation in which the base at the 5' end and a base paring with the base at the 5' end are substituted and a base next to either one of the bases on the 3' side is substituted, as long as the efficiency of introduction of unnatural amino acid is increased. In other words, the mutant tRNA of the present invention is a mutant tRNA that is generally a typical tRNA such as tRNA for *E. coli-* or *Mycoplasma capricolum*-derived tryptophan, in which a base corresponding to the 72nd base from the 5' end is substituted and a base next to the base on the 3' side (i.e., a base corresponding to the 73rd base at the 5' end of tRNA for *E. coli-* or *Mycoplasma capricolum*-derived tryptophan) is substituted. Herein, a base corresponding to the 72nd base indicates a base at a position at which it pairs with the base at the 5' end. Also, a base corresponding to the 73rd base indicates a base next to the 72nd on the 3' side.

According to the present invention, a mutant tRNA into which a single base has been inserted just before the CCA sequence is designated as 73.1A, 73.1C, 73.1G, or 73.1U (provided that A, C, G, or U represents the type of inserted base). When an unnatural amino acid such as a labeled amino acid is allowed to bind to a mutant tRNA so as to be introduced into a protein, the efficiency of introduction of unnatural amino acid becomes high.

An example of the mutant tRNA of the present invention is a mutant tRNA for *Mycoplasma capricolum*-derived tryptophan into which a single base has been inserted just before the CCA sequence.

In addition, the mutant tRNA of the present invention includes a mutant tRNA paring with a 4-base codon, which is used for introduction of an unnatural amino acid with the use of a 4-base codon CGGG. For instance, such mutant tRNA is a mutant tRNA for yeast-derived phenylalanine having an anticodon comprising 4 bases (CCCG) into which a single base (A, C, G, or U) has been inserted just before the CCA sequence at the 3' end.

The above mutant tRNA can be synthesized based on sequence information. In addition, the above mutant tRNA can be produced by introducing a mutation into a tRNA such as the aforementioned tRNA for microorganism-derived tryptophan. A mutant can be produced using a conventional genetic engineering technique.

For instance, a mutation can be introduced into a gene with the use of a mutagenesis kit utilizing site-directed mutagenesis (such as Mutant-K (TAKARA) or Mutant-G (TAKARA)), an LA PCR in vitro Mutagenesis series kit (TAKARA), or the like according to a conventional method

such as the Kunkel method or the Gapped duplex method, or according to a method based on such a method.

The tRNA of the present invention further includes an aminoacyl tRNA obtained by aminoacylating the above tRNA with an amino acid or an analog thereof. Examples of an amino acid or an analog thereof used for aminoacylation include, but are not limited to, natural amino acids, unnatural amino acids, and derivatives thereof. In addition, modified amino acids generated through post-translational modification such as fluorescent substanceylation, methylation, or acetylation are also included.

The term "unnatural amino acid" refers to any artificial compound having an amino skeleton in a single molecule thereof, which does not naturally exist. Such a compound can be prepared by allowing a different labeled compound to bind to an amino acid skeleton. Such an "amino acid skeleton" comprises a carboxyl group, an amino group, and a portion binding therebetween in an amino acid.

An example of an unnatural amino acid is a "labeled amino acid," which is an amino acid bound to a labeled compound. For instance, such unnatural amino acid is an amino acid obtained by allowing a labeled compound to bind to an amino acid having an amino acid skeleton comprising an aromatic ring such as a benzene ring on a side chain. In view of functions, photoresponsive amino acids, photoswitch amino acids, fluorescent probe amino acids, fluorescent-labeled amino acids, and the like can be used.

Examples of derivatives of such amino acids include hydroxy acid, mercapto acid, and carboxylic acid.

Labeled compounds used in the present invention are dye compounds, fluorescent substances, chemiluminescent/bioluminescent substances, enzyme substrates, coenzymes, antigenic substances, and protein binding substances, which are known to persons skilled in the art.

Preferably, fluorescent-labeled compounds have an excitation wavelength in the visible light range (approximately 400 to 700 nm). Further preferably, such compounds have a luminescence wavelength in the visible light range. Particularly preferably, such compounds have a strong luminescence intensity in a water solution.

A chemiluminescent or bioluminescent substance such as luciferin or Lumigen or a derivative thereof can be used as a labeled compound in the present invention.

Further, a compound can be adequately selected from among coenzymes, antigenic substances, substances known to bind to specific proteins, and the like depending on desired functions to be imparted to a target protein so that such compound can be used as a labeled compound in the present invention. For instance, when a substrate for a specific enzyme (e.g., a substrate for alkaline phosphatase, β-galactosidase, or the like) is introduced, detection can be carried out with the use of a color reaction of the enzyme. In addition, a protein labeled with an antigenic protein or a substance that is known to bind to a specific protein can be used for an indirect detection method using an antibody or a protein that binds thereto, which is advantageous in terms of the ease of purification. For instance, a functional protein labeled with biotin by the method of the present invention has a function of binding to avidin streptavidin via biotin. With the use of such function, it becomes possible to establish a system for detection of a specific substance with the use of the binding to avidin or streptavidin labeled by the method of the present invention or with a chemiluminescent compound or the like. In addition to the above examples, persons skilled in the art would be able to understand that it is possible to use, as a labeled compound, a substance that can be detected with the

use of a variety of dyes and different biochemical, chemical, and immunochemical detection methods.

An amino acid is allowed to bind to a labeled compound directly or via a spacer. The term "spacer" indicates a portion that binds between an amino acid portion of a labeled amino acid molecule and a labeled compound. Specifically, an amino acid side chain of a labeled amino acid molecule does not directly bind to a labeled compound. It is thought that when at least one atom exists between an amino acid side chain and a labeled compound, an amino acid portion of the labeled amino acid binds to the labeled compound via a spacer. A spacer may comprise at least one atom of at least one of C, O, N, and S on the main chain thereof. In addition, the main chain structure of a spacer comprises 2 to 10 atoms, preferably 3 to 8 atoms, and more preferably 5, 6, or 7 atoms, which are the above atoms bound to each other in a linear chain. Such linear chain structure contains at least one double bond. Further, a spacer may have 1 to several, preferably 1 to 5, and more preferably 1 to 3 rings such as a benzene ring and/or a cyclohexyl ring. In addition, a spacer may have a ring structure comprising a benzene ring or a cyclohexyl ring or a structure obtained by combining a ring structure and the above linear chain structure. Specific examples of a spacer include: polyolefins such as polyethylene, polypropylene, polyisoprene, polystyrene, polyvinyl, and polyvinyl chloride; polyethers such as polyoxyethylene, polyethylene glycol, and polyvinyl alcohol; polyamide; polyester; polyimide; poly-urthane; and polycarbonate.

Depending on types of labeled compound, it is advantageous to allow an amino acid to bind to a labeled compound via a spacer such that the functions of the labeled compound can be more effectively exhibited in the protein into which the labeled compound has been introduced. For instance, it is considered that, depending on types of labeled compound, steric hindrance in the protein into which the labeled compound has been introduced is more attenuated in the case of binding via a spacer.

Further, it is preferable that the labeled amino acid of the present invention have an aromatic ring on a side chain of an amino acid portion and that a labeled compound bind to the aromatic ring directly or via a spacer.

The term "aromatic ring" used herein generally refers to any unsaturated ring compound. Thus, such compound includes 5- or 6-membered heterocyclic aromatic rings and polycyclic compounds having a structure comprising at least 2 rings, preferably 2 to 5 rings, and more preferably 2 to 3 rings. Particularly preferably, an aromatic ring is a benzene ring. Among natural amino acids, phenylalanine, tryptophan, and tyrosine are natural aromatic amino acids having an aromatic ring on a side chain. An unnatural amino acid derived from such natural aromatic amino acid in which a labeled compound binds to the aromatic ring (directly or via a spacer) is a preferred example of the unnatural amino acid of the present invention.

The binding between an amino acid having an aromatic ring and a labeled compound (directly or via a spacer) may be binding between adequate functional groups. A labeled compound is allowed to bind directly or via a spacer to a different functional group of a natural or unnatural amino acid that is not involved in a peptide elongation reaction upon protein synthesis, such functional group being selected from the group consisting of an amino group, a thiol group, a carboxyl group, a hydroxyl group, an aldehyde group, an allyl group, and a halogenated alkyl group. Examples of a probe used for labeling of an amino group that can be used include compounds such as succinimide ester, isothiocyanate, sulfonyl-chloride, NBD-halide, and dichlorotriazine. Examples of a

probe used for labeling of a thiol group that can be used include compounds such as alkyl halide, maleimide, and aziridine. Examples of a probe used for labeling of a carboxyl group that can be used include a diazomethane compound, aliphatic bromide, and carbodiimide. For instance, succinimide ester is introduced into a labeled compound directly or via a spacer and an amino group is introduced into an aromatic ring of an amino acid. In such a case, the labeled compound and the amino acid can be bound via an amide bond. Examples of an amino acid having an amino group that has been introduced into an aromatic ring include aminophenylalanine. A functional group used in the above case can be adequately selected and introduced. Also, the binding method can be adequately selected. In such a case, it is possible to allow an amino group on a side chain of aminophenylalanine to selectively react by carrying out an amide bond formation reaction at approximately pH 5 even in the presence of another amino group. Alternatively, the other amino groups are protected by butyloxycarbonylation (Boc: butyloxycarbonyl) or the like. In such a case, de-butyloxycarbonylation (Boc: butyloxycarbonyl) can be carried out, followed by a reaction. For such technique, for example, descriptions in "New Biochemistry Experimentation Course (Shin Seika-gaku Jikken Koza) 1, protein VI, Structure-Function Correlation (Kozo-Kino Sokan)" and the like can be referred to.

An aromatic ring may bind to an atom forming an amino acid skeleton directly or indirectly via 1, 2, or 3 atoms of at least one of C, O, N, and S. When an aromatic ring is a benzene ring, the position on a benzene ring at which a spacer or a labeled compound binds to an amino acid is a para position or a meta position in the amino acid skeleton such that the efficiency of incorporation into a ribosome becomes higher, which is preferable. Particularly preferably, the position is a para position.

As described above, a labeled compound is allowed to bind to a functional group of an aromatic ring directly or via a spacer. In the case of aminophenylalanine, paraminophenylalanine and meta-aminophenylalanine are preferably used.

In order to allow an amino acid to bind to tRNA so as to produce aminoacyl tRNA, it is necessary to allow a specific group to first bind to an amino acid. For instance, when dinucleotide (pdCpA) is allowed to first bind to a carboxyl group of an amino acid, it is possible to produce an artificial aminoacyl tRNA by allowing tRNA (tRNA(-CA)) lacking a CA dinucleotide at the 3' end to bind thereto. Such artificial aminoacyl tRNA can be produced in accordance with descriptions in WO2004/009709 and the like. For instance, it is possible to produce aminoacyl pdCpA by a method comprising protecting an amino acid α amino group with a Boc group and an amino acid side chain functional group with Boc or OtBoc, allowing Boc-amino acid to be subjected to cyanomethyl esterification, and carrying out a reaction with pdCpA, or by a method comprising carrying out a reaction between Boc-amino acid and pdCpA with the use of the condensing agent carbonyldiimidazole (CDI). Binding with tRNA(-CA) may be carried out with the use of T4 RNA ligase.

It is possible to introduce an amino acid or a derivative thereof into a protein with the use of the tRNA of the present invention by synthesizing a protein in an intracellular or cell-free translation system with the use of DNA encoding the protein into which an amino acid or a derivative thereof is introduced, such DNA having a stop codon TAG that has been introduced at a codon position corresponding to a position for introduction of an amino acid or a derivative thereof, and the aminoacyl tRNA of the present invention to which an amino acid or a derivative has been bound.

Synthesis in a cell-free translation system can be carried out by mixing in vitro an expression vector containing a gene to be expressed with a necessary reagent so as to allow the gene to be expressed without introducing the expression vector into a host cell (Spirin, A. S. et al., (1988) "A continuous cell-free translation system capable of producing polypeptides in high yield" Science 242, 1162; Kim, D. M. et al., (1996) "A highly efficient cell-free protein synthesis system from *E. coli*" Eur. J. Biochem. 239, 881-886). In some cases, the term "cell-free protein synthesis system" merely indicates a cell-free translation system in which genetic information contained in mRNA is decoded such that protein synthesis is carried out in a ribosome. Also, in some cases, the term indicates both a cell-free transcription system in which RNA synthesis is carried out using DNA as a template and the above cell-free translation system. In a cell-free translation system, an organism extract is used. An organism extract contains components necessary for protein synthesis. Examples of such components include ribosomes, 20 types of aminoacyl tRNA synthases, methionyl-tRNA transformylase, three types of translation initiation factors (IF1, IF2, and IF3), three types of translation elongation factors (EF-G, EF-Tu, and EF-Ts), three types of translation termination factors (RF1, RF2, and RF3), a ribosome recycling factor (RRF), and RNA polymerase. In addition to the above examples, another protein may be added for efficient translation. Persons skilled in the art can determine a protein that can be added for further efficient translation. Examples of an organism extract that can be used include *E. coli*-derived organism extracts, wheat germ-derived organism extracts, rabbit reticulocyte-derived organism extracts, animal cell-derived organism extracts, and insect cell-derived organism extracts. An organism extract can be obtained by, for example, disruption using a French press or glass beads. An example of *E. coli*-derived microorganism extract is an S30 extract, which can be obtained by, for example, the method of Pratt et al. (Pratt, Transcription and Translation—a practical approach, Henes, B. D. and Higgins, S. J. ed., IRL Press, Oxford, 179-209 [1984]). An S30 extract contains ribosome, 20 types of aminoacyl tRNA synthases, methionyl-tRNA transformylase, three types of translation initiation factors (IF1, IF2, and IF3), three types of translation elongation factors (EF-G, EF-Tu, and EF-Ts), three types of translation termination factors (RF1, RF2, and RF3), a ribosome recycling factor (RRF), and the like. In addition to the above organism extract, a cell-free protein synthesis system may contain an ATP regenerating system, a plasmid containing a promoter and a nucleic acid encoding a protein to be expressed (or mRNA encoding a protein to be expressed), tRNA, RNA polymerase, an RNase inhibitor, an energy source such as ATP, GTP, CTP, or UTP, a buffer, amino acids, salts, antimicrobial agents, and the like. The concentration of each component can be adequately determined.

An ATP regenerating system is not limited, and thus a combination of a known phosphate donor and a known kinase can be used. Examples of such a combination include a combination of phosphoenolpyruvate (PEP) and pyruvate kinase (PK), a combination of creatine phosphate (CP) and creatine kinase (CK), and a combination of acetyl phosphate (AP) and acetate kinase (AK). An ATP regenerating system produced based on such a combination may be added to a cell-free protein synthesis system.

It is necessary for a cell-free protein synthesis system to contain mRNA encoding a protein to be produced. For such mRNA, the cell-free protein synthesis system may contain a system that transcribes a nucleic acid into a cell-free protein synthesis system; that is to say, a system that produces mRNA

encoding the protein. In such case, DNA is added. In addition, it is also possible to separately synthesizing mRNA via transcription or the like from template DNA with the use of T7 DNA polymerase or the like so as to add the obtained mRNA to the cell-free protein synthesis system of the present invention. mRNA can be produced with the use of a plasmid containing an adequate promoter and DNA encoding a protein to be produced, such DNA being located downstream of the promoter, and RNA polymerase that acts on the promoter. The plasmid used herein is not limited. A known plasmid can be used by introducing an adequate promoter, a ribosome binding site, and the like thereinto by a known gene engineering technique. Persons skilled in the art can adequately select a plasmid used in the present invention or design and construct such plasmid by themselves. A promoter that can be used may be an endogenous promoter contained in a microorganism used in a cell-free protein synthesis system or an exogenous promoter. Examples of a promoter that can be preferably used include the above Trc promoter, T7 promoter, and Tac promoter, which are superior in terms of efficiency.

A protein can be expressed with the use of a commercially available cell-free expression kit. Examples of such kit include the Rapid Translation System (RTS) (Roche) and the Expressway In Vitro Protein Synthesis System (Invitrogen). In such case, an expression vector to be used is not limited, and thus a vector available for a cell-free translation system can be used. An example of an expression vector for the former kit is pIVEX2.2bNde. Examples of an expression vector for the latter kit are pEXP1 and pEXP2.

With the use of the tRNA of the present invention, it is possible to introduce an unnatural amino acid such as fluorescent substance-labeled amino acid into an arbitrary site on a protein such that a fluorescent-labeled protein can be produced. For instance, PDIPY FL-aminophenylalanine is introduced into the Tyr84 site of streptavidin, such that fluorescent-labeled streptavidin can be produced.

In addition, interaction between a protein and other molecules can be detected by introducing fluorescent-labeled amino acids that can serve as a donor and an acceptor for fluorescence resonance energy transfer into two arbitrary sites on a protein. In such case, either one of fluorescent-labeled amino acids may be introduced by the 4-base codon method. The 4-base codon method can be carried out in accordance with descriptions in Hohsaka T., et al., J. Am. Chem. Soc., 118, 9778-9779, 1996 and Hohsaka T., et al., J. Am. Chem. Soc., 121, 34-40, 1999. Examples of such protein include any protein that interacts molecules so as to cause changes in conformation. Examples of such protein include calmodulin, cGMP-dependent protein kinase, a steroid hormone acceptor, a ligand binding domain of a steroid hormone acceptor, protein kinase C, an inositol-1,4,5-triosephosphate acceptor, recoverin, maltose binding protein, and DNA binding protein. A molecule that interacts with a protein is specified for each protein. Examples of such molecules include organic or inorganic low-molecular-weight molecules such as proteins, nucleic acids, sugars, and ions. In a case in which a protein is calmodulin, examples of such molecule include a calmodulin binding protein and a calcium ion. In addition, an example of a cGMP-dependent protein kinase is cGMP and an example of a maltose binding protein is maltose. Further, in the case of a steroid hormone acceptor or a DNA binding protein, a steroid hormone or DNA specific to the relevant protein is used. Examples of a substance that can serve as an energy donor and a substance that can serve as an energy acceptor for fluorescence resonance energy transfer include any known fluorescent substances such as BODIPY compounds, rhodamine (TAMRA), fluorescein (FITC), Texas

Red, acridine orange, Cyber Green, Cy3, Cy5, and derivatives thereof. Fluorescent substances that can serve as an energy donor and a fluorescent substance that can serve as an energy acceptor for fluorescence resonance energy transfer may be selected in a manner such that the excitation spectrum of a substance that serves as an energy acceptor overlaps the radiation spectrum of a substance that serves as an energy donor. For instance, BODIPY (registered trademark) FL and BODIPY (registered trademark) 558/568 can be selected as an energy donor and an energy acceptor, respectively. Alternatively, a combination of an energy donor and an energy acceptor can be BODIPY FL and BODIPY 576/586, BODIPY FL and TAMRA, BODIPY FL and Cy3, Fluorescein and BODIPY 558/568, Alexa488 and BODIPY 558/568, BODIPY 558/568 and Cy5, or the like.

In the case of the protein of the present invention, it is necessary for the protein to contain an energy donor and an energy acceptor, which are located at positions relative to each other such that an orientation appropriate for induction of fluorescence resonance energy transfer can be maintained when the protein conformation is changed.

For such purpose, a fluorescent-labeled amino acid that is labeled with a fluorescent substance is introduced into a specific site in a protein. The position at which a fluorescent-labeled amino acid is introduced differs depending on the protein into which the fluorescent-labeled amino acid is introduced. The protein conformation is analyzed based on the amino acid sequence of a natural protein and then fluorescent-labeled amino acids are introduced into amino acid sites that are located at positions in the vicinity of each other in the protein conformation. The protein conformation can be predicted with the use of, for example, commercially available protein conformation analysis software. Alternatively, either an energy donor or an energy acceptor is first introduced at the N or C terminal of a protein and the other is introduced into several to several tens of sites in the protein. Then, an introduction position appropriate for the present invention can be selected from among the above sites.

For instance, when a protein is calmodulin, an amino acid labeled with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY FL) and an amino acid labeled with 4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY 558/568) can be introduced into the 40th base and the N terminal or the 99th base and the N terminal of a calmodulin amino acid sequence, respectively. Meanwhile, when an amino acid labeled with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid and an amino acid labeled with 4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid are introduced into the 113th base and the N terminal or the 148th base and the N terminal of a calmodulin amino acid sequence, respectively, the efficiency of sufficient fluorescence resonance energy transfer is not changed even when a protein interacts with other molecules. With the use of the thus obtained protein of the present invention, interaction between the protein and other molecules can be detected. By allowing the protein of the present invention to interact with other molecules, the conformation of the protein of the present invention is changed such that the distance between an energy acceptor and an energy donor and their orientation relative to each other are changed. When the protein in the above state is irradiated with the excitation light of the energy donor, fluorescence resonance energy transfer (FRET) takes place between the energy donor and the energy acceptor. At such time, the distance between the energy acceptor and the energy donor and their orientation relative to each other are

changed so that FRET efficiency is also changed. Changes in the protein conformation can be detected by measuring such changes.

As a light source for excitation light, a light source obtained by allowing broad-wavelength-range ultraviolet light or visible light to have a desired wavelength range with the use of a filter or a spectrometer can be used. Alternatively, a monochromatic light source such as a laser can be used. When a laser light source is used, a helium-cadmium laser (442 nm) is generally used. However, a blue diode laser (405 nm), an argon ion laser (457 nm), an LD excitation solid laser (diode-pumped solid-state laser) (430 nm), and the like may be used. In the case of the two-photon excitation method, a pulse laser (approximately 800 nm) may be used.

The above proteins correspond to the following proteins:

1. A protein comprising a fluorescent-labeled amino acid labeled with a fluorescent substance that serves as a energy donor for fluorescence resonance energy transfer and a fluorescent-labeled amino acid labeled with a fluorescent substance that serves as an energy acceptor for fluorescence resonance energy transfer,

   such protein comprising the two different fluorescent-labeled amino acids located at positions on the protein at which the distance between the fluorescent substance that serves as a energy donor and the fluorescent substance that serves as an energy acceptor and their orientation relative to each other are changed due to changes in the protein conformation caused by the binding between the protein and a molecule capable of binding to a protein, resulting in changes in the efficiency of fluorescence resonance energy transfer;

2. The protein according to 1 above, wherein either the fluorescent-labeled amino acid labeled with a fluorescent substance that serves as a energy donor for fluorescence resonance energy transfer or the fluorescent-labeled amino acid labeled with a fluorescent substance that serves as an energy acceptor for fluorescence resonance energy transfer protein exists at the N or C terminal and the other exists at a non-N- or non-C-terminal site;

3. The protein according to 1 or 2 above, wherein the amino acids each labeled with a fluorescent substance have been introduced by the 4-base codon method;

4. The protein according to any one of 1 to 3 above, wherein the molecule capable of binding to a protein is selected from the group consisting of proteins, nucleic acids, sugars, and low-molecular-weight molecules;

5. The protein according to any one of 1 to 4 above, wherein the fluorescent substance that serves as an energy donor and the fluorescent substance that serves as an energy acceptor are fluorescent substances which have an excitation wavelength and an emission wavelength, respectively, in the visual light range;

6. The protein according to 5 above, wherein the fluorescent substance that serves as an energy donor and the fluorescent substance that serves as an energy acceptor each are a molecule having a chemical structure comprising 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene as a base structure or a salt or derivative thereof;

7. The protein according to 6 above, wherein the fluorescent substance that serves as an energy donor is 4,4-difluoro-5, 7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid or a salt thereof and the fluorescent substance that serves as an energy acceptor is 4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid or a salt thereof;

8. The protein according to 1 to 7 above, wherein the fluorescent substance that serves as an energy donor and the

fluorescent substance that serves as an energy acceptor bind to an amino group located at the para position of p-aminophenylalanine;

9. The protein according to 1 to 8 above, which is calmodulin;

10. The calmodulin according to 9 above, wherein the amino acids labeled with a fluorescent substance that serves as an energy donor and a fluorescent substance that serves as an energy acceptor are located at positions on the calmodulin in a manner such that the distance therebetween and their orientation relative to each other are changed when the calmodulin interacts with a calmodulin-binding protein and a calcium ion, resulting in changes in the efficiency of fluorescence resonance energy transfer;

11. The calmodulin according to 10 above, wherein the fluorescent substance that serves as an energy donor is 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid or a salt thereof, the fluorescent substance that serves as an energy acceptor is 4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid or a salt thereof, and the amino acids labeled with such fluorescent substances are located at positions on the calmodulin in a manner such that the distance therebetween and their orientation relative to each other are changed when the calmodulin interacts with a calmodulin-binding protein and a calcium ion, resulting in changes in the efficiency of fluorescence resonance energy transfer; and

12. A calmodulin containing 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid or a salt thereof and 4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid or a salt thereof at the 40th base and the N terminal or at the 99th base and the N terminal, respectively, of the calmodulin amino acid sequence.

The present invention is hereafter described in greater detail with reference to the following examples, although the technical scope of the present invention is not limited thereto.

EXAMPLE 1

A Method for Producing Fluorescent-Labeled Amino Acid-tRNA

Three oligonucleotides were chemically synthesized based on individual tRNA base sequences. PCR was carried out using a primer comprising a T7 promoter and a base sequence corresponding to the 1st to 31st bases of tRNA and a primer comprising a base sequence corresponding to the 24th to 46th bases of tRNA (provided that KOD Dash buffer, 0.2 mM dNTP, 1.25 units of KOD Dash, and 50 nmol primers are contained in 50 μl). Thus, a double-strand DNA was obtained. The reaction solution (1 μL) was designated as a template and then PCR was carried out under the same conditions as above with the use of a primer having a T7 promoter sequence (CTAATACGACTCACTATA) (SEQ ID NO: 32) and a primer having a base sequence corresponding to the 39th to 74th bases of tRNA, such that the tRNA(-CA) gene was produced. Purification was carried out using a MinElute PCR Purification kit (QIAGEN).

Next, tRNA(-CA) was synthesized by a transcription reaction. A reaction solution (100 μL) contained 40 mM Tris-HCl (pH 8.0), 20 mM MgCl$_2$, 5 mM DTT, 4 mM NTP, 20 mM GMP, 2 mM spermidine, 10 μg/mL BSA, a ribonuclease inhibitor (40 units), inorganic pyrophosphatase (1 unit), T7 RNA polymerase (400 units), and the tRNA(-CA) gene (10 μg). The reaction solution was subjected to a reaction at 37° C. for 18 hours. tRNA(-CA) was purified by a DEAE-cellulose column (Whatman).

Subsequently, 5× Ligation Buffer (275 mM Hepes-Na pH 7.5, 75 mM MgCl$_2$, 16.5 mM DTT, and 5 mM ATP) (4 μL), 200 μM tRNA(-CA) (2.5 μL), a DMSO solution containing BODIPY FL-aminophenylalanine-pdCpA (2 μL), 0.1% BSA (0.4 μL), T4 RNA Ligase (25 units/μL) (1.2 μL), and water (9.9 μL) were mixed and subjected to a reaction at 4° C. for 2 hours. 3M AcOK (pH 4.5, 10 μL) and water (70 μL) were added thereto. Phenol/chloroform (=1/1) (saturated with 0.3 M AcOK, pH 4.5) was added in a volume equal to that of the resultant, followed by agitation and centrifugation. The upper layer was recovered therefrom and chloroform was added in a volume equal to that of the resultant, followed by agitation and centrifugation. The upper layer was recovered therefrom and ethanol (300 μL) was added thereto. The resultant was lightly mixed and allowed to stand at −20° C. for 1 hour, followed by centrifugation at 15000 rpm and 4° C. for 30 minutes. The supernatant was removed therefrom and 70% EtOH (200 μL) preserved at −20° C. was added thereto, followed by centrifugation at 15000 rpm and 4° C. for 5 seconds. The supernatant was removed therefrom, followed by drying under reduced pressure. The resultant was dissolved in 1 mM potassium acetate (pH 4.5, 2 μL).

EXAMPLE 2

Evaluation of Introduction of Fluorescent-Labeled Amino Acid

A reaction solution (10 μL) was mixed with 55 mM Hepes-KOH (pH 7.5), 210 mM potassium glutamate, 6.9 mM ammonium acetate, 1.7 mM dithiothreitol, 1.2 mM ATP, 0.28 mM GTP, 26 mM phosphoenolpyruvate, 1 mM spermidine, 1.9% polyethylene glycol-8000, 35 μg/mL folic acid, 12 mM magnesium acetate, 20 types of 0.1 mM amino acids, streptavidin mRNA (in which a stop codon UAG had been introduced into the Tyr83 site) (8 μg/μL, an E. coli extract (2 μL) (Promega), and a fluorescent-labeled amino acid-tRNA solution (1 μL). A translation reaction was carried out at 37° C. for 1 hour.

Water (9 μL) and 2× sample buffer (10 μL) were added to a translation reaction solution (1 μL), followed by heating at 95° C. for 5 minutes. The resultant was collected in an amount of 5 μL so as to be subjected to 15% SDS-PAGE. The obtained electrophoresis gel was observed with a fluorescence scanner (FMBIO-III, Hitachi Software Engineering Co., Ltd.). The introduction of fluorescent-labeled amino acid was observed (488 nm excitation/520 nm detection). The introduction efficiency was quantified. In addition, with the use of the same electrophoresis gel, Western blot analysis was carried out using an anti-T7tag antibody (Novagen).

The mRNA sequence of streptavidin obtained by substituting the Tyr83 site with UAG (SEQ ID NO: 33) is shown below. The sequence ranges from a start codon to the original stop codon. The underlined part corresponds to the inserted stop codon.

```
AUGGCUAGCAUGACUGGUGGACAGCAAAUGGGUACCGAAUUCCAUAUG

GACCCGUCCAAGGACUCCAAAGCUCAGGUUUCUGCAGCCGAAGCUGGU

AUCACUGGCACCUGGUAUAACCAACUGGGGUCGACUUUCAUUGUGACC

GCUGGUGCGGACGGAGCUCUGACUGGCACCUACGAAUCUGCGGUUGGU

AACGCAGAAUCCCGCUACGUACUGACUGGCCGUUAUGACUCUGCACCU

GCCACCGAUGGCUCUGGGUACCGCUCUGGGGCUGGACUGUGGCUUGGAAA
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AACAACUAGCGUAAUGCGCACAGCGCCACUACGUGGUCUGGCCAAUAC

GUUGGCGGUGCUGAGGCUCGUAUCAACACUCAGUGGCUGUUAACAUCC

GGCACUACCGAAGCGAAUGCAUGGAAAUCGACACUAGUAGGUCAUGAC

ACCUUUACCAAAGUUAAGCCUUCUGCUGCUAGCAUUGAUGCUGCCAAG

AAAGCAGGCGUAAACAACGGUAACCCUCUAGACGCGUGUUCAGCAACAC

CACCACCACCACCACUAA
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EXAMPLE 3

Introduction of Fluorescent-Labeled Amino Acid into UAG with the Use of an E. coli-Derived Mutant tRNA

22 types of tRNAs each having an E. coli-derived tRNA sequence and comprising CUA as an anticodon as a result of substitution were produced according to the method described in Example 1. Subsequently, the introduction of fluorescent-labeled amino acid was evaluated by the method described in Example 2.

FIG. 4 is a fluorescence image of SDS-PAGE in the case involving the addition of tRNA to which fluorescent-labeled amino acid was added. As shown in FIG. 4, it was found that tRNA for tryptophan is appropriately selected from among E. coli tRNAs for the introduction of an unnatural amino acid.

FIG. 6A shows the results of Western blotting in the case involving the use of tRNA to which no amino acid was added. FIG. 6B is a fluorescence image of SDS-PAGE in the case involving the use of tRNA to which fluorescent-labeled amino acid was added. FIG. 6C shows fluorescence band intensities of proteins subjected to introduction with the use of the individual mutants. As shown in FIGS. 6A to 6C, G1-C72, A73 and G1-C72, G73 had high introduction activity. Note that a full-length protein was produced in the case of G1-C72, G73 even without addition of a fluorescent-labeled unnatural amino acid. Thus, it is considered that a natural amino acid is added in a translation system in such case. Accordingly, G1-C72, A73 to which no natural amino acid is introduced is the most preferable.

EXAMPLE 4

Introduction of Fluorescent-Labeled Amino Acid into UAG with the Use of a Mycoplasma capricolum-Derived Mutant tRNA

30 types of tRNAs each having a Mycoplasma capricolum-derived tRNA sequence and comprising CUA as an anticodon as a result of substitution were produced by the method described in Example 1. Subsequently, the introduction of fluorescent-labeled amino acid was evaluated by the method described in Example 2.

FIG. 8 shows the results of introduction of fluorescent-labeled amino acid into UAG with the use of a Mycoplasma capricolum-derived mutant tRNA. As shown in FIG. 8, it was found that tRNA for tryptophan is appropriately selected from among Mycoplasma capricolum-derived mutant tRNAs for the introduction of unnatural amino acid.

FIG. 10A shows a fluorescence image in the case involving the use of tRNAs to which no amino acid was added and tRNAs to which fluorescent-labeled amino acid was added. FIG. 10B shows fluorescence band intensities of proteins subjected to introduction with the use of individual mutants. As shown in FIGS. 10A and 10B, all Mycoplasma capri-

colum-derived mutant tRNAs showed introduction activity at higher levels than that of a mutant tRNA (a mutant G1-C72, A73) for E. coli tryptophan. G1-C72, A73 from which substantially no full-length protein is generated without the addition of fluorescent-labeled amino acid is particularly preferable.

EXAMPLE 5

Introduction of Fluorescent-Labeled Amino Acid into UAG with the Use of a Mutant tRNA (a Mutant G1-C72, A73) for Tryptophan Derived from a Different Organism

31 types of tRNAs each having a sequence of tRNA for tryptophan derived from a different organism and comprising CUA as an anticodon as a result of substitution caused by introduction of a mutant G1-C72, A73 were produced by the method described in Example 1. Subsequently, the introduction of fluorescent-labeled amino acid was evaluated by the method described in Example 2. FIGS. 11A and 11B show sequences of 31 types of mutant tRNAs (SEQ ID NOS: 1 to 31) each having CUA as an anticodon as a result of substitution caused by introduction of tRNA (G1-C72, A73) for tryptophan derived from a different organism. In FIGS. 11A and 11B, "U" is expressed as "T." In addition, the 73^{rd} base is "A" in SEQ ID NOS: 1 to 31. Alternatively, the effects of the present invention can be obtained when "G" replaces "A" (G1-C72, G73).

FIGS. 12A and 12B show the results of introduction of fluorescent-labeled amino acid into UAG with the use of a mutant tRNA (G1-C72, A73) for tryptophan derived from a different organism.

FIG. 13A shows the results of fluorescence imaging in the case involving the use of tRNA to which no amino acid was added and tRNA to which fluorescent-labeled amino acid was added. FIG. 13B shows fluorescence band intensities of proteins subjected to introduction of individual mutants. As shown in FIGS. 13A and 13B, 8 types of tRNAs for tryptophan, including tRNA for Mycoplasma capricolum-derived tryptophan, exhibited high levels of introduction activity. Among them, a Mycoplasma capricolum-derived mutant tRNA (a G1-C72, A73 mutant), which is unlikely to cause generation of a full-length protein to which no fluorescent-labeled amino acid is added, is the most preferable.

EXAMPLE 6

Double Introduction of Fluorescent-Labeled Amino Acids into Maltose Binding Protein (MBP)

(1) Introduction Only by the 4-Base Codon Method

With the use of tRNA for yeast phenylalanine, BODIPY FL-aminophenylalanine-tRNA (having ACCC as an anticodon) and BODIPY 558/568-aminophenylalanine-tRNA (having CCCG as an anticodon) were produced by the method described in Example 1. Subsequently, with the use of mRNA of maltose binding protein (such mRNA having CGGG at the N terminal and GGGU at the C terminal), double introduction of fluorescent-labeled amino acids was carried out by the method described in Example 2. Note that detection of fluorescence in electrophoresis gel was carried out at 488 nm excitation/520 nm detection and, in addition, at 532 nm excitation/605 nm detection.

FIG. 14A shows an outline of a method for double introduction of fluorescent-labeled amino acids into maltose binding protein (MBP) based on the 4-base codon method. FIG.

14B shows the results of double introduction of fluorescent-labeled amino acids into maltose binding protein (MBP) based on the 4-base codon method. tRNA having CCCG introduced fluorescent-labeled amino acids exclusively into the gene having CGGG. Meanwhile, tRNA having ACCC also introduced fluorescent-labeled amino acids into the gene having no GGGU. That is, in the case of this gene, fluorescence-labeled amino acid was introduced into a site other than a 4-base codon GGGU. Thus, it was attempted to use a stop codon UAG instead of a 4-base codon.

The mRNA sequence of a maltose binding protein having CGGG at the N terminal and GGGU at the C terminal is shown below (SEQ ID NO: 34). The sequence ranges from a start codon to the original stop codon. The underlined part corresponds to the inserted codon.

AUGGCUAGCAUGACUGGUGGACAGCAAAUGGGUACU<u>CGGGAGUAAC</u>GA

AUUCAAAAUCGAAGAAGGUAAACUGGUAAUCUGGAUUAACGGCGAUAA

AGGCUAUAACGGUCUCGCUGAAGUCGGUAAGAAAUUCGAGAAAGAUAC

CGGAAUUAAAGUCACCGUUGAGCAUCCGGAUAAACUGGAAGAGAAAUU

CCCACAGGUUGCGGCAACUGGCGAUGGCCCUGACAUUAUCUUCUGGGC

ACACGACCGCUUUGGUGGCUACGCUCAAUCUGGCCUGUUGGCUGAAAU

CACCCCGGACAAAGCGUUCCAGGACAAGCUGUAUCCGUUUACCUGGGA

UGCCGUACGUUACAACGGCAAGCUGAUUGCUUACCCGAUCGCUGUUGA

AGCGUUAUCGCUGAUUUAUAACAAAGAUCUGCUGCCGAACCCGCCAAA

AACCUGGGAAGAGAUCCCGGCGCUGGAUAAAGAACUGAAAGCGAAAGG

UAAGAGCGCGCUGAUGUUCAACCUGCAAGAACCGUACUUCACCUGGCC

GCUGAUUGCUGCUGACGGGGGUUAUGCGUUCAAGUAUGAAAACGGCAA

GUACGACAUUAAAGACGUGGGCGUGGAUAACGCUGGCGCGAAAGCGGG

UCUGACCUUCCUGGUUGACCUGAUUAAAAACAAACACAUGAAUGCAGA

CACCGAUUACUCCAUCGCAGAAGCUGCCUUUAAUAAAGGCGAAACAGC

GAUGACCAUCAACGGCCCGUGGGCAUGGUCCAACAUCGACACCAGCAA

AGUGAAUUAUGGUGUAACGGUACUGCCGACCUUCAAGGGUCAACCAUC

CAAACCGUUCGUUGGCGUGCUGAGCGCAGGUAUUAACGCCGCCAGUCC

GAACAAAGAGCUGGCAAAAGAGUUCCUCGAAAACUAUCUGCUGACUGA

UGAAGGUCUGGAAGCGGUUAAUAAAGACAAACCGCUGGGUGCCGUAGC

GCUGAAGUCUUACGAGGAAGAGUUGGCGAAAGAUCCACGUAUUGCCGC

CACUAUGGAAAACGCCCAGAAAGGUGAAAUCAUGCCGAACAUCCCGCA

GAUGUCCGCUUUCUGGUAUGCCGUGCGUACUGCGGUGAUCAACGCCGC

CAGCGGUCGUCAGACUGUCGAUGAAGCCCUGAAAGACGCGCAGACUCG

UAUCACCAAG<u>GGGUAGC</u>CACCACCACCACCACUAA

(2) Introduction with the Combined Use of the 4-Base Codon Method and the Mutant tRNA of the Present Invention

With the use of tRNA for yeast phenylalanine, BODIPY 558/568-aminophenylalanine-tRNA (having CCCG as an anticodon) was produced by the method described in Example 1. Meanwhile, with the use of a mutant tRNA for *Mycoplasma capricolum*-derived tryptophan (a G1-C72, A73 mutant), BODIPY FL-aminophenylalanine-tRNA (having CUA as an anticodon) was produced by the method described in Example 1. Subsequently, with the use of mRNA (having CGGG at the N terminal portion and UAG at the C terminal portion) of a maltose binding protein, double introduction of

fluorescent-labeled amino acids was carried out by the method described in Example 2. Note that detection of fluorescence in electrophoresis gel was carried out at 488 nm excitation/520 nm detection and, in addition, at 532 nm excitation/605 nm detection.

FIG. 15B shows an outline of a method for double introduction of fluorescent-labeled amino acids into maltose binding protein (MBP) based on the 4-base codon method and a stop codon method with the use of the mutant tRNA of the present invention. FIG. 15B shows the results of double introduction of fluorescent-labeled amino acids into maltose binding protein (MBP) based on the 4-base codon method and a stop codon method with the use of the mutant tRNA of the present invention. A *Mycoplasma capricolum*-derived mutant tRNA (a G1-C72, A73 mutant) was able to introduce fluorescent-labeled amino acids exclusively into the gene having UAG. In addition, the introduction efficiency was also equivalent to that in the case of 4-base codon GGGU. Accordingly, it was found that a combination of CGGG and UAG is also effective for introduction of two types of fluorescent-labeled amino acids.

The mRNA sequence of a maltose binding protein having CGGG at the N terminal portion and UAG at the C terminal portion is shown below (SEQ ID NO: 35). The sequence ranges from a start codon to the original stop codon. The underlined portion corresponds to the inserted codon.

AUGGCUAGCAUGACUGGUGGACAGCAAAUGGGUACU<u>CGGGAGUAAC</u>GA

AUUCAAAAUCGAAGAAGGUAAACUGGUAAUCUGGAUUAACGGCGAUAA

AGGCUAUAACGGUCUCGCUGAAGUCGGUAAGAAAUUCGAGAAAGAUAC

CGGAAUUAAAGUCACCGUUGAGCAUCCGGAUAAACUGGAAGAGAAAUU

CCCACAGGUUGCGGCAACUGGCGAUGGCCCUGACAUUAUCUUCUGGGC

ACACGACCGCUUUGGUGGCUACGCUCAAUCUGGCCUGUUGGCUGAAAU

CACCCCGGACAAAGCGUUCCAGGACAAGCUGUAUCCGUUUACCUGGGA

UGCCGUACGUUACAACGGCAAGCUGAUUGCUUACCCGAUCGCUGUUGA

AGCGUUAUCGCUGAUUUAUAACAAAGAUCUGCUGCCGAACCCGCCAAA

AACCUGGGAAGAGAUCCCGGCGCUGGAUAAAGAACUGAAAGCGAAAGG

UAAGAGCGCGCUGAUGUUCAACCUGCAAGAACCGUACUUCACCUGGCC

GCUGAUUGCUGCUGACGGGGGUUAUGCGUUCAAGUAUGAAAACGGCAA

GUACGACAUUAAAGACGUGGGCGUGGAUAACGCUGGCGCGAAAGCGGG

UCUGACCUUCCUGGUUGACCUGAUUAAAAACAAACACAUGAAUGCAGA

CACCGAUUACUCCAUCGCAGAAGCUGCCUUUAAUAAAGGCGAAACAGC

GAUGACCAUCAACGGCCCGUGGGCAUGGUCCAACAUCGACACCAGCAA

AGUGAAUUAUGGUGUAACGGUACUGCCGACCUUCAAGGGUCAACCAUC

CAAACCGUUCGUUGGCGUGCUGAGCGCAGGUAUUAACGCCGCCAGUCC

GAACAAAGAGCUGGCAAAAGAGUUCCUCGAAAACUAUCUGCUGACUGA

UGAAGGUCUGGAAGCGGUUAAUAAAGACAAACCGCUGGGUGCCGUAGC

GCUGAAGUCUUACGAGGAAGAGUUGGCGAAAGAUCCACGUAUUGCCGC

CACUAUGGAAAACGCCCAGAAAGGUGAAAUCAUGCCGAACAUCCCGCA

GAUGUCCGCUUUCUGGUAUGCCGUGCGUACUGCGGUGAUCAACGCCGC

CAGCGGUCGUCAGACUGUCGAUGAAGCCCUGAAAGACGCGCAGACUCG

UAUCACCAAG<u>UAG</u>CACCACCACCACCACUAA

EXAMPLE 7

Introduction of Fluorescent-Labeled Amino Acid Having TAMRA

With the use of a *Mycoplasma capricolum*-derived mutant tRNA (a G1-C72, A73 mutant), TAMRA-X-aminophenyla-lanine-tRNA was produced by the method described in Example 1. Subsequently, streptavidin mRNA (SA2UAG) in which the 2^{nd} site had been substituted with UAG was used for introduction of TAMRA-labeled amino acid by the method described in Example 2. Note that detection of fluo-rescence in electrophoresis gel was carried out at 532 nm excitation/605 nm detection. For comparison, introduction was carried out in a similar manner with the use of streptavi-din mRNA in which the 2" site had been substituted with CGGG with the use of tRNA for yeast phenylalanine having an anticodon comprising 4 bases (CCCG). FIG. **16**A shows the chemical structure of fluorescent-labeled amino acid hav-ing TAMRA (TAMRAXAF). In FIG. **16**A, the portion indi-cated with 5 and 6 corresponds to a mixture of a substance binding to the site 5 and a substance binding to the side 6. In addition, FIG. **16**B shows the results of fluorescence imaging in the case involving the use of tRNA to which fluorescent-labeled amino acid was added. Further, FIG. **16**C shows fluo-rescence band intensities of proteins subjected to introduc-tion. As shown in FIGS. **16**B and **16**C, a *Mycoplasma capricolum*-derived mutant tRNA (a G1-C72, A73 mutant) caused introduction of fluorescent-labeled amino acid having TAMRA with good efficiency, as with the case of fluorescent-labeled amino acid having BODIPY FL described in Example 4. In addition, higher levels of introduction activity were exhibited compared with the case in which introduction into a 4-base codon CGGG was carried out with the use of tRNA for yeast phenylalanine having an anticodon compris-ing 4 bases (CCCG).

The sequence of streptavidin mRNA in which the 2" site was substituted with UAG is shown below (SEQ ID NO: 36). The sequence ranges from a start codon to the original stop codon. The underlined part corresponds to introduced UAG.

AUGUAGCCGUCCAAGGACUCCAAAGCUCAGGUUUCUGCAGCCGAAGCU

GGUAUCACUGGCACCUGGUAUAACCAACUGGGGUCGACUUUCAUUGUG

ACCGCUGGUGCGGACGGAGCUCUGACUGGCACCUACGAAUCUGCGGUU

GGUAACGCAGAAUCCCGCUACGUACUGACUGGCCGUUAUGACUCUGCA

CCUGCCACCGAUGGCUCUGGUACCGCUCUGGGCUGGACUGUGGCUUGG

AAAAACAACUAGCGUAAUGCGCACAGCGCCACUACGUGGUCUGGCCAA

UACGUUGGCGGUGCUGAGGCUCGUAUCAACACUCAGUGGCUGUUAACA

UCCGGCACUACCGAAGCGAAUGCAUGGAAAUCGACACUAGUAGGUCAU

GACACCUUUACCAAAGUUAAGCCUUCUGCUGCUAGCAUUGAUGCUGCC

AAGAAAGCAGGCGUAAACAACGGUAACCCUCUAGACGCUGUUCAGCAA

CACCACCACCACCACUAA

In addition, the sequence of streptavidin mRNA sequence in which the 2^{nd} site was substituted with CGGG is shown below (SEQ ID NO: 37). The sequence ranges from a start codon to a stop codon. The underlined part corresponds to introduced CGGG.

AUGCGGGCCGUCCAAGGACUCCAAAGCUCAGGUUUCUGCAGCCGAAGC

UGGUAUCACUGGCACCUGGUAUAACCAACUGGGGUCGACUUUCAUUGU

GACCGCUGGUGCGGACGGAGCUCUGACUGGCACCUACGAAUCUGCGGU

UGGUAACGCAGAAUCCCGCUACGUACUGACUGGCCGUUAUGACUCUGC

ACCUGCCACCGAUGGCUCUGGUACCGCUCUGGGCUGGACUGUGGCUUG

GAAAAACAACUAGCGUAAUGCGCACAGCGCCACUACGUGGUCUGGCCA

AUACGUUGGCGGUGCUGAGGCUCGUAUCAACACUCAGUGGCUGUUAAC

AUCCGGCACUACCGAAGCGAAUGCAUGGAAAUCGACACUAGUAGGUCA

UGACACCUUUACCAAAGUUAAGCCUUCUGCUGCUAGCAUUGAUGCUGC

CAAGAAAGCAGGCGUAAACAACGGUAACCCUCUAGACGCUGUUCAGCA

ACACCACCACCACCACUAA

EXAMPLE 8

Introduction of Unnatural Amino Acid with the Use of a Mutant tRNA into which a Single Base has been Inserted Just Before the CCA Sequence at the 3' End

(1) Mutant tRNA for *Mycoplasma capricolum*-Derived Tryp-tophan

A mutant tRNA (G1-C72, A73) for *Mycoplasma capri-colum*-derived tryptophan into which a single base (A, C, G, or U) had been inserted just before the CCA sequence at the 3' end was produced by the method described in Example 1. In the present invention, such mutant tRNA is referred to as single-base-extended tRNA in some cases. For instance, a mutant tRNA (G1-C72, A73) for *Mycoplasma capricolum*-derived tryptophan into which A, C, G, or U has been inserted just before the CCA sequence at the 3' end is referred to as single-base-extended MycW tRNA in some cases. Subse-quently, the introduction of fluorescent-labeled amino acid was evaluated by the method described in Example 2. FIG. **17** shows the structures of the mutant tRNAs used (SEQ ID NOS: 1 and 38). FIG. **17** (right) shows a mutant tRNA for *Mycoplasma capricolum*-derived tryptophan into which A, C, G, or U has been inserted just before the CCA sequence at the 3' end (SEQ ID NO: 38). FIG. **18** shows the results of introduction of BFLAF into SA83UAG with the use of single-base-extended MycW. FIG. **18**A shows the results of fluores-cence imaging in the case involving the use of tRNA to which fluorescent-labeled amino acid was added. FIG. **18**B shows fluorescence band intensities of proteins subjected to intro-duction with the use of the individual mutants. As shown in FIGS. **18**A and **18**B, tRNA into which A, C, G, or U had been inserted just before the CCA sequence at the 3' end exhibited a high level of introduction activity comparable to that of the original tRNA. In addition, this result indicates that a mutant tRNA (G1-C72, A73) for *Mycoplasma capricolum*-derived tryptophan can introduce fluorescent substanceylated tyrosine in addition to fluorescent-labeled amino acid with good efficiency.

Further, tRNA was produced by the method described in Example 1 with the use of fluorescent substanceylated tyrosine instead of fluorescent-labeled amino acid. Subse-quently, the introduction of fluorescent substanceylated tyrosine was evaluated by the method described in Example 2. Note that fluorescence imaging was not carried out and Western blot using an anti-fluorescent substanceylated tyrosine antibody or an anti-T7tag antibody was exclusively

carried out. FIG. **19** shows the results of introduction of fluorescent substanceylated tyrosine (pTyr) into SA2UAG with the use of single-base-extended MycW. FIG. **19**A shows the results of Western blot with the use of streptavidin mRNA in which the 2" site was substituted with UAG and tRNA to which fluorescent substanceylated tyrosine was added. FIG. **19**B shows Western blot band intensities of proteins subjected to introduction. As shown in FIGS. **19**A and **19**B, tRNA into which A, C, G, or U had been introduced just before the CCA sequence at the 3' end exhibited a high level of introduction activity comparable to that of the original tRNA.

Meanwhile, FIG. **20**A shows the results of Western blot with the use of streptavidin mRNA (SA83UAG) in which the 83^{rd} side was substituted with UAG and tRNA to which fluorescent substanceylated tyrosine was added. FIG. **20**B shows Western blot band intensities of proteins subjected to introduction. As shown in FIGS. **20**A and **20**B, tRNA into which A, C, G, or U had been inserted just before the CCA sequence at the 3' end exhibited a higher level of introduction activity than that of the original tRNA. In particular, good efficiency was obtained with the use of tRNA into which a single base C had been inserted.

(2) Mutant tRNA for Yeast-Derived Phenylalanine

A mutant tRNA for yeast-derived phenylalanine having CCCG as an anticodon (YF(CCCG)), in which a base had been inserted or deleted at one terminal, was produced by the method described in Example 1. Subsequently, the introduction of fluorescent-labeled amino acid was evaluated by the method described in Example 2. Note that a streptavidin mRNA used contained CGGG at the Tyr83 site instead of UAG. FIG. **21** shows the structures of mutant tRNAs (SEQ ID NOS: 39 and 40). FIG. **22**A shows the results of fluorescence imaging in the case involving the use of tRNA to which fluorescent-labeled amino acid was added. FIG. **22**B shows the fluorescence band intensities of proteins subjected to introduction with the use of the individual mutants. As shown in FIGS. **22**A and **22**B, tRNA into which a single base had been inserted just before the CCA sequence at the 3' end and tRNA in which a single base located just before the CCA sequence had been removed exhibited high levels of introduction activity comparable to those of the original tRNAs.

Further, with the use of streptavidin mRNA having a T7tag sequence at the N terminal into which CGGG had been inserted just after the T7tag sequence, the introduction of fluorescent-labeled amino acid was evaluated by the method described in Example 2. FIG. **23**A shows the results of fluorescence imaging in the case involving the use of tRNA to which fluorescent-labeled amino acid was added. FIG. **23**B shows the fluorescence band intensities of proteins subjected to introduction with the use of the individual mutants. As shown in FIGS. **23**A and **23**B, tRNA into which a single base had been inserted just before the CCA sequence at the 3' end exhibited a higher level of introduction activity than that of the original tRNA.

The sequence of streptavidin mRNA having a T7tag sequence at the N terminal into which CGGG has been introduced just the T7tag sequence is shown below (SEQ ID NO: 41). The sequence ranges from a start codon to a stop codon. The underlined part corresponds to the inserted CGGG codon.

```
AUGGCUAGCAUGACUGGUGGACAGCAAAUGGGUCCCCGGGAGUAACGA

AUUCCAUAUGGACCCGUCCAAGGACUCCAAAGCUCAGGUUUCUGCAGC

CGAAGCUGGUAUCACUGGCACCUGGUAUAACCAACUGGGGUCGACUUU
```

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CAUUGUGACCGCUGGUGCGGACGGAGCUCUGACUGGCACCUACGAAUC

UGCGGUUGGUAACGCAGAAUCCCGCUACGUACUGACUGGCCGUUAUGA

CUCUGCACCUGCCACCGAUGGCUCUGGUACCGCUCUGGGCUGGACUGU

GGCUUGGAAAAACAACUAUCGUAAUGCGCACAGCGCCACUACGUGGUC

UGGCCAAUACGUUGGCGGUGCUGAGGCUCGUAUCAACACUCAGUGGCU

GUUAACAUCCGGCACUACCGAAGCGAAUGCAUGGAAAUCGACACUAGU

AGGUCAUGACACCUUUACCAAAGUUAAGCCUUCUGCUGCUAGCAUUGA

UGCUGCCAAGAAAGCAGGCGUAAACAACGGUAACCCUCUAGACGCUGU

UCAGCAACACCACCACCACCACCACUAA
```

Industrial Applicability

Functional modification and structural and functional analysis of proteins have been carried out by introducing unnatural amino acids into proteins. An example of an existing method for introducing an unnatural amino acid into a protein is a method wherein a codon located at a target site for introduction has been substituted with a stop codon UAG and translation is carried out in the presence of tRNA having an anticodon CUA and being aminoacylated with an unnatural amino acid. However, when such tRNA translates UAG, it competes with a termination factor. In the case of a conventionally used yeast Phe tRNA, such competition with a termination factor is strongly induced, resulting in a decrease in the efficiency of introduction of unnatural amino acids.

Thus, the present inventors searched for a tRNA that can introduce an unnatural amino acid into a protein with good efficiency with the use of a stop codon UAG and found such tRNA from among tRNAs derived from a variety of living species. Further, they have found tRNAs that can introduce an unnatural amino acid into a protein with good efficiency by modifying the above tRNA. With the use of such tRNAs, it becomes possible to synthesize a fluorescent-labeled protein into which a fluorescent-labeled unnatural amino acid has been introduced (WO2004/009709 A1). It also becomes possible to detect interaction of proteins and other molecules with better efficiency by introducing fluorescent-labeled unnatural amino acids, which can be a donor and an acceptor for fluorescence resonance energy transfer, into specific sites of a protein.

For instance, it becomes possible to synthesize streptavidin into which a fluorescent-labeled unnatural amino acid BODIPY FL-aminophenylalanine has been introduced into the Tyr83 site, and to introduce fluorescent-labeled unnatural amino acids, which can be a donor and an acceptor for fluorescence resonance energy transfer, into a maltose binding protein by combining the amino acids with a 4-base codon CGGG.

Amino acids to be introduced are not limited to fluorescent-labeled unnatural amino acids. Thus, the present invention can be applied to any type of amino acid or amino acid analog that can be introduced into proteins, such as hydroxy acid. In addition, since tRNA exhibits translation activity in different living species, the tRNA of the present invention is not limited to *E. coli* cell-free translation systems and thus can be used for translation in cells or any type of cell-free translation system derived from living species.

All publications, patents, and patent applications cited herein are incorporated herein by reference in their entirety.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 41

<210> SEQ ID NO 1
<211> LENGTH: 75
<212> TYPE: RNA
<213> ORGANISM: Mycoplasma capricolum

<400> SEQUENCE: 1

gggagaguag uucaauggua gaacgucggu cucuaaaacc gagcguugag gguucgauuc   60

cuuucucucc cacca   75

<210> SEQ ID NO 2
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 2

gggggcguag uucaauuggu agagcaccgg ucucuaaaac cgggguguugg gaguucgagu   60

cucuccgccc ccacca   76

<210> SEQ ID NO 3
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Aquifex aeolicus

<400> SEQUENCE: 3

ggggcuguag cucaauuggc agagcgcggg acucuaaauc ccgugggugg ggguucgagu   60

ccucccagcc ccacca   76

<210> SEQ ID NO 4
<211> LENGTH: 74
<212> TYPE: RNA
<213> ORGANISM: Bacillus halodurans

<400> SEQUENCE: 4

gggggcauag uguaacggua gaacagaggu cucuaaaacc uccggugugg guucgauucc   60

uacugccccc acca   74

<210> SEQ ID NO 5
<211> LENGTH: 74
<212> TYPE: RNA
<213> ORGANISM: Bacillus subtilis

<400> SEQUENCE: 5

gggggcauag uuuaacggua gaacagaggu cucuaaaacc uccggugugg guucgauucc   60

uacugccccc acca   74

<210> SEQ ID NO 6
<211> LENGTH: 77
<212> TYPE: RNA
<213> ORGANISM: Borrelia burgdorferi

<400> SEQUENCE: 6

ggggguguag uucaauuugg uagagcaucg gucucuaaaa ccgaaaguug uagguucaaa   60

uccuuccacc cccacca   77

<210> SEQ ID NO 7
<211> LENGTH: 77
<212> TYPE: RNA

-continued

<213> ORGANISM: Buchnera sp

<400> SEQUENCE: 7

ggggguguag uucaauuugg uagagcaucg gucucuaaaa ccgaaaguug uagguucaaa   60

uccuuccacc cccacca   77

<210> SEQ ID NO 8
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Campylobacter jejuni

<400> SEQUENCE: 8

ggggcaauag cuccaacggu agagcgccgg auucuaaauc cgaugguugg ggguucgaau   60

cccucuugcc ccacca   76

<210> SEQ ID NO 9
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Caulobacter crescentus

<400> SEQUENCE: 9

gggaguguag cucagcuggu agagcaucgg ucucuaaaac cgagggccgg ggguucgagu   60

cccuccacuc ccacca   76

<210> SEQ ID NO 10
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Chlamydia pneumoniae

<400> SEQUENCE: 10

ggguguguag cuuagcuggu agagcagugg ccucuaaagc cgccggucgg ggguucgauu   60

cccuucgcac ccacca   76

<210> SEQ ID NO 11
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Chlamydia trachomatis
<220> FEATURE:
<223> OTHER INFORMATION: Chlamydia trachomatis D1

<400> SEQUENCE: 11

ggguguguag cuuagauggu agagcagugg ccucuaaagc cgccggucgg ggguucgaau   60

cccuccgcac ucacca   76

<210> SEQ ID NO 12
<211> LENGTH: 75
<212> TYPE: RNA
<213> ORGANISM: Chlamydia trachomatis
<220> FEATURE:
<223> OTHER INFORMATION: Chlamydia trachomatis D2

<400> SEQUENCE: 12

ggguguguag cuuagauggu agagcagugg ccucuaaagc cgccggucgg ggguucgaau   60

cccucuucac cacca   75

<210> SEQ ID NO 13
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Deinococcus radiodurans

<400> SEQUENCE: 13

```
ggguucuuag cucaguuggu agagcggcgg ucucuaaaac cguaggucga ggguucaagu      60

ccuucagggc ccacca                                                      76
```

<210> SEQ ID NO 14
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Haemophilus influenzae
<220> FEATURE:
<223> OTHER INFORMATION: Haemophilus influenzae Rd

<400> SEQUENCE: 14

```
gggggcguag uucaauuggu agagcaccgg ucucuaaaac cgggguguugg gaguucgagc      60

cucuccgccc ccacca                                                      76
```

<210> SEQ ID NO 15
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Helicobacter pylori

<400> SEQUENCE: 15

```
gggucaguag cuccaauggu agagcgucgg ucucuaaaac cgguuguugg ggguucgagu      60

cccuccuggc ccacca                                                      76
```

<210> SEQ ID NO 16
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Mycobacterium leprae

<400> SEQUENCE: 16

```
gggggcguag cucaacuggc agagcagcgg ucucuaaaac cgcagguugc agguucaaau      60

ccugucgccc ccacca                                                      76
```

<210> SEQ ID NO 17
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 17

```
gggggcguag cucaacuggc agagcagcgg ucucuaaaac cgcagguugc agguucaagu      60

ccugucgccc ccacca                                                      76
```

<210> SEQ ID NO 18
<211> LENGTH: 74
<212> TYPE: RNA
<213> ORGANISM: Mycoplasma genitalium

<400> SEQUENCE: 18

```
gggggguag uuuaguggua gaacaacagu cucuaaaacu gucugugugg guucgauucc      60

uuccaccccc acca                                                        74
```

<210> SEQ ID NO 19
<211> LENGTH: 74
<212> TYPE: RNA
<213> ORGANISM: Mycoplasma pneumoniae
<220> FEATURE:
<223> OTHER INFORMATION: Mycoplasma pneumoniae 1

<400> SEQUENCE: 19

```
ggggguguag uuuaguggca gaacaacagu cucuaaaacu gucugugugg guucgauucc      60

uuccacccccc acca                                                       74
```

```
<210> SEQ ID NO 20
<211> LENGTH: 75
<212> TYPE: RNA
<213> ORGANISM: Mycoplasma pneumoniae
<220> FEATURE:
<223> OTHER INFORMATION: Mycoplasma pneumoniae 2

<400> SEQUENCE: 20

ggggguauag uucaaaggua gaacaucugu cucuaaaaua gaguguugug gguucgaguc        60

cugcuacccc cacca                                                         75


<210> SEQ ID NO 21
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Neisseria meningitidis
<220> FEATURE:
<223> OTHER INFORMATION: Neisseria meningitidis MC58

<400> SEQUENCE: 21

gggccaauag cucaauuggu agaguaucgg ucucuaaaac cgaggguugg ggguucgaga        60

cccucuuggc ccacca                                                        76


<210> SEQ ID NO 22
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Procaryote Trp consensus

<400> SEQUENCE: 22

gggggcguag uucaauuggu agagcagcgg ucucuaaaac cgcagguugg ggguucgagu        60

cccuccgccc ccacca                                                        76


<210> SEQ ID NO 23
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Pseudomonas aeruginosa

<400> SEQUENCE: 23

gggccaguag cucaauuggc agagcggcgg ucucuaaaaa agcagguugg ggguucgauu        60

cccuccuggc ccacca                                                        76


<210> SEQ ID NO 24
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Rickettsia prowazekii

<400> SEQUENCE: 24

gggaguguag cucaauuggu agagcgccgg ucucuaaaac cggaggguugc ggguucgauu       60

ccugucgcuc ccacca                                                        76


<210> SEQ ID NO 25
<211> LENGTH: 74
<212> TYPE: RNA
<213> ORGANISM: Staphylococcus aureus
<220> FEATURE:
<223> OTHER INFORMATION: Staphylococcus aureus N315

<400> SEQUENCE: 25

gggggcauag uucaacggua gaauagaggu cucuaaaacc uuuggugugg guucgauucc        60

uacugccccc acca                                                          74
```

-continued

```
<210> SEQ ID NO 26
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Synechocystis sp.

<400> SEQUENCE: 26

gcguccuuag uucaguuggu agaacgcagg ucucuaaaac cugaugucgg ggguucaagu    60

ccuccagggc gcacca                                                   76


<210> SEQ ID NO 27
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Thermotoga maritima

<400> SEQUENCE: 27

ggggccguag cucaacuggu agagcgccgg ucucuaaaac cguggguugc ggguucgagu    60

ccugccggcc ccacca                                                   76


<210> SEQ ID NO 28
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Treponema pallidum

<400> SEQUENCE: 28

gggucaguag cucuaauggc agagcgucgg ucucuaaaac cgaauguuga agguucgagu    60

ccuuccuggc ccacca                                                   76


<210> SEQ ID NO 29
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Ureaplasma urealyticum

<400> SEQUENCE: 29

gaggguauag uucaauuggu agaacagcag acucuaaauc ugcguguugc ggguucgagu    60

ccuguuaccc ucacca                                                   76


<210> SEQ ID NO 30
<211> LENGTH: 77
<212> TYPE: RNA
<213> ORGANISM: Vibrio cholerae

<400> SEQUENCE: 30

gggggguag cuccaauugg cagagcagcg gauucuaaau ccgcguguug ggaguucgaa    60

ucucuccacc cccacca                                                  77


<210> SEQ ID NO 31
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Xylella fastidiosa

<400> SEQUENCE: 31

gcgucaguag cucaauuggc agagcagcgg ucucuaaaac cgcagguugg ggguucgagu    60

cccuccuggc gcacca                                                   76


<210> SEQ ID NO 32
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer
```

<400> SEQUENCE: 32

ctaatacgac tcactata   18

<210> SEQ ID NO 33
<211> LENGTH: 546
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 33

auggcuagca ugacuggugg acagcaaaug gguaccgaau uccauaugga cccguccaag   60

gacuccaaag cucagguuuc ugcagccgaa gcugguauca cuggcaccug guauaaccaa   120

cuggggucga cuuucauugu gaccgcuggu gcggacggag cucugacugg caccuacgaa   180

ucugcgguug guaacgcaga aucccgcuac guacugacug gccguuauga cucugcaccu   240

gccaccgaug gcucugguac cgcucugggc uggacugugg cuuggaaaaa caacuagcgu   300

aaugcgcaca gcgccacuac guggucuggc caauacguug gcggugcuga ggcucguauc   360

aacacucagu ggcuguuaac auccggcacu accgaagcga augcauggaa aucgacacua   420

guaggucaug acaccuuuac caaaguuaag ccuucugcug cuagcauuga ugcugccaag   480

aaagcaggcg uaaacaacgg uaacccucua gacgcuguuc agcaacacca ccaccaccac   540

cacuaa   546

<210> SEQ ID NO 34
<211> LENGTH: 1190
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 34

auggcuagca ugacuggugg acagcaaaug gguacucggg aguaacgaau ucaaaaucga   60

agaagguaaa cugguaaucu ggauuaacgg cgauaaaggc uauaacgguc ucgcugaagu   120

cgguaagaaa uucgagaaag auaccggaau uaaagucacc guugagcauc cggauaaacu   180

ggaagagaaa uucccacagg uugcggcaac uggcgauggc ccugacauua ucuucugggc   240

acacgaccgc uuugguggcu acgcucaauc uggccuguug gcugaaauca ccccggacaa   300

agcguuccag gacaagcugu auccguuuac cugggaugcc guacguuaca acggcaagcu   360

gauugcuuac ccgaucgcug uugaagcguu aucgcugauu uauaacaaag aucugcugcc   420

gaacccgcca aaaaccuggg aagagauccc ggcgcuggau aaagaacuga agcgaaagg   480

uaagagcgcg cugauguuca accugcaaga accguacuuc accuggccgc ugauugcugc   540

ugacgggggu uaugcguuca aguaugaaaa cggcaaguac gacauuaaag acgugggcgu   600

ggauaacgcu ggcgcgaaag cgggucugac cuuccugguu gaccugauua aaaacaaaca   660

caugaaugca gacaccgauu acuccaucgc agaagcugcc uuuaauaaag gcgaaacagc   720

gaugaccauc aacggcccgu gggcaugguc caacaucgac accagcaaag ugaauuaugg   780

uguaacggua cugccgaccu ucaaggguca accauccaaa ccguucguug gcgugcugag   840

cgcagguauu aacgccgcca guccgaacaa agagcuggca aaagaguucc ucgaaaacua   900

ucugcugacu gaugaagguc uggaagcggu uaauaaagac aaaccgcugg gugccguagc   960

gcugaagucu uacagaggaag aguuggcgaa agauccacgu auugccgcca cuauggaaaa   1020

cgcccagaaa ggugaaauca ugccgaacau cccgcagaug uccgcuuucu gguaugccgu   1080

-continued

```
gcguacugcg gugaucaacg ccgccagcgg ucgucagacu gucgaugaag cccugaaaga    1140

cgcgcagacu cguaucacca aggggguagcc accaccacca ccaccacuaa             1190


<210> SEQ ID NO 35
<211> LENGTH: 1186
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 35

auggcuagca ugacuggugg acagcaaaug gguacucggg aguaacgaau ucaaaaucga      60

agaagguaaa cugguaaucu ggauuaacgg cgauaaaggc uauaacgguc ucgcugaagu     120

cgguaagaaa uucgagaaag auaccggaau uaaagucacc guugagcauc cggauaaacu     180

ggaagagaaa uucccacagg uugcggcaac uggcgauggc ccugacauua ucuucugggc     240

acacgaccgc uuuggguggcu acgcucaauc uggccuguug gcugaaauca ccccggacaa     300

agcguuccag gacaagcugu auccguuuac cugggaugcc guacguuaca acggcaagcu     360

gauugcuuac ccgaucgcug uugaagcguu aucgcugauu uauaacaaag aucugcugcc     420

gaacccgcca aaaaccuggg aagagauccc ggcgcuggau aaagaacuga aagcgaaagg     480

uaagagcgcg cugauguuca accugcaaga accguacuuc accuggccgc ugauugcugc     540

ugacgggggu uaugcguuca aguaugaaaa cggcaaguac gacauuaaag acgugggcgu     600

ggauaacgcu ggcgcgaaag cgggucugac cuuccugguu gaccugauua aaaacaaaca     660

caugaaugca gacaccgauu acuccaucgc agaagcugcc uuuaauaaag gcgaaacagc     720

gaugaccauc aacggcccgu gggcaugguc caacaucgac accagcaaag ugaauuaugg     780

uguaacggua cugccgaccu ucaaggguca accauccaaa ccguucguug gcgugcugag     840

cgcagguauu aacgccgcca guccgaacaa agagcuggca aaagaguucc ucgaaaacua     900

ucugcugacu gaugaagguc uggaagcggu uaauaaagac aaaccgcugg gugccguagc     960

gcugaagucu uacgaggaag aguuggcgaa agauccacgu auugccgcca cuauggaaaa    1020

cgcccagaaa ggugaaauca ugccgaacau cccgcagaug uccgcuuucu gguaugccgu    1080

gcguacugcg gugaucaacg ccgccagcgg ucgucagacu gucgaugaag cccugaaaga    1140

cgcgcagacu cguaucacca aguagcacca ccaccaccac cacuaa                   1186


<210> SEQ ID NO 36
<211> LENGTH: 501
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 36

auguagccgu ccaaggacuc caaagcucag guuucugcag ccgaagcugg uaucacuggc      60

accugguaua accaacuggg gucgacuuuc auugugaccg cuggugcgga cggagcucug     120

acuggcaccu acgaaucugc gguugguaac gcagaauccc gcuacguacu gacuggccgu     180

uaugacucug caccugccac cgauggcucu gguaccgcuc ugggcuggac uguggcuugg     240

aaaaacaacu agcguaaugc gcacagcgcc acuacugggu cuggccaaua cguuggcggu     300

gcugaggcuc guaucaacac ucaguggcug uuaacauccg gcacuaccga agcgaaugca     360

uggaaaucga cacuaguagg ucaugacacc uuuaccaaag uuaagccuuc ugcugcuagc     420

auugaugcug ccaagaaagc aggcguaaac aacgguaacc cucuagacgc uguucagcaa     480
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-continued

```
caccaccacc accaccacua a                                                     501


<210> SEQ ID NO 37
<211> LENGTH: 502
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 37

augcgggccg uccaaggacu ccaaagcuca gguuucugca gccgaagcug guaucacugg        60

caccugguau aaccaacugg ggucgacuuu cauugugacc gcuggugcgg acggagcucu        120

gacuggcacc uacgaaucug cgguugguaa cgcagaaucc cgcuacguac ugacuggccg        180

uuaugacucu gcaccugcca ccgauggcuc ugguaccgcu cugggcugga cuguggcuug        240

gaaaaacaac uagcguaaug cgcacagcgc cacuacgugg ucuggccaau acguuggcgg        300

ugcugaggcu cguaucaaca cucaguggcu guuaacaucc ggcacuaccg aagcgaaugc        360

auggaaaucg acacuaguag gucaugacac cuuuaccaaa guuaagccuu cugcugcuag        420

cauugaugcu gccaagaaag caggcguaaa aacgguaac ccucuagacg cguuucagca        480

acaccaccac caccaccacu aa                                                    502


<210> SEQ ID NO 38
<211> LENGTH: 76
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (73)..(73)
<223> OTHER INFORMATION: n is a, c, g, or u

<400> SEQUENCE: 38

gggagaguag uucaauggua gaacgucggu cucuaaaacc gagcguugag gguucgauuc        60

cuuucucucc cancca                                                          76


<210> SEQ ID NO 39
<211> LENGTH: 77
<212> TYPE: RNA
<213> ORGANISM: Saccharomyces cerevisiae

<400> SEQUENCE: 39

gcggauuuag cucaguuggg agagcgccag acucccgaau cuggaggucc uguguucgau        60

ccacagaauu cgcacca                                                         77


<210> SEQ ID NO 40
<211> LENGTH: 78
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (75)..(75)
<223> OTHER INFORMATION: n is a, c, g, or u

<400> SEQUENCE: 40

gcggauuuag cucaguuggg agagcgccag acucccgaau cuggaggucc uguguucgau        60

ccacagaauu cgcancca                                                        78


<210> SEQ ID NO 41
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<211> LENGTH: 556
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic

<400> SEQUENCE: 41

auggcuagca ugacuggugg acagcaaaug gguccccggg aguaacgaau uccauaugga      60

cccguccaag gacuccaaag cucagguuuc ugcagccgaa gcugguauca cuggcaccug     120

guauaaccaa cuggggucga cuuucauugu gaccgcuggu gcggacggag cucugacugg     180

caccuacgaa ucugcgguug guaacgcaga aucccgcuac guacugacug gccguuauga     240

cucugcaccu gccaccgaug gcucugguac cgcucugggc uggacugugg cuuggaaaaa     300

caacuaucgu aaugcgcaca gcgccacuac guggucuggc caauacguug gcggugcuga     360

ggcucguauc aacacucagu ggcuguuaac auccggcacu accgaagcga augcauggaa     420

aucgacacua guaggucaug acaccuuuac caaaguuaag ccuucugcug cuagcauuga     480

ugcugccaag aaagcaggcg uaaacaacgg uaacccucua gacgcuguuc agcaacacca     540

ccaccaccac cacuaa                                                     556
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The invention claimed is:

1. A mutant tRNA, which is a mutant of tRNA for tryptophan obtained from a microorganism selected from the group consisting of *Mycoplasma capricolum*, *Bacillus halodouranns*, *Bacillus subtillis*, *Borrelia burgdorferi*, *Mycoplasma genitalium*, *Mycoplasma pneumoniae* 1, *Mycoplasma pneumoniae* 2 and *Staphylococcus aureus* N315,

wherein the mutation consists of;

(i) G at the 5' end,

(ii) C at 5th base from the 3' end which pairs with the G of (i) at the 5' end, and A at 4th base from the 3' end which is adjacent to the 3' side of the C of (ii),

wherein the mutant tryptophan tRNA (mtRNAcuA$_{CUA}$^{trp}) pairs with a UAG codon and has CUA as an anticodon; and

where said mtRNA$_{CUA}$^{trp} has a higher efficiency of incorporation of an unnatural amino acid, a modified amino acid or a derivative thereof into a protein in an in-vitro cell-free translation system compared to the wild type tRNA^{trp} or to a tRNA$_{CUA}$^{trP} that does not comprise the mutations of (i), (ii) or (iii).

2. The mutant tRNA of claim 1, which consists of nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20 and SEQ ID NO:25.

3. The mutant tRNA of claim 1 into which a single base A, C, G, or U has been inserted at the 4th position from the 3' end, which is adjacent to 5' side of ACC sequence located at 1st to 3rd position from the 3' end.

4. The mutant tRNA of claim 1, which is aminoacylated with an amino acid derivative and wherein the amino acid derivative is selected from the group consisting of hydroxy acid, mercapto acid, and carboxylic acid.

5. The mutant tRNA of claim 1, wherein the amino acid is fluorescently-labeled.

6. A method for introducing an amino acid selected from the group consisting of an unnatural amino acid, a modified amino acid, or a derivative thereof into a protein, the method comprising:

providing an mRNA of a protein into which said amino acid is introduced; the mutant tRNA of claim 1 and

allowing the mutant tRNA to pair with the UAG codon, wherein the mRNA has a UAG codon that is a codon corresponding to a site at which an amino acid is introduced thereby introducing said amino acid into the protein.

7. A method of producing a protein comprising two different phosphor amino acids in an in-vitro translation system, wherein the method comprises:

preparing an mRNA into which a single 4-base codon and a single UAG codon have been inserted at specified positions;

preparing two mutant tRNA molecules of claim 1 comprising anti-codons that pair with the 4-base codon and the UAG codon, and wherein one of the tRNA molecules is bound to an amino acid labeled with the fluorescent energy donor and the other tRNA is bound to an amino acid labeled with a fluorescent energy acceptor; and

allowing the translation system to synthesize the protein wherein, the protein encoded by said mRNA displays a change in distance and orientation upon interaction with other molecules and results in a change in the efficiency of fluorescence resonance energy transfer.

8. The method of claim 6, wherein protein synthesis is carried out in a cell-free translation system.

9. The method of claim 7, wherein protein synthesis is carried out in a cell-free translation system.

10. A method for introducing an amino acid selected from the group consisting of an unnatural amino acid, a modified amino acid, or a derivative thereof into a protein, the method comprising:

introducing an amino acid into a protein in a manner such that mRNA of a protein into which an amino acid is introduced and the mutant tRNA of claim 3 are employed; and

allowing the mutant tRNA to pair with the UAG codon, wherein the mRNA has a UAG codon that is a codon corresponding to a site at which an amino acid is introduced.

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11. A method of producing a protein comprising two different phosphor amino acids in an in-vitro translation system, wherein the method comprises preparing an mRNA into which a single 4-base codon and a single UAG codon have been inserted at specified positions;

preparing two mutant tRNA molecules of claim **3** comprising anti-codons that pair with the 4-base codon and the UAG codon, and wherein one of the tRNA molecules is bound to an amino acid labeled with the fluorescent energy donor and the other tRNA is bound to an amino acid labeled with a fluorescent energy acceptor; and

allowing the translation system to synthesize the protein wherein, the protein encoded by said mRNA displays a change in distance and orientation upon interaction with other molecules and results in a change in the efficiency of fluorescence resonance energy transfer.

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