inhibited by the native bacterial termination complex, which normally recognizes the stop codon and terminates translation. Release Factor 1 (RF1) is a termination complex protein that facilitates the termination of translation by recognizing the amber codon in an mRNA sequence. RF1 recognition of the amber stop codon can promote pre-mature truncation products at the site of non-native amino acid incorporation, and thus decreased protein yield. Therefore, attenuating the activity of RF1 may increase nnAA incorporation into recombinant proteins.

[0004] It has previously been shown that nnAA incorporation can be increased by attenuating RF1 activity in 3 ways: 1) neutralizing antibody inactivation of RF1, 2) genomic knockout of RF1 (in an RF2 bolstered strain), and 3) site specific removal of RF1 using a strain engineered to express RF1 containing a protein tag for removal by affinity chromatography (Chitin Binding Domain and His Tag). The present disclosure describes a novel method for inactivating RF1 by introducing proteolytic cleavage sites into the RF1 amino acid sequence. The cleavage sites are not accessible to the protease during bacterial cell growth, but are cleaved by the protease when the bacterial cells are lysed to produce cell-free extract. Thus, the yield of full length polypeptides having a nnAA incorporated at an amber codon is increased in bacterial cell extracts expressing modified RF1 variants described herein.

BRIEF SUMMARY OF THE ~~INVENTION~~DISCLOSURE

[0005] The present ~~invention~~ disclosure provides recombinant target proteins that are modified to comprise an Outer Membrane Protein T1 (OmpT1) protease cleavage site. The OmpT1 cleavage site includes a scissile OmpT1 peptide bond. In some embodiments, the target protein is modified to introduce the OmpT1 cleavage site into a surface exposed motif of the target protein. Because OmpT1 cleaves substrates between dibasic residues with high efficiency, the native surface exposed motif is modified to include two adjacent basic amino acids that are positively charged at pH 7.0. The target protein can be an essential protein that is required, for example, for normal cell growth and/or survival.

[0006] In some embodiments, the surface exposed motif has a B factor of at least 50 $Å^2$ when the motif region of the protein is uncomplexed. In some embodiments, surface exposed motif has a total solvent accessible surface area of between about 25 $Å^2$ and about 225 $Å^2$. In some embodiments, the target protein is modified such that the scissile OmpT1 peptide bond

is located in a position of the protein which exhibits a Phi angle of from 0° to -180° or a Psi angle from 0° to +180° in a Ramachadran Plot.

[0007] In one aspect, the ~~invention~~ disclosure provides a bacterial cell expressing both OmpT1 and an essential target protein recombinantly modified to include a scissile OmpT1 peptide bond. In some embodiments, the scissile OmpT1 peptide bond is located within a surface exposed motif and the native motif is modified to include two adjacent basic amino acids that are positively charged at pH 7.0. In some embodiments, the bacterial cell expresses an essential target protein recombinantly modified to include a scissile OmpT1 peptide bond where the bond is in a position of the protein which is located within a surface exposed motif having a B factor of at least 50 $Å^2$ when the protein is uncomplexed. In some embodiments, the bacterial cell expresses an essential target protein recombinantly modified to include a scissile OmpT1 peptide bond where the bond is in a position of the protein which is located within a surface exposed motif having a total solvent accessible surface area of between about 25 $Å^2$ and about 225 $Å^2$. In one embodiment, the bacterial cell expresses an essential target protein recombinantly modified to include a scissile OmpT1 peptide bond where the bond is in a position of the protein which exhibits a Phi angle of from 0° to -180° or a Psi angle from 0° to +180° in a Ramachadran Plot. In one embodiment, the bacterial cell is an *E. coli*.

[0008] In some embodiments, the essential target protein is selected from RF1, RF2, RNAse, thioredoxin reductase, glutarodoxin reductase, glutathione reductase, amino acid degrading enzymes, polyphosphate kinase, or cold shock proteins.

[0009] In a second aspect, the ~~invention~~ disclosure provides a method for reducing the deleterious activity of a modified essential target protein in an *in vitro* cell free synthesis system, the method comprising the steps of:

i) culturing an OmpT1 positive bacteria expressing the modified essential target protein said protein modified to include a scissile OmptT1 peptide bond where the protein is modified to include two adjacent basic amino acids that are positively charged at pH 7.0;

ii) lysing the bacteria to create a cell free synthesis extract;

iii) contacting the essential target protein with OmpT1 in an amount sufficient to reduce intact protein by 50%;

iv) adding a nucleic acid template to the extract where the template codes for a protein of interest; and,

v) allowing the cell free synthesis system to produce the protein of interest.

[0010] In some embodiments of the method, the scissile OmptT1 peptide bond is located within a surface exposed motif having a B factor of at least 50 $Å^2$ when the protein is uncomplexed. In some embodiments, the scissile OmpT1 peptide bond is located within a surface exposed motif having a total solvent accessible surface area of between about 25 $Å^2$ and about 225 $Å^2$. In some embodiments, the scissile OmpT1 peptide bond is located in a position of the protein which exhibits a Phi angle of from 0° to -180° or a Psi angle from 0° to +180° in a Ramachadran Plot.

[0011] In some embodiments of the method, the oxidative phosphorylation system of the bacteria remains active after cell lysis and during the synthesis of the protein of interest. In one embodiment, the cell free synthesis system places a non-native amino acid at an amber codon of the protein of interest.

[0012] In a third aspect, the ~~invention~~ disclosure provides a functional Releasing Factor 1 protein (RF1) that is cleavable by an Outer Membrane Protein T1 (OmpT1), where a scissile OmpT1 peptide bond is located within the switch loop region corresponding to amino acids 287-304 of wild type RF1 (SEQ ID NO:1) and where the switch loop region is modified to include two adjacent basic amino acids that are positively charged at pH 7.0. In some embodiments, the two adjacent basic amino acids are independently selected from arginine and lysine. In other embodiments, the switch loop region is modified to have three adjacent basic amino acids. In some embodiments, the native asparagine at position 296 is substituted for one of the three adjacent basic amino acids. The functional, modified RF1 protein is cleaved by OmpT1 at a faster rate than the wild-type RF1. For example, in one embodiment, the cleavage activity of modified RF1 by OmpT1 is greater than 50% of the wild type RF1 (SEQ ID NO:1) after 30 minutes at 30° C when the modified and wild-type proteins are present at a similar concentration in a cell-free extract from bacteria expressing OmpT1.

[0013] In some embodiments, the functional RF1 that is cleavable by OmpT1 contains an OmpT1 cleavage peptide in the switch loop region.

[0014] In a fourth aspect, the ~~invention~~ disclosure provides a nucleic acid encoding a functional Releasing Factor 1 protein (RF1) that is cleavable by an Outer Membrane Protein T1

(OmpT1) where a scissile OmpT1 peptide bond is located within the switch loop region of RF1 corresponding to amino acids 287-304 of wild type RF1 (SEQ ID NO:1) and where the switch loop region is modified to include two adjacent basic amino acids that are positively charged at pH 7.0.

[0015] In a fifth aspect, the ~~invention~~ disclosure provides a method for reducing RF1 competition at an amber codon in an *in vitro* cell free synthesis system, the method comprising the steps of:

i) culturing an OmpT1 positive bacteria expressing a functional Releasing Factor 1 protein (RF1) that is cleavable by an Outer Membrane Protein T1 (OmpT1)

ii) lysing the bacteria to create a cell free synthesis extract;

iii) contacting the RF1 protein in the extract with OmpT1 in an amount to sufficient to reduce intact RF1 protein by 50%;

iv) adding a nucleic acid template to the extract where the template codes for a protein of interest and includes an amber codon; and,

v) allowing the cell free synthesis system to produce the protein of interest,

where the RF1 protein has a scissile OmpT1 peptide bond is located within the switch loop region corresponding to amino acids 287-304 of wild type RF1 (SEQ ID NO:1) and where the switch loop region is modified to include two adjacent basic amino acids that are positively charged at pH 7.0.

[0016] In one embodiment of the method, the OmpT1 positive bacteria is from *E.* coli. In some embodiments, the oxidative phosphorylation system of the bacteria remains active after cell lysis and during the synthesis of the protein of interest. In other embodiments, the cell free synthesis system places a non-native amino acid at the amber codon of the protein of interest.

[0017] In another aspect, the ~~invention~~ disclosure provides a cell free synthesis system comprising, in a single reaction mixture:

i) components from a bacterial lysate sufficient to translate a nucleic acid template encoding a protein;

ii) a nucleic acid template encoding a protein of interest and having at least one amber codon;

iii) tRNA complementary to the amber codon; and,

iv) a functional Releasing Factor 1 (RF1) protein cleavable by an Outer Membrane Protein T1 (OmpT1), where a scissile OmpT1 peptide bond is located within the

switch loop region of RF1 corresponding to amino acids 287-304 of wild type RF1 (SEQ ID NO:1) and where the switch loop region is modified to include two adjacent basic amino acids that are positively charged at pH 7.0.

[0018] In some embodiments, the cell free synthesis system reaction mixture further comprises a non-natural amino acid and corresponding amino acid synthetase, the synthetase able to charge the tRNA complimentary to the amber codon with the non-natural amino acid. In some embodiments, the cell free synthesis system generates ATP via an active oxidative phosphorylation system.

[0019] In yet another aspect, the ~~invention~~ disclosure provides a method for expressing protein in a cell-free synthesis system, comprising:

i. combining a nucleic acid template with a bacterial extract comprising an RF1 protein that has been cleaved by OmpT1 at a scissile OmpT1 peptide bond located within the switch loop region corresponding to amino acids 287-303 of wild type RF1 (SEQ ID NO:1) and where the switch loop region has been modified to include two adjacent basic amino acids that are positively charged at pH 7.0 under conditions that permit protein synthesis; and

ii. expressing a protein from the nucleic acid template.

[0020] In another aspect, the ~~invention~~ disclosure provides a bacterial cell comprising a genomically integrated sequence coding for a functional RF1 that is cleavable by OmpT1.

[0020a] In another aspect, there is provided a functional Releasing Factor 1 protein (RF1) that is cleavable by an Outer Membrane Protein T1 (OmpT1), where a scissile OmpT1 peptide bond is located within the switch loop region corresponding to amino acids 287-304 of wild type RF1 (SEQ ID NO:1) and where the switch loop region is modified to include three adjacent basic amino acids selected from arginine and lysine that are positively charged at pH 7.0.

[0020b] In another aspect, there is provided a functional Releasing Factor 1 protein (RF1) that is cleavable by an Outer Membrane Protein T1 (OmpT1), where a scissile OmpT1 peptide bond is located within the switch loop region, and wherein the switch loop region corresponding to amino acids 287-304 of wild type RF1 (SEQ ID NO:1) comprises an amino acid sequence selected from the group of sequences consisting of:

QARRGSTRRNLLGSGDRS (SEQ ID NO:26);

QQARRGTRRNLLGSGDRS (SEQ ID NO:27);

QQAARRGRRNLLGSGDRS (SEQ ID NO:28);

QQAEARRGRNLLGSGDRS (SEQ ID NO:29);

QQAEAARRGNLLGSGDRS (SEQ ID NO:30);

QQAEASARRGLLGSGDRS (SEQ ID NO:31);

QQAEASTARRGLGSGDRS (SEQ ID NO:32);

QQAEASTRARRGGSGDRS (SEQ ID NO:33);

QQAEASTRRARRGSGDRS (SEQ ID NO:34);

QQAEASTRRNARRGGDRS (SEQ ID NO:35);

QQAEASTRRNLARRGDRS (SEQ ID NO:36);

QQAEASTRRNLLARRGRS (SEQ ID NO:37);

QQAEASTRRNLLGARRGS (SEQ ID NO:38);

QQAEASTRRNLLGSARRG (SEQ ID NO:39);

QQAEASTRRNLLGSGARR (SEQ ID NO:40);

QQAWLAARRGRGGSGDRS (SEQ ID NO:41);

QQAEWLAARRGRGSGDRS (SEQ ID NO:42);

QQAEAWLAARRGRGGDRS (SEQ ID NO:43);

QQWGGRWARKKGTIGDRS (SEQ ID NO:44);

QQAWGGRWARKKGTIDRS (SEQ ID NO:45);

QQAEWGGRWARKKGTIRS (SEQ ID NO:46);

QQAEASTRRKRLGSGDRS (SEQ ID NO:13);

QQAEASTRRKRKGSGDRS (SEQ ID NO:20); and

QQAEASTRRKRRGSGDRS (SEQ ID NO:21).

[0020c] In another aspect, there is provided a nucleic acid encoding a functional Releasing Factor 1 protein (RF1) that is cleavable by an Outer Membrane Protein T1

(OmpT1) where a scissile OmpT1 peptide bond is located within the switch loop region of RF1 corresponding to amino acids 287-304 of wild type RF1 (SEQ ID NO:1) and where the switch loop region is modified to include three adjacent basic amino acids selected from arginine and lysine that are positively charged at pH 7.0.

[0020d] In another aspect, there is provided a method for reducing RF1 competition at an amber codon in an *in vitro* cell free synthesis system, the method comprising the steps of:

i) culturing an OmpT1 positive bacteria expressing a functional Releasing Factor 1 protein (RF1) that is cleavable by an Outer Membrane Protein T1 (OmpT1);

ii) lysing the bacteria to create a cell free synthesis extract;

iii) contacting the RF1 protein in the extract with OmpT1 in an amount to sufficient to reduce intact RF1 protein by 50%;

iv) adding a nucleic acid template to the extract where the template codes for a protein of interest and includes an amber codon; and,

v) allowing the cell free synthesis system to produce the protein of interest,

where the RF1 protein has a scissile OmpT1 peptide bond is located within the switch loop region corresponding to amino acids 287-304 of wild type RF1 (SEQ ID NO:1) and where the switch loop region is modified to include three adjacent basic amino acids selected from arginine and lysine that are positively charged at pH 7.0.

[0020e] In another aspect, there is provided a cell free synthesis system comprising in a single reaction mixture:

i) components from a bacterial lysate sufficient to translate a nucleic acid template encoding a protein;

ii) a nucleic acid template encoding a protein of interest and having at least one amber codon;

iii) tRNA complementary to the amber codon; and,

iv) a functional Releasing Factor 1 (RF1) protein cleavable by an Outer Membrane Protein T1 (OmpT1), where a scissile OmpT1 peptide bond is located within the switch loop region of RF1 corresponding to amino acids 287-304 of wild type RF1 (SEQ ID

NO:1) and where the switch loop region is modified to include three adjacent basic amino acids selected from arginine and lysine that are positively charged at pH 7.0.

[0020f]In another aspect, there is provided a method for expressing protein in a cell-free synthesis system, comprising:

i. combining a nucleic acid template with a bacterial extract comprising an RF1 protein that has been cleaved by OmpT1 at a scissile OmpT1 peptide bond located within the switch loop region corresponding to amino acids 287-303 of wild type RF1 (SEQ ID NO:1) and where the switch loop region has been modified to include three adjacent basic amino acids selected from arginine and lysine that are positively charged at pH 7.0 under conditions that permit protein synthesis; and

ii. expressing a protein from the nucleic acid template.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] **Figure 1** shows that modified RF1 protein variants containing OmpT cleavage sites have wild-type RF1 activity when added to cell-free extracts lacking OmpT activity. The ability of exogenous RF-1 variants to reduce suppression (increase chain termination) when adding a non-native amino acid at an amber codon introduced at position S378 of the Fc protein (Fc_S378TAG) was determined. Figure 1(A) Lane 1 is a control extract with no exogenously added RF1. The extract contains wild-type RF1, which results in reduced amber suppression, producing a truncated Fc (lower band in gel). Lane 2 is a control extract containing exogenously added wild-type (WT) RF1. The addition of wild-type RF1 results in a further reduction in amber suppression, which produces relatively more truncated Fc. Lanes 3-7 show cell-free extracts containing different RF1 variants of the invention. All five

REPLACEMENT SHEETS

1. A functional Releasing Factor 1 protein (RF1) that is cleavable by an Outer Membrane Protein T1 (OmpT1), where a scissile OmpT1 peptide bond is located within the switch loop region corresponding to amino acids 287-304 of wild type RF1 (SEQ ID NO:1) and where the switch loop region is modified to include three adjacent basic amino acids that are positively charged at pH 7.0.

2. The RF1 protein of claim 1 wherein the three adjacent basic amino acids are independently selected from the group comprising: arginine and lysine.

3. The RF1 protein of claim 1 where the switch loop region is modified to have at least two additional adjacent basic amino acids.

4. The RF1 protein of claim 1 wherein the native asparagine at position 296 is substituted for one of the three adjacent basic amino acids.

5. The RF1 protein of claim 1 wherein the cleavage activity by OmpT1 is greater than 50% of the wild type RF1 (SEQ ID NO:1) after 30 minutes at 30° C when the modified and wild-type proteins are present at a similar concentration in a cell-free extract from bacteria expressing OmpT1.

6. The RF1 protein of claim 1 wherein the switch loop region comprises an amino acid sequence selected from the group of sequences consisting of:

QQAEASTRR**K**LLGSGDRS (SEQ ID NO:5),
QQAEASTRR**R**LLGSGDRS (SEQ ID NO:6),
QQAEASTRR**KK**LGSGDRS (SEQ ID NO:12),
QQAEASTRR**KR**LGSGDRS (SEQ ID NO:13),
QQAEASTRR**KV**LGSGDRS (SEQ ID NO:14),
QQAEASTRR**RK**LGSGDRS (SEQ ID NO:15),
QQAEASTRR**RR**LGSGDRS (SEQ ID NO:16),
QQAEASTRR**RV**LGSGDRS (SEQ ID NO:17),
QQAEASTRR**KKK**GSGDRS (SEQ ID NO:18),

QQAEASTRR**KKR**GSGDRS (SEQ ID NO:19),
QQAEASTRR**KRK**GSGDRS (SEQ ID NO:20),
QQAEASTRR**KRR**GSGDRS (SEQ ID NO:21),
QQAEASTRR**RRR**GSGDRS (SEQ ID NO:22),
QQAEASTRR**RKR**GSGDRS (SEQ ID NO:23),
QQAEASTRR**RRK**GSGDRS (SEQ ID NO:24), and
QQAEASTRR**RKK**GSGDRS (SEQ ID NO:25).

7. A functional Releasing Factor 1 protein (RF1) that is cleavable by an Outer Membrane Protein T1 (OmpT1), wherein the switch loop region corresponding to amino acids 287-304 of wild type RF1 (SEQ ID NO:1) comprises an amino acid sequence selected from the group of sequences consisting of:

QARRGSTRRNLLGSGDRS (SEQ ID NO:26);
QQARRGTRRNLLGSGDRS (SEQ ID NO:27);
QQAARRGRRNLLGSGDRS (SEQ ID NO:28);
QQAEARRGRNLLGSGDRS (SEQ ID NO:29);
QQAEAARRGNLLGSGDRS (SEQ ID NO:30);
QQAEASARRGLLGSGDRS (SEQ ID NO:31);
QQAEASTARRGLGSGDRS (SEQ ID NO:32);
QQAEASTRARRGGSGDRS (SEQ ID NO:33);
QQAEASTRRARRGSGDRS (SEQ ID NO:34);
QQAEASTRRNARRGGDRS (SEQ ID NO:35);
QQAEASTRRNLARRGDRS (SEQ ID NO:36);
QQAEASTRRNLLARRGRS (SEQ ID NO:37);
QQAEASTRRNLLGARRGS (SEQ ID NO:38);
QQAEASTRRNLLGSARRG (SEQ ID NO:39); and
QQAEASTRRNLLGSGARR (SEQ ID NO:40).

8. The RF1 protein of claim 7 wherein the switch loop region comprises an amino acid sequence selected from the group of sequences consisting of:

QQAWLAARRGRGGSGDRS (SEQ ID NO:41);
QQAEWLAARRGRGSGDRS (SEQ ID NO:42);

QQAEAWLAARRGRGGDRS (SEQ ID NO:43);

QQWGGRWARKKGTIGDRS (SEQ ID NO:44);

QQAWGGRWARKKGTIDRS (SEQ ID NO:45); and

QQAEWGGRWARKKGTIRS (SEQ ID NO:46).

9. The RF1 protein of claim 7 wherein the switch loop region comprises an amino acid sequence selected from the group of sequences consisting of:

QQAEASTRR**KR**LGSGDRS (SEQ ID NO:13),

QQAEASTRR**KRK**GSGDRS (SEQ ID NO:20),

QQAEASTRR**KRR**GSGDRS (SEQ ID NO:21), and

QQAWLAARRGRGGSGDRS (SEQ ID NO:41).

10. A bacterial cell expressing both an Outer Membrane Protein T1 (OmpT1) and an essential target protein recombinantly modified to include a scissile OmpT1 peptide bond where the bond is in a position of the protein which is located within a surface exposed motif having a B factor of at least 50 Å2 when the protein is uncomplexed and where the native motif is modified to include two adjacent basic amino acids that are positively charged at pH 7.0.

11. A bacterial cell expressing both an Outer Membrane Protein T1 (OmpT1) and an essential target protein recombinantly modified to include a scissile OmpT1 peptide bond where the bond is in a position of the protein which is located within a surface exposed motif having a total solvent accessible surface area of between about 25 $Å^2$ and about 225 $Å^2$ and where the native motif is modified to include two adjacent basic amino acids that are positively charged at pH 7.0.

12. A bacterial cell expressing both an Outer Membrane Protein T1 (OmpT1) and an essential target protein recombinantly modified to include a scissile OmpT1 peptide bond where the bond is in a position of the protein which exhibits a Phi angle of from 0° to -180° or a Psi angle from 0° to +180° in a Ramachadran Plot and where the native motif is modified to include two adjacent basic amino acids that are positively charged at pH 7.0.

13. The bacterial cell of claims 10-12, wherein the essential target protein is selected from the group consisting of RF1, RF2, RNAse, protease, thioredoxin reductase,

glutarodoxin reductase, glutathione reductase, amino acid degrading enzymes, polyphosphate kinase, and cold shock proteins.

14. The bacterial cell of claims 10-12, wherein the bacterial cell is an *E. coli*.

15. A method for preparing a cell free synthesis extract, the method comprising the steps of:

i) culturing an OmpT1 positive bacteria expressing a modified essential target protein said protein modified to include a scissile OmptT1 peptide bond where the protein is modified to include two adjacent basic amino acids that are positively charged at pH 7.0; and

ii) lysing the bacteria to create a cell free synthesis extract.

16. The method of claim 15, wherein the scissile OmptT1 peptide bond is located within a surface exposed motif having a B factor of at least 50 Å2 when the protein is uncomplexed.

17. The method of claim 15, wherein the scissile OmptT1 peptide bond is located within a surface exposed motif having a total solvent accessible surface area of between about 25 Å2 and about 225 Å2.

18. The method of claim 15, wherein the scissile OmptT1 peptide bond is located in a position of the protein which exhibits a Phi angle of from 0° to -180° or a Psi angle from 0° to +180° in a Ramachadran Plot.

19. The method of claim 15 wherein the OmpT1 positive bacteria is E. coli.

20. The method of claim 15 wherein the oxidative phosphorylation system of the bacteria remains active after cell lysis and during the synthesis of the protein of interest.

21. The method of claim 15 where the cell free synthesis system places a non-native amino acid at an amber codon of the protein of interest.

22. A nucleic acid encoding a functional Releasing Factor 1 protein (RF1) that is cleavable by an Outer Membrane Protein T1 (OmpT1) where a scissile OmpT1 peptide bond is located within the switch loop region of RF1 corresponding to amino acids 287-304 of wild type RF1 (SEQ ID NO:1) and where the switch loop region is modified to include three adjacent basic amino acids that are positively charged at pH 7.0.

23. A method for preparing a cell free synthesis extract, the method comprising the steps of:

i) culturing an OmpT1 positive bacteria expressing a functional Releasing Factor 1 protein (RF1) that is cleavable by an Outer Membrane Protein T1 (OmpT1); and

ii) lysing the bacteria to create a cell free synthesis extract.

24. The method of claim 23 wherein the OmpT1 positive bacteria is from E. coli.

25. The method of claim 23 wherein the oxidative phosphorylation system of the bacteria remains active after cell lysis and during the synthesis of the protein of interest.

26. The method of claim 23 where the cell free synthesis system places a non-native amino acid at the amber codon of the protein of interest.

27. A cell free synthesis system comprising in a single reaction mixture:

i) components from a bacterial lysate sufficient to translate a nucleic acid template encoding a protein;

ii) a nucleic acid template encoding a protein of interest and having at least one amber codon;

iii) tRNA complementary to the amber codon; and,

iv) a functional Releasing Factor 1 (RF1) protein cleavable by an Outer Membrane Protein T1 (OmpT1), where a scissile OmpT1 peptide bond is located within the switch loop region of RF1 corresponding to amino acids 287-304 of wild type RF1 (SEQ ID NO:1) and where the switch loop region is modified to include three adjacent basic amino acids that are positively charged at pH 7.0.

28. The cell free synthesis system of claim 27 wherein the reaction mixture further comprises a non-natural amino acid and corresponding amino acid synthetase, the synthetase able to charge the tRNA complimentary to the amber codon with the non-natural amino acid.

29. The cell free synthesis system of claim 27 wherein the system generates ATP via an active oxidative phosphorylation system.

30. A method for expressing protein in a cell-free synthesis system, comprising:

i. combining a nucleic acid template with a bacterial extract comprising an RF1 protein that has been cleaved by OmpT1 at a scissile OmpT1 peptide bond located within the switch loop region corresponding to amino acids 287-303 of wild type RF1 (SEQ ID NO:1) and where the switch loop region has been modified to include two adjacent basic amino acids that are positively charged at pH 7.0 under conditions that permit protein synthesis; and

ii. expressing a protein from the nucleic acid template.

31. A bacterial cell comprising the functional RF1 of claim 1 or claim 7 incorporated into the bacterial cell's genome.

32. A bacterial extract comprising a modified essential target protein, said protein modified to include a scissile OmptT1 peptide bond where the protein is modified to include two adjacent basic amino acids that are positively charged at pH 7.0.

33. The bacterial extract of claim 32, wherein the essential target protein is a functional Releasing Factor 1 protein (RF1) that is cleavable by OmpT1.

WHAT IS CLAIMED IS:

1. A functional Releasing Factor 1 protein (RF1) that is cleavable by an Outer Membrane Protein T1 (OmpT1), where a scissile OmpT1 peptide bond is located within the switch loop region corresponding to amino acids 287-304 of wild type RF1 (SEQ ID NO:1) and where the switch loop region is modified to include three adjacent basic amino acids selected from arginine and lysine that are positively charged at pH 7.0.

2. The RF1 protein of claim 1 where the switch loop region is modified to have four or five adjacent basic amino acids.

3. The RF1 protein of claim 1 wherein the native asparagine at position 296 is substituted for one of the three adjacent basic amino acids.

4. The RF1 protein of claim 1 wherein the cleavage activity by OmpT1 is greater than 50% of the wild type RF1 (SEQ ID NO:1) after 30 minutes at 30° C when the modified and wild-type proteins are present at a similar concentration in a cell-free extract from bacteria expressing OmpT1.

5. The RF1 protein of claim 1 wherein the switch loop region comprises an amino acid sequence selected from the group of sequences consisting of:

QQAEASTRRKLLGSGDRS (SEQ ID NO:5),
QQAEASTRRRLLGSGDRS (SEQ ID NO:6),
QQAEASTRRKKLGSGDRS (SEQ ID NO:12),
QQAEASTRRKRLGSGDRS (SEQ ID NO:13),
QQAEASTRRKVLGSGDRS (SEQ ID NO:14),
QQAEASTRRRKLGSGDRS (SEQ ID NO:15),
QQAEASTRRRRLGSGDRS (SEQ ID NO:16),
QQAEASTRRRVLGSGDRS (SEQ ID NO:17),
QQAEASTRRKKKGSGDRS (SEQ ID NO:18),
QQAEASTRRKKRGSGDRS (SEQ ID NO:19),
QQAEASTRRKRKGSGDRS (SEQ ID NO:20),
QQAEASTRRKRRGSGDRS (SEQ ID NO:21),
QQAEASTRRRRRGSGDRS (SEQ ID NO:22),
QQAEASTRRRKRGSGDRS (SEQ ID NO:23),
QQAEASTRR**RRK**GSGDRS (SEQ ID NO:24), and
QQAEASTRR**RKK**GSGDRS (SEQ ID NO:25).

6. A functional Releasing Factor 1 protein (RF1) that is cleavable by an Outer Membrane Protein T1 (OmpT1), where a scissile OmpT1 peptide bond is located within the switch loop region, and wherein the switch loop region corresponding to amino acids 287-304 of wild type RF1 (SEQ ID NO:1) comprises an amino acid sequence selected from the group of sequences consisting of:

QARRGSTRRNLLGSGDRS (SEQ ID NO:26);
QQARRGTRRNLLGSGDRS (SEQ ID NO:27);
QQAARRGRRNLLGSGDRS (SEQ ID NO:28);
QQAEARRGRNLLGSGDRS (SEQ ID NO:29);
QQAEAARRGNLLGSGDRS (SEQ ID NO:30);
QQAEASARRGLLGSGDRS (SEQ ID NO:31);
QQAEASTARRGLGSGDRS (SEQ ID NO:32);
QQAEASTRARRGGSGDRS (SEQ ID NO:33);
QQAEASTRRARRGSGDRS (SEQ ID NO:34);
QQAEASTRRNARRGGDRS (SEQ ID NO:35);
QQAEASTRRNLARRGDRS (SEQ ID NO:36);
QQAEASTRRNLLARRGRS (SEQ ID NO:37);
QQAEASTRRNLLGARRGS (SEQ ID NO:38);
QQAEASTRRNLLGSARRG (SEQ ID NO:39);
QQAEASTRRNLLGSGARR (SEQ ID NO:40);
QQAWLAARRGRGGSGDRS (SEQ ID NO:41);
QQAEWLAARRGRGSGDRS (SEQ ID NO:42);
QQAEAWLAARRGRGGDRS (SEQ ID NO:43);
QQWGGRWARKKGTIGDRS (SEQ ID NO:44);
QQAWGGRWARKKGTIDRS (SEQ ID NO:45);
QQAEWGGRWARKKGTIRS (SEQ ID NO:46);
QQAEASTRRKRLGSGDRS (SEQ ID NO:13);
QQAEASTRRKRKGSGDRS (SEQ ID NO:20); and
QQAEASTRRKRRGSGDRS (SEQ ID NO:21).

7. A nucleic acid encoding a functional Releasing Factor 1 protein (RF1) that is cleavable by an Outer Membrane Protein T1 (OmpT1) where a scissile OmpT1 peptide bond is located within the switch loop region of RF1 corresponding to amino acids 287-304

of wild type RF1 (SEQ ID NO:1) and where the switch loop region is modified to include three adjacent basic amino acids selected from arginine and lysine that are positively charged at pH 7.0.

8. A method for reducing RF1 competition at an amber codon in an *in vitro* cell free synthesis system, the method comprising the steps of:

i) culturing an OmpT1 positive bacteria expressing a functional Releasing Factor 1 protein (RF1) that is cleavable by an Outer Membrane Protein T1 (OmpT1);

ii) lysing the bacteria to create a cell free synthesis extract;

iii) contacting the RF1 protein in the extract with OmpT1 in an amount to sufficient to reduce intact RF1 protein by 50%;

iv) adding a nucleic acid template to the extract where the template codes for a protein of interest and includes an amber codon; and,

v) allowing the cell free synthesis system to produce the protein of interest,

where the RF1 protein has a scissile OmpT1 peptide bond is located within the switch loop region corresponding to amino acids 287-304 of wild type RF1 (SEQ ID NO:1) and where the switch loop region is modified to include three adjacent basic amino acids selected from arginine and lysine that are positively charged at pH 7.0.

9. The method of claim 8 wherein the OmpT1 positive bacteria is from *E. coli*.

10. The method of claim 8 wherein the oxidative phosphorylation system of the bacteria remains active after cell lysis and during the synthesis of the protein of interest.

11. The method of claim 8 where the cell free synthesis system places a non-native amino acid at the amber codon of the protein of interest.

12. A cell free synthesis system comprising in a single reaction mixture:

i) components from a bacterial lysate sufficient to translate a nucleic acid template encoding a protein;

ii) a nucleic acid template encoding a protein of interest and having at least one amber codon;

iii) tRNA complementary to the amber codon; and,

iv) a functional Releasing Factor 1 (RF1) protein cleavable by an Outer Membrane Protein T1 (OmpT1), where a scissile OmpT1 peptide bond is located within the switch loop region of RF1 corresponding to amino acids 287-304 of wild type RF1 (SEQ ID

NO:1) and where the switch loop region is modified to include three adjacent basic amino acids selected from arginine and lysine that are positively charged at pH 7.0.

13. The cell free synthesis system of claim 12 wherein the reaction mixture further comprises a non-natural amino acid and corresponding amino acid synthetase, the synthetase able to charge the tRNA complimentary to the amber codon with the non-natural amino acid.

14. The cell free synthesis system of claim 12 wherein the system generates ATP via an active oxidative phosphorylation system.

15. A method for expressing protein in a cell-free synthesis system, comprising:

i. combining a nucleic acid template with a bacterial extract comprising an RF1 protein that has been cleaved by OmpT1 at a scissile OmpT1 peptide bond located within the switch loop region corresponding to amino acids 287-303 of wild type RF1 (SEQ ID NO:1) and where the switch loop region has been modified to include three adjacent basic amino acids selected from arginine and lysine that are positively charged at pH 7.0 under conditions that permit protein synthesis; and

ii. expressing a protein from the nucleic acid template.

16. A bacterial cell comprising the functional RF1 of claim 1 or claim 6 incorporated into the bacterial cell's genome.