

CLAIMS

WHAT IS CLAIMED IS:

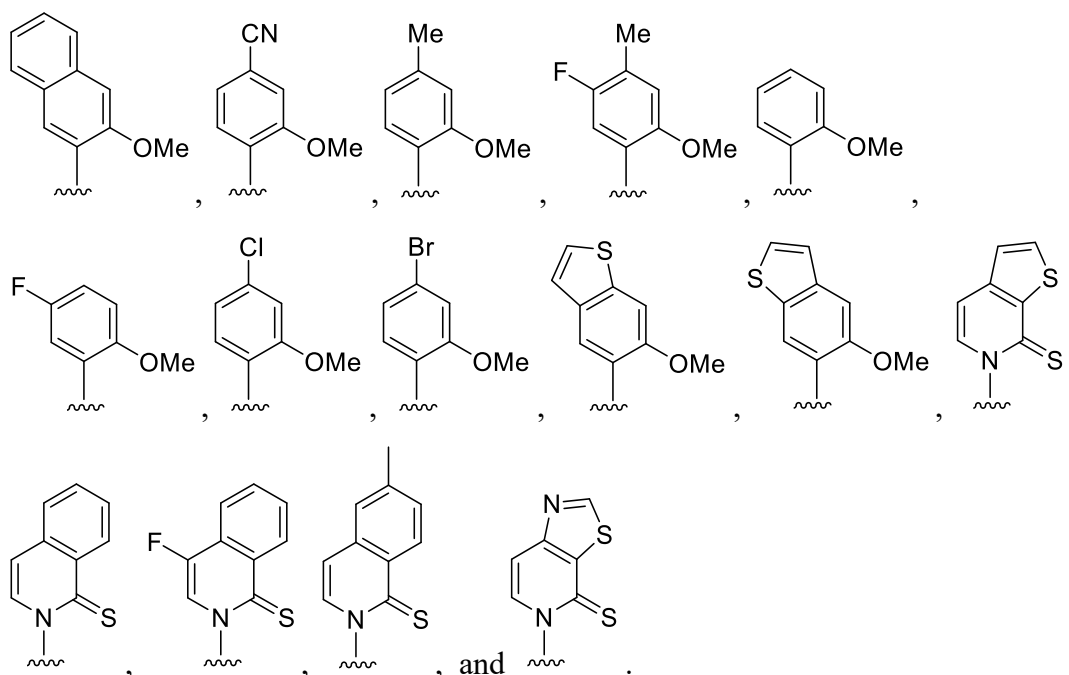
1. A method of synthesizing an unnatural polypeptide in an isolated cell or in a cell-free system, comprising:
 - a. providing at least one unnatural deoxyribonucleic acid (DNA) molecule comprising at least four unnatural base pairs, wherein the at least one unnatural DNA molecule encodes:
 - (i) a messenger ribonucleic acid (mRNA) molecule encoding an unnatural polypeptide that comprises at least a first unnatural amino acid and a second unnatural amino acid, wherein the first unnatural amino acid and the second unnatural amino acid are different, and wherein the mRNA molecule comprises at least a first unnatural codon that encodes the first unnatural amino acid and a second unnatural codon that encodes the second unnatural amino acid; and
 - (ii) at least first and second transfer RNA (tRNA) molecules, the first tRNA molecule comprising a first unnatural anticodon and the second tRNA molecule comprising a second unnatural anticodon, wherein the first unnatural anticodon and the second unnatural anticodon are different, and wherein the nucleotides of the first and second unnatural codons are complementary to the nucleotides of the first and second unnatural anticodons, respectively;wherein the first and second unnatural codons each independently comprises: (A) a nucleic acid sequence NNX; or (B) a nucleic acid sequence NXN, wherein at least one N is G or C; wherein the first and second unnatural anticodons each independently comprises a nucleic acid sequence XNN or NYN, to form an unnatural codon-anticodon pair comprising NNX-XNN or NXN-NYN; and wherein each N is independently a natural nucleotide, X is a first unnatural nucleotide, and Y is a second unnatural nucleotide different from the first unnatural nucleotide, with X-X or X-Y forming an unnatural base pair of the at least four unnatural base pairs in the DNA molecule;
 - b. transcribing the at least one unnatural DNA molecule to afford the mRNA molecule and the at least first and second tRNA molecules; and
 - c. synthesizing the unnatural polypeptide by translating the mRNA molecule of (b) utilizing the at least first and second tRNA molecules of (b), wherein the at least first and second tRNA molecules are charged by at least two different tRNA synthetases comprising a pyrrolysyl tRNA synthetase from the *Methanosarcina* genus and a

tyrosyl tRNA synthetase from *Methanocaldococcus jannaschii*, or derivatives thereof including chimeric pyrrolysyl tRNA synthetases, wherein each of the at least first and second unnatural anticodons direct site-specific incorporation of an unnatural amino acid into the unnatural polypeptide.

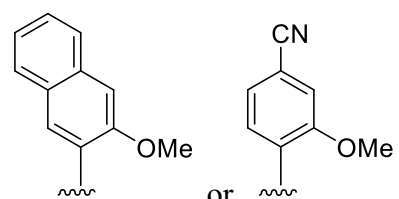
2. The method of claim 1, wherein the first unnatural codon and/or the second unnatural codon comprises a nucleic acid sequence NXN with at least one N being G or C, and the first unnatural anticodon and/or the second unnatural anticodon comprises a nucleic acid sequence NYN with at least one N being complementary C or G.
3. The method of claim 1, wherein the first unnatural codon and/or the second unnatural codon comprises a nucleic acid sequence NNX; and the first unnatural anticodon and/or the second unnatural anticodon comprises a nucleic acid sequence XNN.
4. The method of any one of claims 1-3, wherein X and Y are each independently a nucleotide or each independently comprise a base, wherein the nucleotide or the base is selected from:
 - (i) 2-thiouracil, 2'-deoxyuridine, 4-thio-uracil, uracil-5-yl, hypoxanthin-9-yl (I), 5-halouracil; 5-propynyl-uracil, 6-azo-uracil, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, pseudouracil, uracil-5-oxacetic acid methylester, uracil-5-oxacetic acid, 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, 5-methyl-2-thiouracil, 4-thiouracil, 5-methyluracil, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, or dihydrouracil;
 - (ii) 5-hydroxymethyl cytosine, 5-trifluoromethyl cytosine, 5-halocytosine, 5-propynyl cytosine, 5-hydroxycytosine, cyclocytosine, cytosine arabinoside, 5,6-dihydrocytosine, 5-nitrocytosine, 6-azo cytosine, azacytosine, N4-ethylcytosine, 3-methylcytosine, 5-methylcytosine, 4-acetylcytosine, 2-thiocytosine, phenoxazine cytidine([5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1, 4]benzothiazin-2(3H)-one), phenoxazine cytidine (9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5- b]indol-2-one), or pyridoindole cytidine (H-pyrido [3',2':4,5]pyrrolo [2,3-d]pyrimidin-2-one);

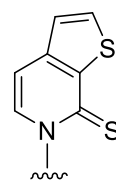
- (iii) 2-aminoadenine, 2-propyl adenine, 2-amino-adenine, 2-F-adenine, 2-amino-propyl-adenine, 2-amino-2'-deoxyadenosine, 3-deazaadenine, 7-methyladenine, 7-deaza-adenine, 8-azaadenine, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, and 8-hydroxyl substituted adenines, N6-isopentenyladenine, 2-methyladenine, 2,6-diaminopurine, 2-methylthio-N6- isopentenyladenine, or 6-aza-adenine;
- (iv) 2-methylguanine, 2-propyl and alkyl derivatives of guanine, 3-deazaguanine, 6-thio-guanine, 7-methylguanine, 7-deazaguanine, 7-deazaguanosine, 7-deaza-8-azaguanine, 8-azaguanine, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, and 8-hydroxyl substituted guanines, 1-methylguanine, 2,2-dimethylguanine, 7-methylguanine, or 6-aza-guanine; and
- (v) hypoxanthine, xanthine, 1-methylinosine, queosine, beta-D-galactosylqueosine, inosine, beta-D-mannosylqueosine, wybutoxosine, hydroxyurea, 2-aminopyridine, or 2-pyridone.

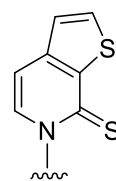
5. The method of any one of claims 1-3, wherein X and Y each comprise a base independently selected from:



6. The method of claim 5, wherein the X comprises a base





7. The method of claim 5 or 6, wherein the Y comprises a base .
8. The method of any one of claims 1-7, wherein at least one NNX-XNN is independently selected from: UUX-XAA, UGX-XCA, CGX-XCG, AGX-XCU, GAX-XUC, CAX-XUG, AUX-XAU, CUX-XAG, GUX-XAC, and UAX-XUA; and/or wherein at least one NXN-NYN is independently selected from: GXU-AYC, CXU-AYG, GXG-CYC, AXG-CYU, GXC-GYC, AXC-GYU, GXA-UYC, CXC-GYG, and UXC-GYA.
9. The method of any one of claims 1-8, wherein the at least first and second tRNA molecules comprise a pyrrolysyl tRNA molecule from the *Methanosarcina* genus and a tyrosyl tRNA molecule from *Methanocaldococcus jannaschii*, or derivatives thereof.
10. The method of any one of claims 1-9, wherein the at least two different tRNA synthetase are selected from chimeric PylRS (chPylRS) and *M. jannaschii* AzFRS (*MjpAzFRS*).
11. The method of any one of claims 1-10, wherein the unnatural polypeptide comprises two, three, or more unnatural amino acids.
12. The method of any one of claims 1-11, wherein the unnatural polypeptide further comprises a third unnatural amino acid and the unnatural polypeptide comprises at least two unnatural amino acids that are the same.
13. The method of any one of claims 1-12, wherein at least one of the first and second unnatural amino acids comprises
 - a lysine analogue;
 - an aromatic side chain;
 - an azido group;
 - an alkyne group; or
 - an aldehyde or ketone group.
14. The method of any one of claims 1-12, wherein at least one of the first and second unnatural amino acids does not comprise an aromatic side chain.

15. The method of any one of claims 1-12, wherein at least one of the first and second unnatural amino acids is selected from N6-((2-azidoethoxy-carbonyl-L-lysine (AzK), N6-propargylethoxy-carbonyl-L-lysine (PraK), N6-(propargyloxy)-carbonyl-L-lysine (PrK), p-azido-phenylalanine(*p*AzF), BCN-L-lysine, norbornene lysine, TCO-lysine, methyltetrazine lysine, allyloxycarbonyllysine, 2-amino-8-oxononanoic acid, 2-amino-8-oxooctanoic acid, p-acetyl-L-phenylalanine, p-azidomethyl-L-phenylalanine (pAMF), p-iodo-L-phenylalanine, m-acetylphenylalanine, 2-amino-8-oxononanoic acid, p-propargyloxyphenylalanine, p-propargyl-phenylalanine, 3-methyl-phenylalanine, L-Dopa, fluorinated phenylalanine, isopropyl-L-phenylalanine, p-azido-L-phenylalanine, p-acyl-L-phenylalanine, p-benzoyl-L-phenylalanine, p-bromophenylalanine, p-amino-L-phenylalanine, isopropyl-L-phenylalanine, O-allyltyrosine, O-methyl-L-tyrosine, O-4-allyl-L-tyrosine, 4-propyl-L-tyrosine, phosphonotyrosine, tri-O-acetyl-GlcNAcp-serine, L-phosphoserine, phosphoserine, L-3-(2-naphthyl)alanine, 2-amino-3-((2-((3-(benzyloxy)-3-oxopropyl)amino)ethyl)selanyl)propanoic acid, 2-amino-3-(phenylselanyl)propanoic, selenocysteine, N6-(((2-azidobenzyl)oxy)carbonyl)-L-lysine, N6-(((3-azidobenzyl)oxy)carbonyl)-L-lysine, and N6-(((4-azidobenzyl)oxy)carbonyl)-L-lysine.
16. The method of any one of claims 1-15, wherein the at least one unnatural DNA molecule is in the form of a plasmid.
17. The method of any one of claims 1-15, wherein the at least one unnatural DNA molecule is integrated into the genome of the isolated cell.
18. The method of any one of claims 1-17, wherein the isolated cell or cell-free system comprises the at least one unnatural DNA molecule, and wherein the method comprises replication and transcription of the at least one unnatural DNA molecule in the isolated cell or the cell-free system and translation of the transcribed mRNA molecule in the isolated cell or the cell-free system.
19. The method of claim 18, wherein the isolated cell is a prokaryote.
20. The method of claim 19, wherein the isolated cell is a bacterium.

21. The method of claim 20, wherein the isolated cell is a gram-positive bacterium.
22. The method of claim 20, wherein the isolated cell is a gram-negative bacterium.
23. The method of claim 22, wherein the isolated cell is *Escherichia coli*.
24. The method of any one of claims 1-3 and 5-23, wherein the at least four unnatural base pairs comprise base pairs each independently selected from dCNMO-dTPT3, dNaM-dTPT3, dCNMO-dTAT1, and dNaM-dTAT1.
25. The method of any one of claims 19-24, wherein the isolated cell comprises a nucleoside triphosphate transporter.
26. The method of claim 25, wherein the nucleoside triphosphate transporter comprises the amino acid sequence of *PtNTT2*.
27. The method of claim 26, wherein the nucleoside triphosphate transporter comprises a truncated amino acid sequence of *PtNTT2*.
28. The method of claim 27, wherein the truncated amino acid sequence of *PtNTT2* is at least about 80% identical to SEQ ID NO: 1.
29. The method of claim 18, wherein the at least one unnatural DNA molecule is in the form of a plasmid.
30. The method of claim 18, wherein the at least one unnatural DNA molecule is integrated into the genome of the isolated cell.
31. The method of any one of claims 1-16 or 18, wherein the method is performed in a cell-free system.
32. The method of any one of claims 1-31, wherein the at least four unnatural base pairs comprise at least one unnatural nucleotide comprising an unnatural sugar moiety.

33. The method of claim 32, wherein the unnatural sugar moiety comprises a modification selected from:
- a modification at the 2' position comprising:
- OH, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, or NH₂F; or O-alkyl, S-alkyl, or N-alkyl; or O-alkenyl, S-alkenyl, or N-alkenyl; or O-alkynyl, S-alkynyl, or N-alkynyl; or O-alkyl-O-alkyl, 2'-F, 2'-OCH₃, or 2'-O(CH₂)₂OCH₃, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁-C₁₀, alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, -O[(CH₂)_nO]_mCH₃, -O(CH₂)_nOCH₃, -O(CH₂)_nNH₂, -O(CH₂)_nCH₃, -O(CH₂)_n-NH₂, or -O(CH₂)_nON[(CH₂)_nCH₃]₂, wherein n and m are from 1 to 10;
- a modification at the 5' position comprising:
- 5'-vinyl, or 5'-methyl (R or S); or
- a modification at the 4' position comprising: 4'-S, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving at least one pharmacokinetic property of an oligonucleotide, or a group for improving at least one pharmacodynamic property of an oligonucleotide; or
- any combination thereof.
34. A cell comprising at least one unnatural DNA molecule comprising at least four unnatural base pairs, wherein the cell is a prokaryotic cell, and wherein the at least one unnatural DNA molecule encodes:
- (i) a messenger ribonucleic acid (mRNA) molecule encoding an unnatural polypeptide that comprises at least a first unnatural amino acid and a second unnatural amino acid, wherein the first unnatural amino acid and the second unnatural amino acid are different, and wherein the mRNA molecule comprises at least a first unnatural codon that encodes the first unnatural amino acid and a second unnatural codon that encodes the second unnatural amino acid; and
- (ii) at least first and second transfer RNA (tRNA) molecules, the first tRNA molecule comprising a first unnatural anticodon and the second tRNA molecule comprising a second unnatural anticodon, wherein the first unnatural anticodon and the second unnatural anticodon are different, and wherein the nucleotides of the first and second unnatural codons are complementary to the nucleotides of the first and second unnatural

anticodons, respectively;

wherein the first and second unnatural codons each independently comprises: (A) a nucleic acid sequence NNX; or (B) a nucleic acid sequence NXN, wherein at least one N is G or C; wherein the first and second unnatural anticodons each independently comprises a nucleic acid sequence XNN or NYN, to form an unnatural codon-anticodon pair comprising NNX-XNN or NXN-NYN; and

wherein each N is independently a natural nucleotide, X is a first unnatural nucleotide, and Y is a second unnatural nucleotide different from the first unnatural nucleotide, with X-X or X-Y forming an unnatural base pair of the at least four unnatural base pairs in the DNA molecule; and

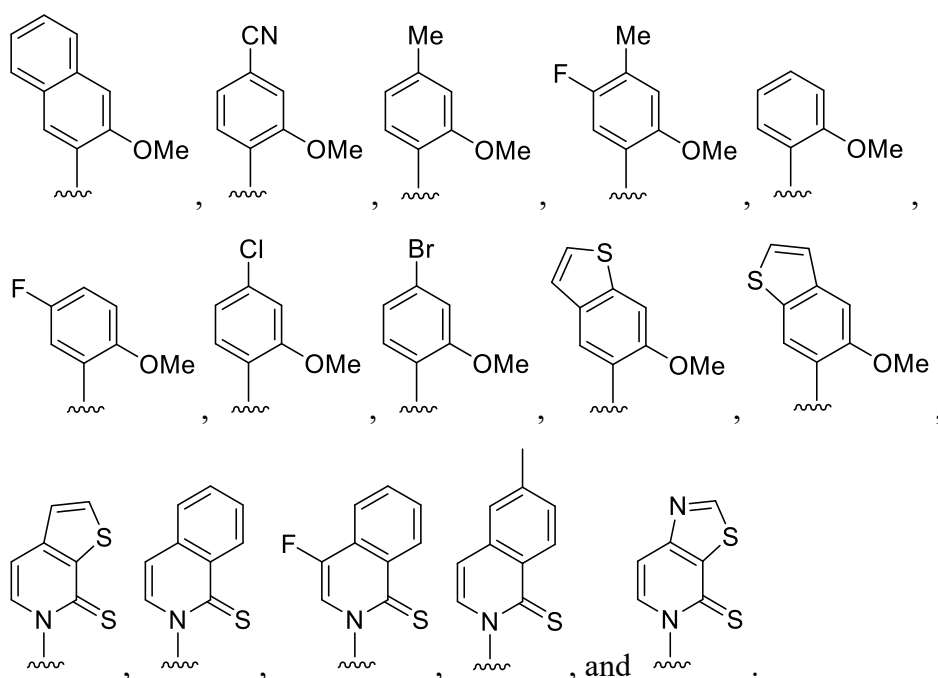
wherein the cell expresses at least two different tRNA synthetases comprising a pyrrolysyl tRNA synthetase from the *Methanosarcina* genus and/or a tyrosyl tRNA synthetase from *Methanocaldococcus jannaschii*, or derivatives thereof including chimeric pyrrolysyl tRNA synthetases, that charge the at least first and second tRNA molecules.

35. The cell of claim 34, wherein the first unnatural codon and/or the second unnatural codon comprises a nucleic acid sequence NXN with at least one N being G or C; and the first unnatural anticodon and/or the second unnatural anticodon comprises a nucleic acid sequence NYN with at least one N being complementary C or G.
36. The cell of claim 34, wherein the first unnatural codon and/or the second unnatural codon comprises a nucleic acid sequence NNX; and the first unnatural anticodon and/or the second unnatural anticodon comprises a nucleic acid sequence XNN.
37. The cell of any one of claims 34-36, further comprising the mRNA molecule encoded by the at least one unnatural DNA molecule and the at least first and second tRNA molecules encoded by the at least one unnatural DNA molecule, wherein the mRNA molecule and the at least first and second tRNA molecules are transcription products of the at least one unnatural DNA molecule.
38. The cell of any one of claims 34-37, wherein the at least first and second tRNA molecules are each covalently linked to a different unnatural amino acid.

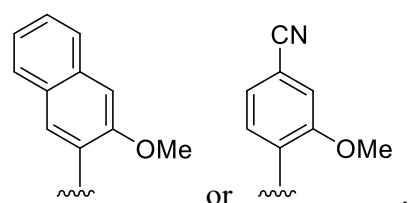
39. The cell of any one of claims 34-38, further comprising the unnatural polypeptide encoded by the mRNA molecule, wherein the unnatural polypeptide is a translation product of the mRNA molecule.
40. The cell of any one of claims 34-39, wherein X and Y are each independently a nucleotide or each independently comprise a base, wherein the nucleotide or the base is selected from:
- (i) 2-thiouracil, 2'-deoxyuridine, 4-thio-uracil, uracil-5-yl, hypoxanthin-9-yl (I), 5-halouracil; 5-propynyl-uracil, 6-azo-uracil, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, pseudouracil, uracil-5-oxacetic acid methylester, uracil-5-oxacetic acid, 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, 5-methyl-2-thiouracil, 4-thiouracil, 5-methyluracil, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, or dihydrouracil;
 - (ii) 5-hydroxymethyl cytosine, 5-trifluoromethyl cytosine, 5-halocytosine, 5-propynyl cytosine, 5-hydroxycytosine, cyclocytosine, cytosine arabinoside, 5,6-dihydrocytosine, 5-nitrocytosine, 6-azo cytosine, azacytosine, N4-ethylcytosine, 3-methylcytosine, 5-methylcytosine, 4-acetylcytosine, 2-thiocytosine, phenoxazine cytidine([5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), phenoxazine cytidine (9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), or pyridoindole cytidine (H-pyrido [3',2':4,5]pyrrolo [2,3-d]pyrimidin-2-one);
 - (iii) 2-aminoadenine, 2-propyl adenine, 2-amino-adenine, 2-F-adenine, 2-amino-propyl-adenine, 2-amino-2'-deoxyadenosine, 3-deazaadenine, 7-methyladenine, 7-deaza-adenine, 8-azaadenine, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, and 8-hydroxyl substituted adenines, N6-isopentenyladenine, 2-methyladenine, 2,6-diaminopurine, 2-methylthio-N6- isopentenyladenine, or 6-aza-adenine;
 - (iv) 2-methylguanine, 2-propyl and alkyl derivatives of guanine, 3-deazaguanine, 6-thio-guanine, 7-methylguanine, 7-deazaguanine, 7-deazaguanosine, 7-deaza-8-azaguanine, 8-azaguanine, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, and 8-hydroxyl substituted guanines, 1-methylguanine, 2,2-dimethylguanine, 7-methylguanine, or 6-aza-guanine; and

(v) hypoxanthine, xanthine, 1-methylinosine, queosine, beta-D-galactosylqueosine, inosine, beta-D-mannosylqueosine, wybutoxosine, hydroxyurea, 2-aminopyridine, or 2-pyridone.

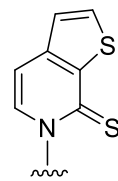
41. The cell of any one of claims 34-39, wherein the unnatural nucleotide of the first unnatural codon and the unnatural nucleotide of the second unnatural codon comprise first and second bases, respectively, independently selected from



42. The cell of claim 41, wherein the X comprises a base



43. The cell of claim 41 or 42, wherein the Y comprises a base



44. The cell of any one of claims 34-43, wherein at least one NNX-XNN is independently selected from: UUX-XAA, UGX-XCA, CGX-XCG, AGX-XCU, GAX-XUC, CAX-XUG, AUX-XAU, CUX-XAG, GUX-XAC, and UAX-XUA; and/or wherein at least one NXN-NYN is independently selected from: GXU-AYC, CXU-AYG, GXG-CYC, AXG-CYU, GXC-GYC, AXC-GYU, GXA-UYC, CXC-GYG, and UXC-GYA.

45. The cell of any one of claims 34-39 or 41-44, wherein the at least four unnatural base pairs are each independently selected from: the group dCNMO/dTPT3, dNaM/dTPT3, dCNMO/dTAT1, and dNaM/dTAT1.
46. The cell of any one of claims 34-45, wherein the at least one unnatural DNA molecule is in the form of a plasmid.
47. The cell of any one of claims 34-45, wherein the at least one unnatural DNA molecule is integrated into genome of the cell.
48. The cell of any one of claims 34-47, wherein the cell expresses a nucleoside triphosphate transporter.
49. The cell of claim 48, wherein the nucleoside triphosphate transporter comprises the amino acid sequence of *PtNTT2*.
50. The cell of claim 49, wherein the nucleoside triphosphate transporter comprises a truncated amino acid sequence of *PtNTT2*.
51. The cell of claim 50, wherein the truncated amino acid sequence of *PtNTT2* is at least about 80% identical to SEQ ID NO: 1.
52. The cell of any one of claims 34-51, wherein the at least first and second tRNA molecules comprise a pyrrolysyl tRNA molecule from the *Methanosarcina* genus and a tyrosyl tRNA molecule from *Methanocaldococcus jannaschii*, or derivatives thereof.
53. The cell of any one of claims 34-52, wherein the at least two different tRNA synthetases are chimeric PylRS (chPylRS) and *M. jannaschii* AzFRS (*MjpAzFRS*).
54. The cell of any one of claims 34 to 53, wherein the cell and/or the at least four unnatural base pairs comprises at least one unnatural nucleotide comprising an unnatural sugar moiety.

55. The cell of claim 54, wherein the unnatural sugar moiety comprises a modification selected from:
- a modification at the 2' position comprising
 - OH, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, or NH₂F; or
 - O-alkyl, S-alkyl, or N-alkyl; or
 - O-alkenyl, S-alkenyl, or N-alkenyl; or
 - O-alkynyl, S-alkynyl, or N-alkynyl; or
 - O-alkyl-O-alkyl, 2'-F, 2'-OCH₃, 2'-O(CH₂)₂OCH₃ wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁-C₁₀, alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, -O[(CH₂)_nO]_mCH₃, -O(CH₂)_nOCH₃, -O(CH₂)_nNH₂, -O(CH₂)_nCH₃, -O(CH₂)_n-NH₂, or -O(CH₂)_nON[(CH₂)_nCH₃]₂, wherein n and m are from 1 to 10;
 - a modification at the 5' position comprising:
 - 5'-vinyl, 5'-methyl (R or S); or
 - a modification at the 4' position comprising: 4'-S, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving at least one pharmacokinetic property of an oligonucleotide, or a group for improving at least one pharmacodynamic property of an oligonucleotide; or
 - any combination thereof.
56. The cell of any one of claims 34 to 55, wherein the cell comprises an RNA polymerase that recognizes at least one of the unnatural nucleotides or unnatural bases during transcription.
57. The cell of any one of claims 34 to 56, wherein the cell synthesizes at least one unnatural polypeptide comprising the at least first and second unnatural amino acids.
58. The cell of any one of claims 34-57, wherein at least one of the first and second unnatural amino acids comprises
- a lysine analogue;
 - an aromatic side chain;
 - an azido group;
 - an alkyne group; or

an aldehyde or ketone group.

59. The cell of any one of claims 34-57, wherein at least one of the first and second unnatural amino acids does not comprise an aromatic side chain.
60. The cell of any one of claims 37 to 57, wherein the at least first and second unnatural amino acids are each independently selected from: N6-((2-azidoethoxy-carbonyl-L-lysine (AzK), N6-propargylethoxy-carbonyl-L-lysine (PraK), N6-(propargyloxy)-carbonyl-L-lysine (PrK), p-azido-phenylalanine(pAzF), BCN-L-lysine, norbornene lysine, TCO-lysine, methyltetrazine lysine, allyloxycarbonyllysine, 2-amino-8-oxononanoic acid, 2-amino-8-oxooctanoic acid, p-acetyl-L-phenylalanine, p-azidomethyl-L-phenylalanine (pAMF), p-iodo-L-phenylalanine, m-acetylphenylalanine, 2-amino-8-oxononanoic acid, p-propargyloxyphenylalanine, p-propargyl-phenylalanine, 3-methyl-phenylalanine, L-Dopa, fluorinated phenylalanine, isopropyl-L-phenylalanine, p-azido-L-phenylalanine, p-acyl-L-phenylalanine, p-benzoyl-L-phenylalanine, p-bromophenylalanine, p-amino-L-phenylalanine, isopropyl-L-phenylalanine, O-allyltyrosine, O-methyl-L-tyrosine, O-4-allyl-L-tyrosine, 4-propyl-L-tyrosine, phosphonotyrosine, tri-O-acetyl-GlcNAcp-serine, L-phosphoserine, phosphoserine, L-3-(2-naphthyl)alanine, 2-amino-3-((2-((3-(benzyloxy)-3-oxopropyl)amino)ethyl)selanyl)propanoic acid, 2-amino-3-(phenylselanyl)propanoic, selenocysteine, N6-(((2-azidobenzyl)oxy)carbonyl)-L-lysine, N6-(((3-azidobenzyl)oxy)carbonyl)-L-lysine, and N6-(((4-azidobenzyl)oxy)carbonyl)-L-lysine.
61. The cell of any one of claims 34-60, wherein the unnatural polypeptide comprises two, three, or more unnatural amino acids.
62. The cell of any one of claims 34 to 61, wherein the cell is an isolated cell.
63. The cell of any one of claims 34-62, wherein the cell is a bacterium.
64. The cell of claim 63, wherein the cell is a gram-positive bacterium.
65. The cell of claim 63, wherein the cell is a gram-negative bacterium.
66. The cell of claim 65, wherein the gram-negative bacterium is *Escherichia coli*.

67. The cell of any one of claims 34-66, wherein:

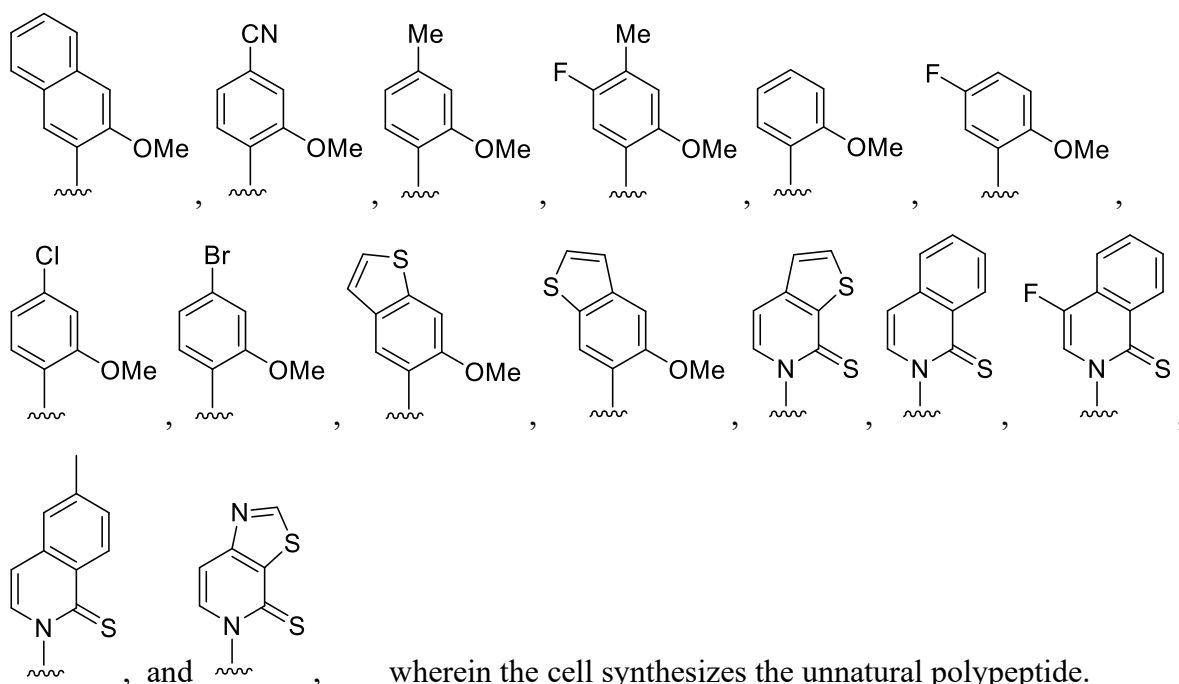
- the unnatural polypeptide encoded by the mRNA molecule comprises at least a third unnatural amino acid, and
- the mRNA molecule comprises at least a third unnatural codon that encodes the third unnatural amino acid, wherein the third unnatural codon independently comprises a nucleic acid sequence NNX or NXN, and the third unnatural anticodon independently comprises a nucleic acid sequence XNN or NYN, to form an unnatural codon-anticodon pair comprising NNX-XNN or NXN-NYN;

wherein each N is independently any natural nucleotide, X is a first unnatural nucleotide, and Y is a second unnatural nucleotide different from the first unnatural nucleotide, with X-X or X-Y forming an unnatural base pair of the at least four unnatural base pairs in the DNA molecule.

68. A cell line comprising the cell of any one of claims 34 to 67.

69. A method of synthesizing an unnatural polypeptide comprising at least two unnatural amino acids, comprising:

contacting the cell of any one of claims 34-67 with unnatural nucleoside triphosphates, wherein the unnatural nucleoside triphosphates each comprise a base independently selected from



70. The method of claim 69, further comprising contacting the cell with unnatural amino acids that correspond to the at least two unnatural amino acids, wherein the first and second unnatural codons encode the at least two unnatural amino acids.

71. The method of claim 69 or 70, wherein the unnatural polypeptide comprises at least three unnatural amino acids.

72. The method of claim 71, further comprising contacting the cell with unnatural amino acids that correspond to the at least three unnatural amino acids, wherein the first, second, and