## SUMMARY

**[0008]** There remains a need for optimal methods for efficiently producing recombinant two chain proteins on a preparative scale. In particular, the integration into the prokaryotic host cell chromosome of translational unit(s) encoding chaperone proteins, and/or the integration of non-native promoters to drive expression of native chaperone proteins, would allow for a single host cell that could be used to express a variety of recombinant protein products and simplify the plasmid engineering and protein purification protocols required for production.

**[0009]** To meet these and other demands, provided herein are prokaryotic host cells and methods of using the same in order to produce two-chain polypeptides. Advantageously, these host cells and methods allow for more efficient production of two-chain polypeptides, *e.g.*, without requiring up-front time and cost to optimize chaperone expression plasmids or downstream purification steps to remove chaperone proteins.

**[0010]** In one example, provided herein are methods of producing a polypeptide comprising two chains in a prokaryotic host cell comprising a host cell chromosome, the methods comprising: (a) culturing the host cell to express the two chains of the polypeptide in a culture medium under conditions suitable for expression of the two chains of the polypeptide, whereby upon expression the two chains fold and assemble to form a biologically active polypeptide in the host cell; wherein the host cell comprises: (1) a first polynucleotide comprising a first translational unit encoding a first chain of the polypeptide; (2) a second polynucleotide comprising a second translational unit encoding a second chain of the polypeptide, wherein the first and second polynucleotides are part of one or more extra-chromosomal polynucleotides; and (3) a third polynucleotide comprising a third translational unit encoding a chaperone protein selected from the group consisting of peptidyl-prolyl isomerases and protein disulfide oxidoreductases, wherein the third translational unit is part of the host cell chromosome, wherein the third translational unit is in operable combination with a promoter that is integrated in the host cell chromosome and drives transcription of the third translational unit, and wherein the combination of the third translational unit and the promoter is non-native to the host cell chromosome; and (b) recovering the biologically active polypeptide from the host cell.

**[0011]** In some examples, the promoter is an inducible promoter. In some examples, the inducible promoter is a Pho promoter that drives transcription of the third translational unit when phosphate in the culture medium has been depleted. In some examples, the inducible

promoter is non-native to the host cell chromosome; and (b) recovering the biologically active polypeptide from the host cell.

**[0012a]** In one aspect, the present disclosure refers to a method of producing a polypeptide comprising three chains in a prokaryotic host cell comprising a host cell chromosome, the method comprising: (a) culturing the host cell to express the three chains of the polypeptide in a culture medium under conditions suitable for expression of the three chains of the polypeptide, whereby upon expression the two chains fold and assemble to form a biologically active polypeptide in the host cell; wherein the host cell comprises: (1) a first polynucleotide comprising a first translational unit encoding a first chain of the polypeptide; (2) a second polynucleotide comprising a second translational unit encoding a second chain of the polypeptide, wherein the first and second polynucleotides are part of one or more extra-chromosomal polynucleotides; (3) a third polynucleotide comprising a third translational unit encoding a protein disulfide oxidoreductase, wherein the third translational unit is part of and native to the host cell chromosome, wherein the third translational unit is in operable combination with a first promoter that is integrated in the host cell chromosome and drives transcription of the third translational unit, and wherein the combination of the third translational unit and the first promoter is non-native to the host cell chromosome; and (4) a fourth polynucleotide comprising a fourth translational unit encoding a peptidyl-prolyl isomerase, wherein the fourth translational unit is part of and native to the host cell chromosome, wherein the fourth translational unit is in operable combination with a second promoter that is integrated in the host cell chromosome and drives transcription of the fourth translational unit, and wherein the combination of the fourth translational unit and the second promoter is non-native to the host cell chromosome; (5) a fifth polynucleotide comprising a fifth translational unit encoding a second protein disulfide oxidoreductase, wherein the fifth translational unit is part of and native to the host cell chromosome, wherein the fifth translational unit is in operable combination with a third promoter that is integrated in the host cell chromosome and drives transcription of the fifth translational unit, and wherein the combination of the fifth translational unit and the third promoter is non-native to the host cell chromosome; and (6) a sixth polynucleotide comprising a sixth translational unit encoding a third chain of the polypeptide, wherein the sixth polynucleotide is part of the one or more extra-chromosomal polynucleotides; whereby upon expression the three chains fold and assemble to form a biologically active polypeptide in the host cell; and (b) recovering the biologically active polypeptide from the host cell.

**[0013]** In some examples, the first and second promoters are both inducible promoters. In some examples, the first and second promoters are both Pho promoters that drive transcription of the third and fourth translational units, respectively, when phosphate in the culture medium has been depleted. In some examples, one of the first and second promoters is an inducible promoter, and the other of the first and second promoters is a constitutive promoter. In some examples, the first promoter is a Pho promoter that drives transcription of the third translational unit when phosphate in the culture medium has been depleted, and the second promoter is a CP25 promoter. In some examples, the second promoter is an inducible promoter, and the first promoter is a constitutive promoter. In some examples, one or both of the third translational unit and fourth translational unit are native to the host cell chromosome. In some examples, the third translational unit and the fourth translational unit are both native to the host cell chromosome. In some examples, one or both of the third translational unit and fourth translational unit are non-native to the host cell chromosome. In some examples, the protein disulfide oxidoreductase is a DsbC protein. In some examples, the protein disulfide oxidoreductase is *E. coli* DsbC. In some examples, the peptidyl-prolyl isomerase is an FkpA protein. In some examples, the FkpA is *E. coli* FkpA. In some examples, the protein disulfide oxidoreductase is *E. coli* DsbC, wherein the first promoter is a Pho promoter that drives transcription of the third translational unit when phosphate in the culture medium has been depleted, wherein the peptidyl-prolyl isomerase is *E. coli* FkpA, and wherein the second promoter is a CP25 promoter. In some examples, the protein disulfide oxidoreductase is *E. coli* DsbC, wherein the first promoter is a Pho promoter that drives transcription of the third translational unit when phosphate in the culture medium has been depleted, wherein the peptidyl-prolyl isomerase is *E. coli* FkpA, and wherein the second promoter is a Pho promoter that drives transcription of the fourth translational unit when phosphate in the culture medium has been depleted. In some examples, the host cell further comprises: (5) a fifth polynucleotide comprising a fifth translational unit encoding a second protein disulfide oxidoreductase, wherein the fifth translational unit is part of the host cell chromosome, wherein the fifth translational unit is in operable combination with a third promoter that is integrated in the host cell chromosome and drives transcription of the fifth translational

polynucleotide comprising a first translational unit encoding a peptidyl-prolyl isomerase, wherein the first translational unit is part of the host cell chromosome, wherein the first translational unit is in operable combination with a first promoter that is integrated in the host cell chromosome and drives transcription of the first translational unit, wherein the combination of the first translational unit and the first promoter is non-native to the host cell chromosome; and (2) a second polynucleotide comprising a second translational unit encoding a protein disulfide oxidoreductase, wherein the second translational unit is part of the host cell chromosome, wherein the second translational unit is in operable combination with a second promoter that is integrated in the host cell chromosome and drives transcription of the second translational unit, wherein the combination of the second translational unit and the second promoter is non-native to the host cell chromosome.

**[0017a]** In one aspect, the present disclosure refers to a prokaryotic host cell comprising a host cell chromosome, wherein the prokaryotic host cell comprises: (1) a first polynucleotide comprising a first translational unit encoding a peptidyl-prolyl isomerase, wherein the first translational unit is part of and native to the host cell chromosome, wherein the first translational unit is in operable combination with a first promoter that is integrated in the host cell chromosome and drives transcription of the first translational unit, and wherein the combination of the first translational unit and the first promoter is non-native to the host cell chromosome; (2) a second polynucleotide comprising a second translational unit encoding a protein disulfide oxidoreductase, wherein the second translational unit is part of and native to the host cell chromosome, wherein the second translational unit is in operable combination with a second promoter that is integrated in the host cell chromosome and drives transcription of the second translational unit, and wherein the combination of the second translational unit and the second promoter is non-native to the host cell chromosome, (3) a third polynucleotide comprising a third translational unit encoding a second protein disulfide oxidoreductase, wherein the third translational unit is part of the host cell chromosome, and wherein the third translational unit is in operable combination with a third promoter that is integrated in the host cell chromosome and drives transcription of the third translational unit, wherein the combination of the third translational unit and the third promoter is non-native to the host cell chromosome; (4) a fourth polynucleotide comprising a fourth translational unit encoding a peptidyl-prolyl isomerase, wherein the fourth translational unit is part of and native to the host cell chromosome, wherein the fourth translational unit is in operable combination with a second promoter that is integrated in the host cell chromosome and drives transcription of the fourth translational unit, and wherein the combination of the fourth translational unit and the second promoter is non-native to the host cell chromosome; (5) a fifth polynucleotide comprising a fifth translational unit encoding a third protein disulfide oxidoreductase, wherein the fifth translational unit is part of and native to the host cell chromosome, wherein the fifth translational unit is in operable combination with a third promoter that is integrated in the host cell chromosome and drives transcription of the fifth translational unit, and wherein the combination of the fifth translational unit and the third promoter is non-native to the host cell chromosome; and (6) a sixth polynucleotide comprising a sixth translational unit encoding a third chain of the polypeptide, wherein the sixth polynucleotide is part of the one or more extra-chromosomal polynucleotides; whereby upon expression the three chains fold and assemble to form a biologically active polypeptide in the host cell.

**[0018]** In some examples, one or both of the first translational unit and the second translational unit are native to the prokaryotic host cell chromosome. In some examples, the first translational unit and the second translational unit are both native to the prokaryotic host cell chromosome. In some examples, one or both of the first translational unit and the second translational unit are non-native to the prokaryotic host cell chromosome. In some examples, the first promoter is a first inducible promoter. In some examples, the first inducible promoter is a Pho promoter. In some examples, the first inducible promoter is an isopropyl beta-D-thiogalactoside (IPTG)-inducible promoter. In some examples, the first promoter is a first constitutive promoter. In some examples, the first constitutive promoter is a CP25 promoter. In some examples, the second promoter is a second inducible promoter. In some examples, the second inducible promoter is a Pho promoter. In some examples, the second inducible promoter is an isopropyl beta-D-thiogalactoside (IPTG)-inducible promoter. In some examples, the second promoter is a second constitutive promoter. In some examples, the second constitutive promoter is a CP25 promoter. In some examples, the peptidyl-prolyl isomerase is an FkpA protein. In some examples, the FkpA is *E. coli* FkpA. In some examples, the protein disulfide oxidoreductase is a DsbC protein. In some examples, the protein disulfide oxidoreductase is *E. coli* DsbC. In some examples, the peptidyl-prolyl isomerase is an FkpA protein, wherein the first promoter is a CP25 promoter, wherein the protein disulfide oxidoreductase is a DsbC protein, and wherein the second promoter is a Pho promoter. In some examples, the peptidyl-prolyl isomerase is an FkpA protein,

## CLAIMS

What is claimed is:

1. A method of producing a polypeptide comprising three chains in a prokaryotic host cell comprising a host cell chromosome, the method comprising:

(a) culturing the host cell to express the three chains of the polypeptide in a culture medium under conditions suitable for expression of the two chains of the polypeptide, whereby upon expression the three chains fold and assemble to form a biologically active polypeptide in the host cell;

wherein the host cell comprises:

(1) a first polynucleotide comprising a first translational unit encoding a first chain of the polypeptide;

(2) a second polynucleotide comprising a second translational unit encoding a second chain of the polypeptide, wherein the first and second polynucleotides are part of one or more extra-chromosomal polynucleotides;

(3) a third polynucleotide comprising a third translational unit encoding a protein disulfide oxidoreductase, wherein the third translational unit is part of and native to the host cell chromosome, wherein the third translational unit is in operable combination with a first promoter that is integrated in the host cell chromosome and drives transcription of the third translational unit, and wherein the combination of the third translational unit and the first promoter is non-native to the host cell chromosome; and

(4) a fourth polynucleotide comprising a fourth translational unit encoding a peptidyl-prolyl isomerase, wherein the fourth translational unit is part of and native to the host cell chromosome, wherein the fourth translational unit is in operable combination with a second promoter that is integrated in the host cell chromosome and drives transcription of the fourth translational unit, and wherein the combination of the fourth translational unit and the second promoter is non-native to the host cell chromosome;

(5) a fifth polynucleotide comprising a fifth translational unit encoding a second protein disulfide oxidoreductase, wherein the fifth translational unit is part of and native to the host cell chromosome, wherein the fifth translational unit is in operable combination with a third promoter that is integrated in the host cell chromosome and drives transcription of the fifth translational

unit, and wherein the combination of the fifth translational unit and the third promoter is non-native to the host cell chromosome; and

(6) a sixth polynucleotide comprising a sixth translational unit encoding a third chain of the polypeptide, wherein the sixth polynucleotide is part of the one or more extra-chromosomal polynucleotides; whereby upon expression the three chains fold and assemble to form a biologically active polypeptide in the host cell; and

(b) recovering the biologically active polypeptide from the host cell.

2. The method of claim 1, wherein the first and second promoters are both inducible promoters.

3. The method of claim 2, wherein the first and second promoters are both Pho promoters that drive transcription of the third and fourth translational units, respectively, when phosphate in the culture medium has been depleted.

4. The method of claim 1, wherein one of the first and second promoters is an inducible promoter, and the other of the first and second promoters is a constitutive promoter.

5. The method of claim 4, wherein the first promoter is an inducible promoter, and the second promoter is a constitutive promoter.

6. The method of claim 5, wherein the first promoter is a Pho promoter that drives transcription of the third translational unit when phosphate in the culture medium has been depleted, and the second promoter is a CP25 promoter.

7. The method of claim 1, wherein the protein disulfide oxidoreductase is *E. coli* DsbC, wherein the first promoter is a Pho promoter that drives transcription of the third translational unit when phosphate in the culture medium has been depleted, wherein the peptidyl-prolyl isomerase is *E. coli* FkpA, and wherein the second promoter is a CP25 promoter.

8. The method of claim 1, wherein the protein disulfide oxidoreductase is *E. coli* DsbC, wherein the first promoter is a Pho promoter that drives transcription of the third translational unit when phosphate in the culture medium has been depleted, wherein the peptidyl-prolyl isomerase is *E. coli* FkpA, and wherein the second promoter is a Pho promoter that drives

transcription of the fourth translational unit when phosphate in the culture medium has been depleted.

9. The method of any one of claims 1-8, wherein the third promoter is an inducible promoter.

10. The method of claim 9, wherein the third promoter is an isopropyl beta-D-thiogalactoside (IPTG)-inducible promoter that drives transcription of the fifth translational unit when IPTG is present in the culture medium.

11. The method of any one of claims 1-8, wherein the first protein disulfide oxidoreductase is *E. coli* DsbC, wherein the first promoter is an isopropyl beta-D-thiogalactoside (IPTG)-inducible promoter that drives transcription of the third translational unit when IPTG is present in the culture medium, wherein the peptidyl-prolyl isomerase is *E. coli* FkpA, wherein the second promoter is a CP25 promoter, wherein the second protein disulfide oxidoreductase is *E. coli* DsbA, wherein the third promoter is an isopropyl beta-D-thiogalactoside (IPTG)-inducible promoter that drives transcription of the fifth translational unit when IPTG is present in the culture medium.

12. The method of any one of claims 9-11, wherein the first translational unit encodes an immunoglobulin heavy chain, wherein the second translational unit encodes an immunoglobulin light chain, wherein the sixth translational unit encodes an immunoglobulin Fc fragment, and wherein the three chains fold and assemble to form a biologically active monovalent antibody.

13. The method of any one of claims 1-11, wherein the polypeptide is a half antibody in which the first chain and the second chain comprise an immunoglobulin heavy chain and an immunoglobulin light chain.

14. The method of any one of claims 1-13, wherein the prokaryotic host cell is *E. coli*.

15. The method of claim 14, wherein

(a) the *E. coli* is of a strain deficient in endogenous protease activity; and/or

(b) the *E. coli* is of a strain with enhanced LacI production or activity.

16. The method of claim 14, wherein the *E. coli* is of the strain Δ*fhuA* Δ*phoA ilvG*2096 (IlvG+; Valr) Δ*prc* spr43H1 Δ*manA lacI*$^{Q}$ Δ*ompT* Δ*menE*742 *degP*S210A.

17. A method of producing a bispecific antibody comprising a first half antibody capable of binding a first antigen and a second half antibody capable of binding a second antigen, the method comprising:

producing the first half antibody according to the method of any one of claims 1-11 or 14-16, wherein the first translational unit encodes the heavy chain of the first half antibody and the second translational unit encodes the light chain of the first half antibody, and wherein the first half antibody comprises at least one knob-forming mutation;

producing the second half antibody according to the method of any one of claims 1-11, or 14-16, wherein the first translational unit encodes the heavy chain of the second half antibody and the second translational unit encodes the light chain of the second half antibody, and wherein the second half antibody comprises at least one hole-forming mutation;

combining, in a reducing condition, the first half antibody with the second half antibody to produce the bispecific antibody.

18. The method of claim 17, wherein the first antigen and the second antigen are different antigens.

19. The method of claim 17 or claim 18, further comprising the step of adding a reducing agent to achieve the reducing condition.

20. The method of claim 19, wherein the reducing agent is glutathione.

21. A prokaryotic host cell comprising a host cell chromosome, wherein the prokaryotic host cell comprises:

(1) a first polynucleotide comprising a first translational unit encoding a peptidyl-prolyl isomerase, wherein the first translational unit is part of and native to the host cell chromosome, wherein the first translational unit is in operable combination with a first promoter that is integrated in the host cell chromosome and drives transcription of the first translational unit, and wherein the combination of the first translational unit and the first promoter is non-native to the host cell chromosome;

(2) a second polynucleotide comprising a second translational unit encoding a protein disulfide oxidoreductase, wherein the second translational unit is part of and native to the host cell chromosome, wherein the second translational unit is in operable combination with a second promoter that is integrated in the host cell chromosome and drives transcription of the second translational unit, and wherein the combination of the second translational unit and the second promoter is non-native to the host cell chromosome,
(3) a third polynucleotide comprising a third translational unit encoding a second protein disulfide oxidoreductase, wherein the third translational unit is part of the host cell chromosome, and wherein the third translational unit is in operable combination with a third promoter that is integrated in the host cell chromosome and drives transcription of the third translational unit, wherein the combination of the third translational unit and the third promoter is non-native to the host cell chromosome;
(4) a fourth polynucleotide comprising a fourth translational unit encoding a peptidyl-prolyl isomerase, wherein the fourth translational unit is part of and native to the host cell chromosome, wherein the fourth translational unit is in operable combination with a second promoter that is integrated in the host cell chromosome and drives transcription of the fourth translational unit, and wherein the combination of the fourth translational unit and the second promoter is non-native to the host cell chromosome;
(5) a fifth polynucleotide comprising a fifth translational unit encoding a third protein disulfide oxidoreductase, wherein the fifth translational unit is part of and native to the host cell chromosome, wherein the fifth translational unit is in operable combination with a third promoter that is integrated in the host cell chromosome and drives transcription of the fifth translational unit, and wherein the combination of the fifth translational unit and the third promoter is non-native to the host cell chromosome; and
(6) a sixth polynucleotide comprising a sixth translational unit encoding a third chain of the polypeptide, wherein the sixth polynucleotide is part of the one or more extra-chromosomal polynucleotides; whereby upon expression the three chains fold and assemble to form a biologically active polypeptide in the host cell.

22. The prokaryotic host cell of claim 21, wherein at least one of the peptidyl-prolyl isomerases is an FkpA protein, wherein the first promoter is a CP25 promoter, wherein at least

one of the protein disulfide oxidoreductases is a DsbC protein, and wherein the second promoter is a Pho promoter.

23. The prokaryotic host cell of claim 21, wherein at least one of the peptidyl-prolyl isomerases is an FkpA protein, wherein the first promoter is a Pho promoter, wherein at least one of the protein disulfide oxidoreductases is a DsbC protein, and wherein the second promoter is a Pho promoter.

24. The prokaryotic host cell of claim 21, wherein at least one of the peptidyl-prolyl isomerases is an FkpA protein, wherein the first promoter is a CP25 promoter, wherein the first protein disulfide oxidoreductase is a DsbC protein, wherein the second promoter is an isopropyl beta-D-thiogalactoside (IPTG)-inducible promoter, wherein the second protein disulfide oxidoreductase is a DsbA protein, and wherein the third promoter is an isopropyl beta-D-thiogalactoside (IPTG)-inducible promoter.

25. The prokaryotic host cell of claim 24, wherein the prokaryotic host cell is *E. coli*.

26. The prokaryotic host cell of claim 25, wherein

(a) the *E. coli* is of a strain deficient in endogenous protease activity; and/or

(b) the *E. coli* is of a strain with enhanced LacI production or activity.

27. The prokaryotic host cell of claim 25, wherein the *E. coli* is of the strain Δ*fhuA* Δ*phoA* *ilvG*2096 (IlvG+; Valr) Δ*prc* spr43H1 Δ*manA* $lacI^{Q}$ Δ*ompT* Δ*menE*742 *degP*S210A.

28. The prokaryotic host cell of any one of claims 21-27, further comprising an extra-chromosomal expression vector that comprises:

(a) a first extra-chromosomal polynucleotide comprising a first extra-chromosomal translational unit encoding a first chain of a two-chain polypeptide; and

(b) a second extra-chromosomal polynucleotide comprising a second extra-chromosomal translational unit encoding a second chain of the two-chain polypeptide;

whereby upon expression the two chains fold and assemble to form a biologically active two-chain polypeptide in the host cell.

29. The prokaryotic host cell of claim 28, wherein the extra-chromosomal expression vector further comprises a third extra-chromosomal polynucleotide comprising a third extra-chromosomal translational unit encoding a third chain of a two-chain polypeptide, whereby upon expression the three chains fold and assemble to form a biologically active polypeptide in the host cell.

30. The prokaryotic host cell of claim 29, wherein the first extra-chromosomal translational unit encodes an immunoglobulin heavy chain, wherein the second extra-chromosomal translational unit encodes an immunoglobulin light chain, wherein the third extra-chromosomal translational unit encodes an immunoglobulin Fc fragment, and wherein the three chains fold and assemble to form a biologically active monovalent antibody.