CLAIMS

WHAT IS CLAIMED IS:

1. A method for producing a recombinant ultralong CDR3 knob peptide, the method comprising: culturing a Pseudomonadales host cell in a culture medium and expressing the recombinant ultralong CDR3 knob peptide in the periplasm of the Pseudomonadales host cell from an expression construct comprising a nucleic acid encoding the recombinant ultralong CDR3 knob peptide directly and operably linked to a periplasmic secretion leader; wherein the recombinant ultralong CDR3 knob peptide is produced by secretion into the periplasm of the Pseudomonadales host cell, and wherein the secreted recombinant ultralong CDR3 knob peptide is present in soluble form, active form, and/or in properly processed form.

2. The method of claim 1, wherein the recombinant ultralong CDR3 knob peptide is about 3 to about 8 kDa; optionally wherein

i. the recombinant ultralong CDR3 knob peptide is about 25 to about 90 amino acids in length and comprises a cysteine motif, wherein the cysteine motif comprises 2-20 cysteine residues capable of forming 1-10 disulfide bonds; and/or

ii. the recombinant ultralong CDR3 knob peptide further comprises a first stalk-forming amino acid sequence and a second stalk-forming amino acid sequence, wherein the first and second stalk-forming sequences are, respectively, first and second β-strands of about 1 to about 15 amino acids in length, wherein

a. the first β-strands are anti-parallel to the second β-strands; and/or

b. the cysteine motif is positioned between the first and second stalk-forming amino acid sequences.

3. The method of claim 1 or 2, wherein the periplasmic secretion leader has at least 85% identity to an amino acid sequence selected from SEQ ID NOS: 24, 26, 28, 30, 32, 34, 38, and 40.

4. The method of any one of claims 1 to 3, wherein the nucleic acid encoding the recombinant ultralong CDR3 knob peptide is operably linked to a ribosome binding site sequence (RBS), optionally wherein

i. the sequence of the RBS is aggaggt or ggagcgt;

ii. the sequence of the nucleic acid encoding the recombinant ultralong CDR3 knob peptide is not directly and/or operably linked to a nucleic acid sequence encoding a fusion partner, a cleavable linker, or both; and/or

iii. the nucleic acid encoding the recombinant ultralong CDR3 knob peptide is optimized for expression in the host cell.

5. The method of any one of claims 1 to 4, further comprising: measuring the quality of the amount of the recombinant ultralong CDR3 knob peptide produced; optionally wherein the quality is measured by a target binding assay;

wherein the assay optionally comprises comparison to a reference, wherein the reference may be a negative control or a corresponding recombinant ultralong CDR3 knob peptide produced from a fusion construct.

6. The method of any one of claims 1 to 5, wherein the produced recombinant ultralong CDR3 knob peptide is present in the periplasm in soluble form at a yield of about 0.5 g/L to about 8 g/L; optionally wherein at least 80% of the produced recombinant ultralong CDR3 knob peptide present in the periplasm in soluble form is active.

7. The method of any one of claims 1 to 6, wherein the *Pseudomonadales* host cell is Pseudomonas fluorescens; optionally wherein the *Pseudomonadales* host cell is deficient in expression of one or more protease, overexpresses one or more folding modulator, overexpresses one or more inactivated protease, or a combination thereof; wherein

i. the one or more protease is selected from: Lon, HslU, HslV, DegP1, DegP2, DegP2 S219A, Prc1, Prc2, MepM1, a serralysin, and AprA; further optionally wherein the one or more protease comprises DegP2;

ii. the one or more protease is selected from: Prc1, Prc2, HslU, HslV, MepM1, and a serralysin; optionally wherein the serralysin is RXF04495.2; and/or

iii. the one or more folding modulator is selected from: SecB, DsbA, DsbC, Skp, and FklB2.

8. The method of claim 7, wherein the host cell

i. overexpresses: a) SecB; b) DsbA, DsbC and Skp; c) DsbA and DsbC; or d) DsbC and FklB2;

ii. is deficient in expression of DegP2, and overexpresses SecB;

iii. is deficient in expression of DegP2, and overexpresses DsbA, DsbC, and Skp;

iv. is deficient in expression of DegP2, and overexpresses DsbA and DsbC; or

v. overexpresses DsbC and FklB2.

9. The method of any one of claims 1 to 8, wherein the host strain

i. has a phenotype and genotype as set forth for any host strain in any one of Tables 3, 4, 6, and 9;

ii. has a phenotype, genotype, and expression construct sequence elements as set forth for any host strain in any one of Tables 3, 4, 6, and 9; or

iii. is any as set forth in any one of Tables 3, 4, 6, and 9.

10. The method of any one of claims 1 to 6, wherein

i. the periplasmic secretion leader has at least 85% identity to an amino acid sequence of SEQ ID NO: 26; and the host cell is deficient in expression of DegP2; and overexpresses SecB;

ii. the periplasmic secretion leader has at least 85% identity to an amino acid sequence of SEQ ID NO: 40; and the host cell is deficient in expression of DegP2; and overexpresses a) SecB, or b) DsbA, DsbC and Skp; or

iii. the periplasmic secretion leader has at least 85% identity to an amino acid sequence of SEQ ID NO: 30; and the host cell is deficient in expression of DegP2; and overexpresses SecB.

11. The method of any one of claims 1 to 10, wherein an amount of the ultralong CDR3 knob peptide secreted into the periplasm is released to the culture medium.

12. The method of any one of claims 1 to 11, further comprising purifying the produced recombinant ultralong CDR3 knob peptide; optionally wherein purifying the produced recombinant ultralong CDR3 knob peptide comprises: separating the cultured Pseudomonadales host cell expressing the recombinant ultralong CDR3 knob peptide from the culture medium to obtain separated Pseudomonadales host cell and separated culture medium; obtaining a cell lysate from the separated Pseudomonadales host cell; performing ultrafiltration of the cell lysate and/or the separated culture medium, to obtain an ultrafiltration permeate and an ultrafiltration concentrate; and

performing chromatographic separation of the ultrafiltration permeate to obtain the purified recombinant ultralong CDR3 knob peptide.

13. The method of claim 12, wherein the ultrafiltration comprises passing the cell lysate and/or the separated culture medium through one or more molecular weight cut offs (MWCO) of about 5 to about 50 kDA; optionally wherein performing chromatographic separation of the ultrafiltration permeate comprises performing cation exchange chromatography on the ultrafiltration permeate.

14. The method of claim 12, wherein purifying the recombinant ultralong CDR3 knob peptide comprises: separating the cultured Pseudomonadales host cell expressing the recombinant ultralong CDR3 knob peptide from the culture medium to obtain separated Pseudomonadales host cell and separated culture medium; obtaining a cell lysate from the separated Pseudomonadales host cell; performing a first chromatographic separation of the cell lysate and/or the separated culture medium, to obtain a first eluate containing the recombinant ultralong CDR3 knob peptide; and

performing a second chromatographic separation of the first eluate to obtain the purified recombinant ultralong CDR3 knob peptide.

15. The method of claim 14, wherein performing the first chromatographic separation of the cell lysate and/or the separated culture medium comprises performing cation exchange chromatography on the cell lysate and/or the separated culture medium; optionally wherein performing the second chromatographic separation of the first eluate comprises performing size exclusion chromatography on the first eluate.