BACTERIAL HOSTS FOR RECOMBINANT PROTEIN EXPRESSION

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 63/209,239 filed June 10, 2021, the contents of which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

[0002] Microbial host cell expression systems are used for production of recombinant proteins with varying degrees of success. Recombinant protein degradation and low yield remain challenges. Alterations of the host cell genome to optimize the production of high-quality protein often result in frustratingly low host cell growth and production yields. Recombinant host cells that achieve both high quality and high yield of recombinant proteins remain needed.

SUMMARY OF THE INVENTION

[0002a] In one aspect, provided herein is a recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell for recombinant protein expression, wherein the host cell is: (a) deficient in a first protease activity, wherein the first protease activity is tail-specific protease activity, wherein the deficient first protease activity results from a mutation in a gene encoding a Prc1 tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 33, a Prc1 tail-specific protease related protein having an amino acid sequence at least 90% similar to SEQ ID NO: 33, a Prc2 tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 35, or a Prc2 tail-specific protease related protein having an amino acid sequence at least 90% similar to SEQ ID NO: 35; and (b) deficient in a second protease activity, wherein the second protease activity is murein DD-endopeptidase activity, wherein the deficient second protease activity results from a mutation in at least one gene encoding a murein DD-endopeptidase; and wherein the host cell further (c) is deficient in at least one additional protease activity, wherein the deficient additional protease activity results from a mutation in at least one gene encoding an additional protease, wherein the additional protease is different from the proteases of (a) and (b); (d) is deficient in one or more autolytic factor activity, wherein the deficient autolytic factor activity results from a mutation in at least one gene encoding an autolytic factor; (e) overexpresses one or more inactivated protease; (f) overexpresses one or more folding modulator; or (g) any combination of (c), (d), (e) and (f).

[0002b] In one aspect, provided herein is a method for producing a recombinant protein of interest comprising: (a) recovering the recombinant protein of interest from the recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell as described herein cultured under suitable fermentation conditions, wherein the recombinant *Pseudomonas fluorescens* gram-negative host cell is transformed with a plasmid comprising a nucleic acid encoding the recombinant protein of interest.

[0002c] In one aspect, provided herein is a recombinant *Pseudomonas fluorescens* gram-negative host cell, wherein the host cell is: (a) deficient in a first protease activity, wherein the deficient first protease activity results from mutations in (i) a gene encoding a Prc1 tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 33; and, (ii) a gene encoding a Prc2 tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 35; and (b) deficient in a second protease activity, wherein the deficient second protease activity results from a mutation in a gene encoding a MepM1 murein DD-endopeptidase having the amino acid sequence set forth as SEQ ID NO: 1; and additionally wherein the host cell is further: (c) deficient in one additional protease activity, wherein the deficient additional protease activity results from a mutation of a gene encoding a serralysin precursor having the amino acid sequence set forth as SEQ ID NO: 9; (d) deficient in one or more autolytic factor activity, wherein the deficient autolytic factor activity results from a mutation in at least one gene encoding an autolytic factor independently selected from: a linear gramicidin synthase subunit D, or a hemolysin precursor; (e) overexpresses a mutant periplasmic serine endoprotease; (f) overexpresses a disulfide isomerase folding modulator having at least 60% similarity or at least 60% identity to an amino acid sequence set forth as SEQ ID NO: 27; or (g) any combination of (c), (d), (e) and (f).

[0003] The present invention provides recombinant bacterial host cells and methods for their use to produce high quality recombinant proteins at high yield. In particular, the compositions and methods provided by the invention are useful for producing recombinant proteins that are sensitive to proteolysis, including antibodies and antibody fragments. The invention includes recombinant gram-negative bacterial host cells comprising combinations of genetic modifications that result in reduced degradation of a recombinant protein while allowing growth to high cell density. In some embodiments, a recombinant gram-negative bacterial host cell of the present invention is deficient in first protease activity and a second protease activity, wherein the first protease activity is a tail-specific protease activity and the second protease activity is a murein DD-endopeptidase activity. In some embodiments, the invention relates to a recombinant gram-negative bacterial host cell that is deficient in tail-specific protease activity and a murein DD-endopeptidase activity, wherein the host cell produces high quality, undegraded, recombinant protein, and grows to a high cell density. In some embodiments, the murein DD-endopeptidase activity is a MepM activity. In some embodiments, the gram-negative bacterial host cell is not deficient in a MepS activity. In some embodiments, the recombinant gram-negative bacterial host cell deficient in a first protease activity and a second protease activity, wherein the first protease activity is a tail-specific protease activity, and the second protease activity is a MepM murein DD-endopeptidase activity, is further deficient in at least one additional protease activity, at least one autolytic factor activity, or both. In some embodiments, the at least one additional protease activity is a serralysin precursor activity. The deficiency in a protein activity, e.g., the first protease, the second protease, an additional protease, or an autolytic factor, can result from a

CLAIMS

WHAT IS CLAIMED IS:

1. A recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell for recombinant protein expression, wherein the host cell is:

(a) deficient in a first protease activity, wherein the first protease activity is tail-specific protease activity, wherein the deficient first protease activity results from a mutation in a gene encoding a Prc1 tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 33, a Prc1 tail-specific protease related protein having an amino acid sequence at least 90% similar to SEQ ID NO: 33, a Prc2 tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 35, or a Prc2 tail-specific protease related protein having an amino acid sequence at least 90% similar to SEQ ID NO: 35; and

(b) deficient in a second protease activity, wherein the second protease activity is murein DD-endopeptidase activity, wherein the deficient second protease activity results from a mutation in at least one gene encoding a murein DD-endopeptidase;

and wherein the host cell further

(c) is deficient in at least one additional protease activity, wherein the deficient additional protease activity results from a mutation in at least one gene encoding an additional protease, wherein the additional protease is different from the proteases of (a) and (b);

(d) is deficient in one or more autolytic factor activity, wherein the deficient autolytic factor activity results from a mutation in at least one gene encoding an autolytic factor;

(e) overexpresses one or more inactivated protease;

(f) overexpresses one or more folding modulator; or

(g) any combination of (c), (d), (e) and (f).

2. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 1, wherein the deficient murein DD-endopeptidase activity results from a mutation in a gene encoding a MepM1 murein DD-endopeptidase having the amino acid sequence set forth as SEQ ID NO: 1, or a murein DD-endopeptidase related protein having an amino acid sequence at least 30% similar to SEQ ID NO: 1.

3. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 1or 2, wherein: the host cell of (c) is deficient in 1 to 10 different additional protease activities; the host cell of (d) is deficient in 1-5 different autolytic factor activities; the host cell of (e) overexpresses 1 to 10 different inactivated proteases, wherein each inactivated protease is different; the host cell of (f) overexpresses 1-10 different folding modulators, or any combination thereof.

4. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 2, wherein:

the one or more deficient additional protease activity of (c) results from a mutation of a serralysin precursor having the amino acid sequence set forth as SEQ ID NO: 9; a serralysin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9; a serralysin precursor having the amino acid sequence set forth as SEQ ID NO: 47; or a serralysin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 47; the one or more deficient autolytic factor activity of (d) results from a mutation of at least one gene encoding an autolytic factor independently selected from: a linear gramicidin synthase subunit D, a hemolysin precursor, a leukotoxin, and a porin; the one or more inactivated protease of (e) is a mutant periplasmic serine endoprotease; and the one or more folding modulator of (f) is a disulfide isomerase.

5. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 2, wherein the gram-negative bacterial host cell further comprises a functional protease activity, wherein the functional protease activity is the activity of: a MepS1 having the amino acid sequence set forth as SEQ ID NO: 5; or a MepS1 related protein having at least 50% sequence similarity to the *P. fluorescens* MepS1 protease amino acid sequence set forth as SEQ ID NO: 5.

6. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of any preceding claim, wherein the gram-negative bacterial host cell further comprises a functional protease activity, wherein the functional protease activity is: a MepS2 having the amino acid

sequence set forth as SEQ ID NO: 7; or a MepS2 related protein having at least 50% sequence similarity to the *P. fluorescens* MepS2 protease amino acid sequence set forth as SEQ ID NO: 7.

7. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 1, wherein the deficient first protease activity results from a mutation of a coding sequence and/or noncoding sequence.

8. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 1, wherein the second protease activity is deficient due to a mutation that results in a conservative or non-conservative substitution in an active site amino acid or an allosteric site amino acid of a protease having the second protease activity.

9. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 1, wherein the deficient second protease activity results from at least one mutation of the second protease gene, wherein the mutation results in a disruption of the amino acid sequence at a position corresponding to: (i) any one or more of residues 134 to 145 of SEQ ID NO: 1; (ii) any one or more of residues 319 to 411 of SEQ ID NO: 1; (iii) one or more of residues any 361 to 378 of SEQ ID NO: 1; (iv) any one or more residue selected from 248, 319, 330, 332, 334, 337, 378, 410, and 411 of SEQ ID NO: 1; or any combination of (i), (ii), (iii), and (iv).

10. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of any preceding claim, wherein the bacterial host cell is *Pseudomonas fluorescens*, wherein the deficient second protease activity results from a gene mutation that results in an amino acid substitution of SEQ ID NO: 1 selected from: Y248stop, G332S, D334N, A337T, H411Y, P410L, and any conservative or non-conservative amino acid substitution of any one of R319, H330, D334, H378, and H411.

11. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 1, wherein the host cell is capable of high-density cell growth in culture.

12. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 1, further comprising at least one expression construct, each expression construct comprising at least one nucleic acid sequence encoding a recombinant protein of interest.

13. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 12, wherein the recombinant protein of interest is selected from: an antibody, antibody fragment, or derivative of an antibody or antibody fragment.

14. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 13, wherein the antibody, antibody fragment, or derivative thereof is selected from: a monoclonal antibody; a complementarity determining region (CDR) fragment; a CDR-grafted antibody; a single-chain antibody; a single chain antibody fragment; modified antibody, a bispecific antibody, a chimeric antibody; a diabody; a triabody; a tetrabody; a minibody; a linear antibody; a chelating recombinant antibody; a bibody; a tribody; an intrabody; a nanobody; a small modular immunopharmaceutical (SMIP); an antigen-binding-domain immunoglobulin fusion protein; a camelid antibody; a shark single domain antibody, an avian antibody (e.g., a chicken antibody), a VHH-containing antibody; a F(ab); a F(ab)'; F(ab)'$_2$; scFv; an Fc fragment generated from the heavy chain constant region of an antibody; a reduced IgG fragment (e.g., generated by reducing the hinge region disulfide bonds of IgG); an Fc fusion protein (e.g., comprising the Fc domain of IgG fused together with a protein or peptide of interest); a domain antibody; a VL; a VNAR; a VH; and a VHH.

15. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 12, wherein the antibody, antibody fragment, or derivative thereof, binds to a target selected from: a cytokine; a chemokine; a drug; a cell-surface protein, e.g., a receptor, cell-surface marker, pathogen surface-protein, etc.; a growth factor; a growth factor receptor; immune checkpoint molecule, and a blood factor.

16. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 12, wherein the antibody, antibody fragment, or derivative thereof is a Fab'.

17. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 16, wherein the Fab' binds to a target selected from: Carcinoembryonic antigen (CEA); CD22; fibrin II, beta chain; TNF-alpha; and NCA-90 (granulocyte antigen).

18. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 12, wherein the at least one expression construct encoding the antibody, antibody fragment, or derivative thereof comprises at least one nucleic acid sequence encoding a heavy chain, at least one nucleic acid sequence encoding a light chain, or both, wherein the heavy chain is full-length or a heavy chain fragment, and the light chain is full-length or a light chain fragment.

19. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 18, wherein each heavy chain-encoding nucleic acid sequence and each light chain-encoding nucleic acid sequence is individually operably linked to an independently selected nucleic acid sequence encoding a periplasmic secretion signal.

20. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 19, wherein the Fab' is certolizumab.

21. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 1, wherein the host cell is deficient in:

(i) the first protease activity;

(ii) the second protease activity;

(iii) the activity of a serralysin precursor having the amino acid sequence set forth as SEQ ID NO: 9, a homologue of the serralysin precursor having the amino acid sequence set forth as SEQ ID NO: 9, or a serralysin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9;

(iv) an HslU protease having the amino acid sequence set forth as SEQ ID NO: 37, a homologue of the HslU protease having the amino acid sequence set forth as SEQ ID NO: 37, or a HslU related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 37; and

(v) an HslV protease having the amino acid sequence set forth as SEQ ID NO: 38, a homologue of the HslV protease having the amino acid sequence set forth as SEQ ID NO: 38, or a HslV related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 38.

22. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 1, wherein the cell is:

(i) lsc::lacIQ1;

(ii) Prc1-

(ii) Prc2-

(iii) HslU-

(iv) HslV-

(v) MepM1-

(vi) PyrF-

and (vii) deficient in a serralysin precursor that is: a serralysin precursor having the amino acid sequence set forth as SEQ ID NO: 9; a homologue of the serralysin precursor having the amino acid sequence set forth as SEQ ID NO: 9; or a serralysin precursor related protein having at least 60% similarity or at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 9; wherein the serralysin precursor deficiency results from a mutation in a gene encoding the serralysin precursor.

23. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 22, further comprising an expression vector comprising a nucleic acid sequence encoding disulfide isomerase PDIA6 (SEQ ID NO: 27).

24. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 1, further comprising an expression vector encoding a recombinant protein.

25. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 24, wherein the expression vector encodes a Fab'.

26. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 1, wherein the recombinant *Pseudomonas fluorescens* gram-negative bacterial host has the genotype of strain STR94976.

27. The recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 26, further comprising an expression construct for use in producing a recombinant anti-TNF-alpha Fab'.

28. A method for producing a recombinant protein of interest comprising: (a) recovering the recombinant protein of interest from the recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell of claim 1 cultured under suitable fermentation conditions, wherein the recombinant *Pseudomonas fluorescens* gram-negative host cell is transformed with a plasmid comprising a nucleic acid encoding the recombinant protein of interest.

29. The method of claim 28, wherein the recombinant gram-negative bacterial host cell can grow to high cell density.

30. The method of claim 28, wherein the suitable fermentation conditions comprise induction of the inducible promoter at: an OD575 of 80 to 160, a culture pH of 5.8 to 7.0, a temperature of 28-33 deg C, fed-batch, and a titer range of 0.2 to 5 g/L.

31. The method of claim 28, wherein the recombinant *Pseudomonas fluorescens* gram-negative bacterial host cell grows to a cell density that is increased in comparison to a control cell grown under the same fermentation conditions.

32. The method of claim 31, wherein the increase in cell density is 2-fold to 15-fold.

33. The method of claim 28, further comprising: (b) measuring the yield of intact, soluble, and/or active, recombinant protein of interest recovered from the recombinant gram-negative bacterial host cell.

34. The method of claim 28, wherein the measured yield of intact, soluble, and/or active, recombinant protein is 0.1 to 10 g/L.

35. The method of claim 34, further comprising: (c) measuring the yield of recombinant protein of interest recovered from a control cell that is intact, soluble, active, or a combination thereof.

36. The method of claim 35, further comprising (d) comparing the yield measured in step (b) to the yield measured in step (c), wherein the yield measured in step (b) is 2-fold to 100-fold higher than that measured in step (c).

37. A recombinant *Pseudomonas fluorescens* gram-negative host cell, wherein the host cell is:

(a) deficient in a first protease activity, wherein the deficient first protease activity results from mutations in

(i) a gene encoding a Prc1 tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 33; and,

(ii) a gene encoding a Prc2 tail-specific protease having the amino acid sequence set forth as SEQ ID NO: 35; and

(b) deficient in a second protease activity, wherein the deficient second protease activity results from a mutation in a gene encoding a MepM1 murein DD-endopeptidase having the amino acid sequence set forth as SEQ ID NO: 1; and additionally wherein the host cell is further:

(c) deficient in one additional protease activity, wherein the deficient additional protease activity results from a mutation of a gene encoding a serralysin precursor having the amino acid sequence set forth as SEQ ID NO: 9;

(d) deficient in one or more autolytic factor activity, wherein the deficient autolytic factor activity results from a mutation in at least one gene encoding an autolytic factor independently selected from: a linear gramicidin synthase subunit D, or a hemolysin precursor;

(e) overexpresses a mutant periplasmic serine endoprotease;

(f) overexpresses a disulfide isomerase folding modulator-having at least 60% similarity or at least 60% identity to an amino acid sequence set forth as SEQ ID NO: 27; or

(g) any combination of (c), (d), (e) and (f).

38. The recombinant *Pseudomonas fluorescens* gram-negative host cell of claim 37, wherein the host is (a) and (b).

39. The recombinant *Pseudomonas fluorescens* gram-negative host cell of claim 37, wherein the host cell is (a), (b), and (e), wherein the mutant serine peptidase of (e) is as set forth in SEQ ID NO: 29.

40. The recombinant gram-negative *Pseudomonas fluorescens* host cell of claim 37, wherein the host cell is (a), (b), (e), and (f), wherein the mutant serine peptidase of (e) is as set forth in SEQ ID NO: 29.