In another aspect, there is provided a method of expressing properly folded, biologically active proteins in a bacterial cell free synthesis system comprising the steps of:

i) combining a bacterial extract with a nucleic acid encoding a protein of interest; and

ii) incubating the bacterial extract with the nucleic acid under conditions permitting the expression and proper folding of the protein of interest,

wherein the bacterial extract comprises biologically functioning tRNA, amino acids, ribosomes necessary for cell free protein synthesis, a protein disulfide isomerase and a peptidyl-prolyl cis-trans isomerase, wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present at a concentration sufficient to improve the expression of properly folded biologically active proteins.

In another aspect, there is provided a method for preparing a bacterial extract, comprising:

i) culturing a bacteria that expresses an exogenous protein disulfide isomerase and an exogenous peptidyl-prolyl cis-trans isomerase, and

ii) preparing an extract having an active oxidative phosphorylation system, and comprising biologically functioning tRNA, amino acids and ribosomes necessary for cell free protein synthesis,

wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present in a total concentration of at least about 1 g/liter in the extract.

In another aspect, there is provided a bacterial cell free extract for expressing biologically active proteins comprising an active oxidative phosphorylation system containing biologically functioning tRNA, amino acids and ribosomes necessary for cell free protein synthesis, and further including a protein disulfide isomerase and a peptidyl-prolyl cis-trans isomerase

wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present in a total concentration of at least about 1 g/liter in the extract.

In another aspect, there is provided a method of improving the expression levels of biologically active proteins in a bacterial cell free synthesis system comprising the steps of:

i) combining a bacterial S30 extract prepared from *E.coli* that expresses an exogenous disulfide isomerase and an exogenous prolyl isomerase at a total concentration of at

least 1 g/liter of extract with a nucleic acid encoding a protein of interest to yield a bacterial cell free synthesis system; and,

ii) incubating the bacterial cell free synthesis system under conditions permitting the expression of the protein of interest to a concentration of at least about 100 mg/L,

wherein the bacterial extract has an active oxidative phosphorylation system and comprises biologically functioning tRNA, amino acids, ribosomes necessary for cell free protein synthesis, and the exogenous disulfide isomerase and the exogenous prolyl isomerase, where the exogenous disulfide isomerase is DsbA, DsbB, DsbC, DsbD, DsbG, yeast PDI (yPDI), human PDI (hPDI), or Trr1, and the exogenous prolyl isomerase is FkpA, trigger factor (tig), SlyD, SurA, PpiD, Cpr6, or Fpr1.

In another aspect, there is provided bacterial cell free synthesis system for expressing biologically active proteins comprising:

i) a cell free extract of bacteria having an active oxidative phosphorylation system, containing biologically functioning tRNA, amino acids, ribosomes necessary for cell free protein synthesis, an exogenous disulfide isomerase and an exogenous prolyl isomerase, wherein the exogenous disulfide isomerase and the exogenous prolyl isomerase were expressed in the bacteria at a concentration of at least 1 g/liter of extract; and

ii) a nucleic acid encoding a protein of interest,

where said bacterial cell free synthesis system expresses a protein of interest to a concentration of at least 100 mg/L, and

where the exogenous disulfide isomerase is DsbA, DsbB, DsbC, DsbD, DsbG, yeast PDI (yPDI), human PDI (hPDI), or Trr1, and the exogenous prolyl isomerase is FkpA, trigger factor (tig), SlyD, SurA, PpiD, Cpr6, or Fpr1.

In another aspect, there is provided method of expressing properly folded, biologically active proteins in a bacterial cell free synthesis system comprising the steps of:

i) combining a bacterial extract with a nucleic acid encoding a protein of interest; and

ii) incubating the bacterial extract with the nucleic acid under conditions permitting the expression and proper folding of the protein of interest,

wherein the bacterial extract comprises biologically functioning tRNA, amino acids, ribosomes necessary for cell free protein synthesis, a protein disulfide isomerase and a peptidyl-prolyl cis-trans isomerase, wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present at a concentration sufficient to improve the expression of properly folded biologically active proteins, and

the protein disulfide isomerase is DsbA, DsbB, DsbC, DsbD, DsbG, yeast PDI (yPDI), human PDI (hPDI), or Trr1, and the peptidyl-prolyl cis-trans isomerase is FkpA, trigger factor (tig), SlyD, SurA, PpiD, Cpr6, or Fpr1.

In another aspect, there is provided a bacterial cell free synthesis system for expressing biologically active proteins comprising:

i) a cell free extract of bacteria having an active oxidative phosphorylation system, containing biologically functioning tRNA, amino acids and ribosomes necessary for cell free protein synthesis and further including a protein disulfide isomerase and a peptidyl-prolyl cis-trans isomerase,

wherein the protein disulfide isomerase and the peptidyl-prolyl cis/trans isomerase are present at a concentration sufficient to improve the expression of properly folded biologically active proteins; and

ii) a nucleic acid encoding a protein of interest,

wherein said bacterial cell free synthesis system expresses a protein of interest to a concentration of at least 100 mg/L and

the protein disulfide isomerase is DsbA, DsbB, DsbC, DsbD, DsbG, yeast PDI (yPDI), human PDI (hPDI), or Trr1, and the peptidyl-prolyl cis-trans isomerase is FkpA, trigger factor (tig), SlyD, SurA, PpiD, Cpr6, or Fpr1.

In another aspect, there is provided a method for preparing a bacterial extract, comprising:

i) culturing a bacteria that expresses an exogenous protein disulfide isomerase and an exogenous peptidyl-prolyl cis-trans isomerase, and

ii) preparing an extract having an active oxidative phosphorylation system, and comprising biologically functioning tRNA, amino acids, ribosomes necessary for cell free

protein synthesis, the exogenous protein disulfide isomerase, and the exogenous peptidyl-prolyl cis-trans isomerase,

wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present in a total concentration of at least 1 g/liter in the extract, and

the protein disulfide isomerase is DsbA, DsbB, DsbC, DsbD, DsbG, yeast PDI (yPDI), human PDI (hPDI), or Trr1, and the peptidyl-prolyl cis-trans isomerase is FkpA, trigger factor (tig), SlyD, SurA, PpiD, Cpr6, or Fpr1.

In another aspect, there is provided a bacterial cell free extract for expressing biologically active proteins comprising an active oxidative phosphorylation system containing biologically functioning tRNA, amino acids and ribosomes necessary for cell free protein synthesis, and further including a protein disulfide isomerase and a peptidyl-prolyl cis-trans isomerase,

wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present in a total concentration of at least 1 g/liter in the extract.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] **Figure 1** shows that eukaryotic PDI and bacterial DsbC are functionally interchangeable.

[0012] **[0001]** **Figure 2A** shows a schematic illustration of the chaperone sequential expression screen described in the Examples.

[0013] **Figure 2B** shows that IgG titer can be improved by adding bacterial cell free system expressed protein chaperones to the bacterial cell free synthesis system.

[0014] **Figure 3** shows that the protein chaperones Skp, SlyD and FkpA improve the solubility and/or amount of properly assembled IgG.

[0015] **Figure 4** shows that the protein chaperone FkpA improves the solubility and folding of IgG proteins.

[0016] **Figure 5** shows that the addition of purified FkpA to an extract containing DsbC promotes IgG folding.

[0017] **Figure 6** shows that the addition of exogenous DsbC protein added to an extract containing FkpA increases the IgG titer.

[0018] **Figure 7** shows the amount of GMCSF protein produced by the CFPS in extracts from the indicated bacterial strains that express the chaperones DsbC or FkpA.

[0019] **Figure 8** shows the growth rate of bacterial strains transformed with plasmids that express 1X or 2X copies of DsbC under the control of a constitutive promoter (upper panel). The lower panel shows the amount of DsbC protein present in the periplasmic lysate.

[0020] **Figure 9** shows the amount of DsbC protein produced by bacterial strains overexpressing 1X or 2X copies of DsbC. The upper panel shows the intracellular concentration. The lower panel shows the extract concentration.

[0021] **Figure 10** shows the growth rate of bacterial strains transformed with plasmids that express 1X or 2X copies of FkpA under the control of a constitutive promoter (upper panel). The lower left panel shows the amount of FkpA protein present in total extracts prepared

CLAIMS

1. A method of improving the expression levels of biologically active proteins in a bacterial cell free synthesis system comprising the steps of:

i) combining a bacterial S30 extract prepared from *E.coli* that expresses an exogenous disulfide isomerase and an exogenous prolyl isomerase at a total concentration of at least 1 g/liter of extract with a nucleic acid encoding a protein of interest to yield a bacterial cell free synthesis system; and,

ii) incubating the bacterial cell free synthesis system under conditions permitting the expression of the protein of interest to a concentration of at least about 100 mg/L,

wherein the bacterial extract has an active oxidative phosphorylation system and comprises biologically functioning tRNA, amino acids, ribosomes necessary for cell free protein synthesis, and the exogenous disulfide isomerase and the exogenous prolyl isomerase, where the exogenous disulfide isomerase is DsbA, DsbB, DsbC, DsbD, DsbG, yeast PDI (yPDI), human PDI (hPDI), or Trr1, and the exogenous prolyl isomerase is FkpA, trigger factor (tig), SlyD, SurA, PpiD, Cpr6, or Fpr1.

2. The method of claim 1, wherein the extract is prepared from a bacteria that further expresses and comprises an exogenous deaggregase.

3. The method of claim 2, wherein the bacteria from which the extract is prepared was co-transformed with genes encoding the disulfide isomerase and the prolyl isomerase, or the deaggregase.

4. The method of claims 2 or 3, wherein the exogenous disulfide isomerase is DsbC, the exogenous prolyl isomerase is FkpA or SlyD, and the exogenous deaggregase is Skp.

5.

6. The method of any one of claims 2-4, wherein the bacteria from which the extract is prepared expresses the exogenous disulfide isomerase, the exogenous prolyl isomerase, or the exogenous deaggregase from a gene operably linked to a constitutive promoter.

7. The method of claim 1 wherein the protein of interest has at least one disulfide bond in its biologically active conformation.

8. The method of claim 1 wherein the protein of interest has at least two proline residues.

9. The method of claim 1 wherein the protein of interest is an antibody or antibody fragment.

10. The method of claim 1 wherein the bacterial cell free synthesis system has a volume of between 0.5 liter and 500 liters and the incubation is of a time period lasting from 1-36 hours.

11. A bacterial cell free synthesis system for expressing biologically active proteins comprising:

i) a cell free extract of bacteria having an active oxidative phosphorylation system, containing biologically functioning tRNA, amino acids, ribosomes necessary for cell free protein synthesis, an exogenous disulfide isomerase and an exogenous prolyl isomerase, wherein the exogenous disulfide isomerase and the exogenous prolyl isomerase were expressed in the bacteria at a concentration of at least 1 g/liter of extract; and

ii) a nucleic acid encoding a protein of interest,

where said bacterial cell free synthesis system expresses a protein of interest to a concentration of at least 100 mg/L, and

where the exogenous disulfide isomerase is DsbA, DsbB, DsbC, DsbD, DsbG, yeast PDI (yPDI), human PDI (hPDI), or Trr1, and the exogenous prolyl isomerase is FkpA, trigger factor (tig), SlyD, SurA, PpiD, Cpr6, or Fpr1.

12. The system of claim 11, wherein the bacteria from which the extract is prepared was co-transformed with genes encoding the disulfide isomerase and prolyl isomerase.

13. The system of claim 11 or 13, wherein the exogenous disulfide isomerase is DsbC, and the exogenous prolyl isomerase is FkpA or SlyD.

14. The system of claim 11 or 13, wherein the bacteria is Escherichia coli.

15. The system of claim 11, 13 or 15, wherein the bacteria from which the extract is prepared expresses at least one of the exogenous disulfide isomerase and the exogenous prolyl isomerase from a gene operably linked to a constitutive promoter.

16. The system of claim 11, 13, or 16, wherein the extract is an S30 extract of E. coli.

17. A method of expressing properly folded, biologically active proteins in a bacterial cell free synthesis system comprising the steps of:

i) combining a bacterial extract with a nucleic acid encoding a protein of interest; and

ii) incubating the bacterial extract with the nucleic acid under conditions permitting the expression and proper folding of the protein of interest,

wherein the bacterial extract comprises biologically functioning tRNA, amino acids, ribosomes necessary for cell free protein synthesis, a protein disulfide isomerase and a peptidyl-prolyl cis-trans isomerase, wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present at a concentration sufficient to improve the expression of properly folded biologically active proteins, and

the protein disulfide isomerase is DsbA, DsbB, DsbC, DsbD, DsbG, yeast PDI (yPDI), human PDI (hPDI), or Trr1, and the peptidyl-prolyl cis-trans isomerase is FkpA, trigger factor (tig), SlyD, SurA, PpiD, Cpr6, or Fpr1.

18. The method of claim 18, wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present at a total concentration of at least 1 g /liter in the extract.

19. The method of claim 18, wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present at a total concentration of between 1 g /liter and 14 g /liter in the extract.

20. The method of claim 18, wherein the expression of the protein of interest is improved to a concentration above that concentration where one but not both of the protein

disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present, and the incubation conditions are otherwise the same.

21. The method of claim 18, wherein the protein disulfide isomerase is DsbC, and the peptidyl-prolyl cis/trans isomerase is FkpA or SlyD.

22. The method of claim 18, wherein the bacteria from which the extract is prepared expresses at least one of the protein disulfide isomerase and peptidyl-prolyl cis-trans isomerase from a gene operably linked to a constitutive promoter.

23. The method of claim 18, wherein the bacteria is Escherichia coli.

24. The method of claim 18, wherein the protein of interest has at least one disulfide bond in its biologically active conformation.

25. The method of claim 18, wherein the protein of interest has at least two proline residues.

26. The method of claim 18, wherein the protein of interest is an antibody or antibody fragment.

27. A bacterial cell free synthesis system for expressing biologically active proteins comprising:

i) a cell free extract of bacteria having an active oxidative phosphorylation system, containing biologically functioning tRNA, amino acids and ribosomes necessary for cell free protein synthesis and further including a protein disulfide isomerase and a peptidyl-prolyl cis-trans isomerase,

wherein the protein disulfide isomerase and the peptidyl-prolyl cis/trans isomerase are present at a concentration sufficient to improve the expression of properly folded biologically active proteins; and

ii) a nucleic acid encoding a protein of interest,

wherein said bacterial cell free synthesis system expresses a protein of interest to a concentration of at least 100 mg/L and

the protein disulfide isomerase is DsbA, DsbB, DsbC, DsbD, DsbG, yeast PDI (yPDI), human PDI (hPDI), or Trr1, and the peptidyl-prolyl cis-trans isomerase is FkpA, trigger factor (tig), SlyD, SurA, PpiD, Cpr6, or Fpr1.

28. The system of claim 28, wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present at a total concentration of at least 1 g /liter in the extract.

29. The system of claim 28, wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present at a total concentration of between 1 g /liter and 14 g /liter in the extract.

30. The system of claim 28, wherein the expression of the protein of interest is improved to a concentration above that concentration where one but not both of the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present, and the incubation conditions are otherwise the same.

31. The system of claim 28, wherein the protein disulfide isomerase is DsbC, and the peptidyl-prolyl cis-trans isomerase is FkpA or SlyD.

32. The system of claim 28, wherein the bacteria from which the extract is prepared expresses at least one of the protein disulfide isomerase and peptidyl-prolyl cis-trans isomerase from a gene operably linked to a constitutive promoter.

33. The system of claim 28, wherein the bacteria is Escherichia coli.

34. The system of claim 28, wherein the protein of interest has at least one disulfide bond in its biologically active conformation.

35. The system of claim 28, wherein the protein of interest has at least two proline residues.

36. The system of claim 28, wherein the protein of interest is an antibody or antibody fragment.

37. A method for preparing a bacterial extract, comprising:

i) culturing a bacteria that expresses an exogenous protein disulfide isomerase and an exogenous peptidyl-prolyl cis-trans isomerase, and

ii) preparing an extract having an active oxidative phosphorylation system, and comprising biologically functioning tRNA, amino acids, ribosomes necessary for cell free protein synthesis, the exogenous protein disulfide isomerase, and the exogenous peptidyl-prolyl cis-trans isomerase,

wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present in a total concentration of at least 1 g/liter in the extract, and

the protein disulfide isomerase is DsbA, DsbB, DsbC, DsbD, DsbG, yeast PDI (yPDI), human PDI (hPDI), or Trr1, and the peptidyl-prolyl cis-trans isomerase is FkpA, trigger factor (tig), SlyD, SurA, PpiD, Cpr6, or Fpr1.

38. A bacterial cell free extract for expressing biologically active proteins comprising an active oxidative phosphorylation system containing biologically functioning tRNA, amino acids and ribosomes necessary for cell free protein synthesis, and further including a protein disulfide isomerase and a peptidyl-prolyl cis-trans isomerase,

wherein the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase are present in a total concentration of at least 1 g/liter in the extract.

39. The method of claims 1-10, wherein said protein of interest is expressed at a concentration of 100 mg/L to 1000 mg/L.

40. The method of claims 1-10, wherein said protein of interest is expressed at a concentration greater than 600 mg/L.

41. The method of claims 1-10, wherein said exogenous disulfide isomerase and exogenous prolyl isomerase are expressed in the bacteria at a total concentration of 1 g/liter to 20 g/liter of extract.

42. The system of claim 11 or 13-17, wherein said protein of interest is expressed at a concentration of 100 mg/L to 1000 mg/L.

43. The system of claim 11 or 13-17, wherein said protein of interest is expressed at a concentration greater than 600 mg/L.

44. The system of claim 11 or 13-17, wherein said exogenous disulfide isomerase and exogenous prolyl isomerase are expressed in the bacteria at a concentration of 1 g/liter to 20 g/liter of extract.

45. The method of claims 18-26, wherein said protein of interest is expressed at a concentration of 100 mg/L to 1000 mg/L.

46. The method of claims 18-26, wherein said protein of interest is expressed at a concentration greater than 600 mg/L.

47. The method of claim 22, wherein said protein disulfide isomerase and peptidyl-prolyl cis-trans isomerase are expressed in the bacteria at a concentration of 1 g/liter to 20 g/liter of extract.

48. The system of claims 27-36, wherein said protein of interest is expressed at a concentration of 100 mg/L to 1000 mg/L.

49. The system of claims27-36, wherein said protein of interest is expressed at a concentration greater than 600 mg/L.

50. The system of claim 32, wherein said exogenous disulfide isomerase and exogenous prolyl isomerase are expressed in the bacteria at a concentration of 1 g/liter to 20 g/liter of extract.

51. The method of claims 1-10, where the concentration of the protein of interest is greater than the sum of the concentration expressed by bacteria that separately express the exogenous disulfide isomerase and the exogenous prolyl isomerase, and the incubation conditions are otherwise the same.

52. The system of claims 11-16, where the concentration of the protein of interest is greater than the sum of the concentration expressed by bacteria that separately express

the exogenous disulfide isomerase and the exogenous prolyl isomerase, and the incubation conditions are otherwise the same.

53. The method of claims 17-26, where the concentration of the properly folded biologically active proteins is greater than the sum of the concentration expressed by bacteria that separately express the exogenous disulfide isomerase and the exogenous prolyl isomerase, and the incubation conditions are otherwise the same.

54. The system of claims 27-36, where the concentration of the protein of interest is greater than the sum of the concentration expressed by bacteria that separately express the exogenous disulfide isomerase and the exogenous prolyl isomerase, and the incubation conditions are otherwise the same.

55. The method of claims 1-10, where the growth rate of the bacteria used to prepare the extract is similar to the growth rate of bacteria that do not overexpress the exogenous disulfide isomerase and the exogenous prolyl isomerase.

56. The system of claims 11-16, where the growth rate of the bacteria that express the exogenous disulfide isomerase and the exogenous prolyl isomerase is similar to the growth rate of bacteria that do not overexpress the exogenous disulfide isomerase and the exogenous prolyl isomerase.

57. The method of claim 22, wherein the growth rate of the bacteria from which the extract is prepared is similar to the growth rate of bacteria that do not express at least one of the protein disulfide isomerase and peptidyl-prolyl cis-trans isomerase from a gene operably linked to a constitutive promoter.

58. The method of claim 32, wherein the growth rate of the bacteria from which the extract is prepared is similar to the growth rate of bacteria that do not express at least one of the disulfide isomerase and peptidyl-prolyl cis-trans isomerase from a gene operably linked to a constitutive promoter.

59. The method of claim 37, wherein the growth rate of the bacteria that expresses the exogenous protein disulfide isomerase and the exogenous peptidyl-prolyl cis-trans

isomerase is similar to the growth rate of bacteria that do not overexpress the exogenous disulfide isomerase and the exogenous prolyl isomerase.