EXPRESSION OF BIOLOGICALLY ACTIVE PROTEINS IN A BACTERIAL CELL-FREE SYNTHESIS SYSTEM USING BACTERIAL CELLS TRANSFORMED TO EXHIBIT ELEVATED LEVELS OF CHAPERONE EXPRESSION

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims benefit of priority to US Patent Application No. 61/813,914, filed April 19, 2013, and US Patent Application No. 61/937,069, filed February 7, 2014, the disclosure of each of which is incorporated by reference herein in its entirety.

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED AS AN ASCII TEXT FILE

[0002] The Sequence Listing written in file -58-2PC.TXT, created on April 16, 2014, 73,728 bytes, machine format IBM-PC, MS-Windows operating system, is hereby incorporated by reference in its entirety for all purposes.

BACKGROUND OF THE INVENTION

[0003] The expression of proteins in bacterial cell free synthesis systems is a well established technique for expressing recombinant target proteins. Extracts can be made from bacteria expressing or overexpressing proteins of interest to provide bacterial cell free synthesis systems having altered properties depending on the protein. However, overexpression of proteins during bacterial growth frequently results in slower growth rates for the bacteria and lower protein synthetic activity in extracts prepared from the bacteria.

[0004] Further, expression of recombinant proteins from such extracts often leads to improper folding and loss of biological activity. The use of protein chaperones can improve the proper folding and biological activity of proteins Thus, there remains a need for improved bacterial cell extracts for expressing recombinant proteins that are prepared from bacteria overexpressing chaperones where such extracts can synthesize large amounts of properly folded protein. These and other needs are provided by the present invention, as set forth below.

BRIEF SUMMARY OF THE INVENTION

[0005] The present disclosure provides methods and systems for improving the expression of biologically active and/or properly folded proteins of interest in a cell free synthesis system. The cell free synthesis system comprises a bacterial extract having an active oxidative phosphorylation system and the components necessary for cell free protein synthesis. The cell free synthesis system further comprises an exogenous protein chaperone. In some embodiments, the exogenous protein chaperone is expressed by the bacteria used to prepare the bacterial extract.

[0006] Thus, in one aspect, a method of improving the expression levels of biologically active proteins in a bacterial cell free synthesis system is described, the method comprising the steps of:

i) preparing a bacterial extract having an active oxidative phosphorylation system and comprising biologically functioning tRNA, amino acids and ribosomes necessary for cell free protein synthesis, wherein the bacteria from which the extract is prepared expresses an exogenous protein chaperone at a concentration of at least about 1 gm/liter of extract;

ii) combining the bacterial extract with a nucleic acid encoding a protein of interest to yield a bacterial cell free synthesis system; and,

iii) 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.

[0007] In a second aspect, a bacterial cell free synthesis system for expressing biologically active proteins is described, the system 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 wherein an exogenous protein chaperone was expressed in the bacteria at a level of at least 1 gm/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 about 100 mg/L.

[0008] In a third aspect, a method of expressing properly folded, biologically active proteins in a bacterial cell free synthesis system is described, the method comprising the steps of:

i) preparing a bacterial extract comprising 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;

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

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

[0009] In a fourth aspect, a bacterial cell free synthesis system for expressing biologically active proteins is described, the system 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 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 about 100 mg/L.

[0010] In a fifth aspect, a method of improving the vitality and/or growth rate of an *E coli* cell culture is described, the method comprising the steps of:

i) transforming an *E. coli* cell with a nucleic acid expressing the protein DsbC operably linked to a constitutive promoter; and

ii) culturing the transformed *E. coli* cell under conditions that permit the overexpression of the DsbC protein to an intracellular concentration of at least 1 mg/ml.

BRIEF DESCRIPTION OF THE DRAWINGS

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

[0012] **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

from the bacteria expressing 1X and 2X copies of FkpA. The lower right panel shows the doubling time of the bacterial strains.

[0022] **Figure 11** shows the quantitation of FkpA concentration in extracts from bacteria expressing 1X and 2X copies of FkpA.

[0023] **Figure 12** shows the results of adding a C-terminal His tag to FkpA. (**a**) shows that extract levels of FkpA prepared from bacteria that overexpress FkpA-His (2XFkpA-His (e49)) were increased by a centrifugal spin after extract activation (pre-incubation) at 30°C. (**b**) shows that extracts containing FkpA-His produced more total IgG than extracts containing wild-type FkpA (compare 2XFkpA (e44) to 2XFkpA-His (e49)), and that the total amount of correctly assembled IgG was increased by centrifuging the extract after activation (compare 2XFkpA final spin to 2XFkpA-His final spin). Con 1 and Con 2 are control extracts prepared from bacteria that do not express FkpA.

[0024] **Figure 13** shows that overexpression of chaperones improves the yield of multiple IgGs in an Open Cell Free Synthesis system. (A) Trastuzumab, the CD30 antigen binding brentuximab, and the germline Heavy Chains VH3-7 and VH3-23 in combination with the germline Light Chain Vk3-20 were expressed in SBJY001, 2xDsbC, and 2xD + 2xF extracts in the presence of ^{14}C-leucine and visualized by SDS-PAGE and autoradiography. (B) Assembled IgG expressed in the different extracts was quantified as described in the Examples.

DEFINITIONS

[0025] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art. See, *e.g.*, Lackie, DICTIONARY OF CELL AND MOLECULAR BIOLOGY, Elsevier (4th ed. 2007); Sambrook *et al.*, MOLECULAR CLONING, A LABORATORY MANUAL, Cold Springs Harbor Press (Cold Springs Harbor, NY 1989); Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley and Sons (Hoboken, NY 1995). The term "a" or "an" is intended to mean "one or more." The term "comprise" and variations thereof such as "comprises" and "comprising," when preceding the recitation of a step or an element, are intended to mean that the addition of further steps or elements is optional and not excluded. Any methods, devices and materials similar or equivalent to those described herein can be used in the practice of this invention. The following definitions are provided to facilitate understanding of certain terms used frequently herein and are not meant to limit the scope of the present disclosure.

[0026] The term "active oxidative phosphorylation system" refers to a bacterial lysate that exhibits active oxidative phosphorylation during protein synthesis. For example, the bacterial lysate can generate ATP using ATP synthase enzymes and reduction of oxygen. It will be understood that other translation systems known in the art can also use an active oxidative phosphorylation during protein synthesis. The activation of oxidative phosphorylation can be demonstrated by inhibition of the pathway using specific inhibitors, such as electron transport chain inhibitors.

[0027] The term "antibody" refers to a protein functionally defined as a binding protein and structurally defined as comprising an amino acid sequence that is recognized by one of skill as being derived from the framework region of an immunoglobulin encoding gene of an animal producing antibodies. An antibody can consist of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

[0028] A typical immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (VL) and variable heavy chain (VH) refer to these light and heavy chains respectively.

[0029] Antibodies exist as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce $F(ab)'_2$, a dimer of Fab which itself is a light chain joined to VH-CH1 by a disulfide bond. The $F(ab)'_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the $(Fab')_2$ dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (see, *Fundamental Immunology*, W.E. Paul, ed., Raven Press, N.Y. (1993), for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill

will appreciate that such Fab' fragments may be synthesized de novo either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein also includes antibody fragments either produced by the modification of whole antibodies or synthesized de novo using recombinant DNA methodologies. Antibodies also include single chain antibodies (antibodies that exist as a single polypeptide chain), and single chain Fv antibodies (sFv or scFv) in which a variable heavy and a variable light chain are joined together (directly or through a peptide linker) to form a continuous polypeptide. The single chain Fv antibody is a covalently linked VH-VL heterodimer which may be expressed from a nucleic acid including VH- and VL- encoding sequences either joined directly or joined by a peptide-encoding linker. Huston, et al. (1988) *Proc. Nat. Acad. Sci. USA*, 85: 5879-5883. While the VH and VL are connected to each as a single polypeptide chain, the VH and VL domains associate non-covalently. The first functional antibody molecules to be expressed on the surface of filamentous phage were single-chain Fv's (scFv); however, alternative expression strategies have also been successful. For example Fab molecules can be displayed on phage if one of the chains (heavy or light) is fused to g3 capsid protein and the complementary chain exported to the periplasm as a soluble molecule. The two chains can be encoded on the same or on different replicons; the important point is that the two antibody chains in each Fab molecule assemble post-translationally and the dimer is incorporated into the phage particle via linkage of one of the chains to g3p (see, *e.g.*, U.S. Patent No: 5733743). The scFv antibodies and a number of other structures converting the naturally aggregated, but chemically separated light and heavy polypeptide chains from an antibody V region into a molecule that folds into a three dimensional structure substantially similar to the structure of an antigen-binding site are known to those of skill in the art (see, *e.g.*, U.S. Patent Nos. 5,091,513, 5,132,405, and 4,956,778). Antibodies also includes all those that have been displayed on phage (*e.g.*, scFv, Fv, Fab and disulfide linked Fv (Reiter et al. (1995) Protein Eng. 8: 1323-1331). Antibodies can also include diantibodies, miniantibodies and scFv-Fc fusions.

[0030] The term "bacterial derived cell free extract" refers to preparation of *in vitro* reaction mixtures able to transcribe DNA into mRNA and/or translate mRNA into polypeptides. The mixtures include ribosomes, ATP, amino acids, and tRNAs. They may be derived directly from lysed bacteria, from purified components or combinations of both.

[0031] The term "bacterial cell free synthesis system" refers to the *in vitro* synthesis of polypeptides in a reaction mix comprising biological extracts and/or defined reagents. The

reaction mix will comprise a template for production of the macromolecule, *e.g.* DNA, mRNA, *etc.*; monomers for the macromolecule to be synthesized, *e.g.* amino acids, nucleotides, *etc.*; and co-factors, enzymes and other reagents that are necessary for the synthesis, *e.g.* ribosomes, uncharged tRNAs, tRNAs charged with unnatural amino acids, polymerases, transcriptional factors, tRNA synthetases, *etc.*

[0032] The term "biologically active protein" refers to a protein that retains at least some of the biological activity of the protein of interest. The biological activity can be determined by comparing the activity, function and/or structure of the protein of interest expressed by the methods described herein to the activity of a reference protein of interest. For example, if the reference protein of interest is an IgG, a biologically active protein will comprise a properly folded and assembled IgG molecule. In some embodiments, the reference protein can be a protein expressed by a bacterial cell free synthesis system that does not contain an exogenous protein chaperone. The biological activity can also be determined using an *in vitro* or *in vivo* assay that is appropriate for the protein of interest. The biological activity of the protein of interest can be expressed as the biological activity per unit volume of the cell-free protein synthesis reaction mixture. In some embodiments, the biological activity of a protein produced by the methods described herein is at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% of the activity of a reference protein.

[0033] The term "constitutive promoter" refers to a nucleic acid sequence that, under appropriate conditions, allows for continual transcription of a nucleic acid sequence or gene that is operably connected or linked to the promoter sequence. The appropriate conditions include transcription factors, such as RNA polymerase, that bind to the promoter sequence, and ribonucleotides that are incorporated into the transcribed RNA. Constitutive promoters are typically unregulated promoters in that they promote continual transcription under normal cellular conditions.

[0034] The term "disulfide isomerase" or "protein disulfide isomerase" (PDI) refers to a family of proteins comprising multiple domains, each having a typical thioredoxin (Trx) fold. The PDI molecule has two or more active sites comprising a CXXC motif that are the sites for isomerase activity. *In vitro*, PDI catalyzes the oxidative formation, reduction, or isomerization of disulfide bonds depending on the redox potential of the environment. PDIs are members of a class of folding catalysts, also called foldases. Folding catalysts assist folding by accelerating certain rate-limiting steps in the protein folding process, thereby

reducing the concentration of aggregated protein folding intermediates. In addition to the isomerase function of catalyzing the formation of disulfide bonds, PDI also promotes the folding of polypeptides into their native configuration, and thus acts as a chaperone. The C-terminal region of PDI comprises the polypeptide binding region, and is believed to be responsible for the chaperone activity. The isomerase and chaperone activities of PDI are separate and independent activities, and both activities appear to be required for reactivation of reduced and denatured proteins containing disulfide bonds.

[0035] In gram-negative bacteria, disulfide bond formation, reduction and isomerization are catalyzed by the Dsb (disulfide bond formation) family of proteins, including DsbA, DsbB, DsbC, and DsbD. DsbA catalyzes the oxidative formation of disulfide bonds by transferring its active site disulfide to the target protein, which leaves DsbA in a reduced form. DsbB re-oxidizes DsbA, and passes its electrons to the respiratory chain to regenerate oxidized DsbB. DsbC catalyzes the rearrangement of disulfide bonds and is recognized as a counterpart of eukaryotic PDI. DsbC is maintained in its reduced form by DsbD. DsbC is a homodimer having four thiol groups is each 23 kDa subunit monomer, two in the active site - Cys^{98}-Gly-Tyr-Cys^{101} (SEQ ID NO:29), and the other two a Cys^{141} and Cys^{163}. Similar to PDI, DsbC has chaperone activity that is independent from its isomerase activity. (*See, e.g.*, Chen et al., *J. Biol. Chem.* 274:19601-19605, 1999; and Kolag, O., et al., *Microbial Cell Factories*, 2009, 8:9). Each monomer consists of an N-terminal dimerization domain with a cystatin fold and a C-terminal catalytic domain with a thioredoxin fold (McCarthy A.A., et al., *Nat. Struct. Biol.* 7:196-199, 2000). Other Dsb proteins include DsbE abd DsbG.

[0036] The term "exogenous protein chaperone" generally refers to a protein chaperone (*e.g.*, a recombinant protein chaperone) that is not normally expressed by the bacterial strain used to prepare the bacterial extract, or a recombinant protein chaperone that is expressed by a nucleic acid construct that is not present in the native bacterial strain. For example, if the native bacterial strain used to prepare the bacterial extract naturally expresses low levels of the endogenous protein chaperone (*e.g.*, at levels not sufficient to improve the expression levels of a biologically active protein of interest), the exogenous protein chaperone can be expressed from a non-native nucleic acid construct, such that the nucleic acid sequences encoding the exogenous protein chaperone are under the control of different regulatory sequences than the endogenous sequences encoding the chaperone. For example, the protein chaperones DsbC and FkpA are naturally occurring *E. coli* proteins, but their expression levels are below the limit of detection using the ELISA assays described herein to detect

proteins in bacterial extracts. Thus, the term “exogenous” is synonymous with “heterologous,” which refers to a protein chaperone not normally expressed by the bacterial strain used to prepare the bacterial extract, or a nucleic acid encoding the protein chaperone that is not present in the native bacterial strain. In some embodiments, the term refers to recombinant protein chaperones that are added to a bacterial cell free extract, and thus are not expressed by the bacteria from which the extract was made.

[0037] The terms "identical," “essentially identical” or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same (*e.g.*, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region, as measured using the BLAST and PSI-BLAST algorithms, which are described in Altschul *et al.* (*J. Mol. Biol.* 215:403-10, 1990), and Altschul *et al.* (*Nucleic Acids Res.*, 25:3389-3402, 1997), respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (*see* the internet at ncbi.nlm.nih.gov). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al. supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as

defaults a wordlength of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915-10919, 1992).

[0038] "Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

[0039] A "comparison window," as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art.

[0040] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see*, *e.g.*, Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873-87, 1993). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, typically less than about 0.01, and more typically less than about 0.001.

[0041] When percentage of sequence identity is used in reference to a polypeptide, it is recognized that one or more residue positions that are not otherwise identical can differ by a conservative amino acid substitution, in which a first amino acid residue is substituted for another amino acid residue having similar chemical properties such as a similar charge or hydrophobic or hydrophilic character and, therefore, does not change the functional properties of the polypeptide. Where polypeptide sequences differ in conservative substitutions, the percent sequence identity can be adjusted upwards to correct for the

conservative nature of the substitution. Such an adjustment can be made using well-known methods, for example, scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions can be calculated using the algorithm described in Pearson *et al.* (*Meth. Mol. Biol.* 24:307-331, 1994). Alignment also can be performed by simple visual inspection and manual alignment of sequences.

[0042] The term "conservatively modified variation," when used in reference to a particular polynucleotide sequence, refers to different polynucleotide sequences that encode identical or essentially identical (e.g., at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity over a specified region) amino acid sequences, or where the polynucleotide does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical polynucleotides encode any given polypeptide. For instance, the codons CGU, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleotide sequence variations are "silent variations," which can be considered a species of "conservatively modified variations." As such, it will be recognized that each polynucleotide sequence disclosed herein as encoding a protein variant also describes every possible silent variation. It will also be recognized that each codon in a polynucleotide, except AUG, which is ordinarily the only codon for methionine, and UUG, which is ordinarily the only codon for tryptophan, can be modified to yield a functionally identical molecule by standard techniques. Accordingly, each silent variation of a polynucleotide that does not change the sequence of the encoded polypeptide is implicitly described herein.

[0043] Furthermore, it will be recognized that individual substitutions, deletions or additions that alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 10%, and generally less than 1%) in an encoded sequence can be considered conservatively modified variations, provided the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative amino acid substitutions providing functionally similar amino acids are well known in the art, including the following

six groups, each of which contains amino acids that are considered conservative substitutes for each another:

[0044] 1) Alanine (Ala, A), Serine (Ser, S), Threonine (Thr, T);

[0045] 2) Aspartic acid (Asp, D), Glutamic acid (Glu, E);

[0046] 3) Asparagine (Asn, N), Glutamine (Gln, Q);

[0047] 4) Arginine (Arg, R), Lysine (Lys, K)

[0048] 5) Isoleucine (Ile, I), Leucine (Leu, L), Methionine (Met, M), Valine (Val, V); and

[0049] 6) Phenylalanine (Phe, F), Tyrosine (Tyr, Y), Tryptophan (Trp, W).

[0050] Two or more amino acid sequences or two or more nucleotide sequences are considered to be "substantially similar" if the amino acid sequences or the nucleotide sequences share at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with each other, or with a reference sequence over a given comparison window. Two or more proteins are also considered substantially similar if they incorporate conservative amino acid substitutions providing functionally similar amino acids into the amino acid sequence.

[0051] The term “incubation conditions are otherwise the same” refers to experimental conditions that, for comparison purposes, are the same except that the control or reference extract does not contain or express an exogenous protein chaperone. The term also includes a comparison between a control extract that expresses or contains one class of exogenous protein chaperone (*e.g.*, a PDI) and an extract that expresses or contains two different classes of exogenous protein chaperones (*e.g.*, a PDI and a PPIase). For example, the extract can be prepared from a bacterial strain that expresses or overexpresses one class of protein chaperone (*e.g.*, a PDI or DsbC) and a purified protein from the other class of protein chaperone (*e.g.*, a purified PPIase such as FkpA) can be added to the extract. The conditions can also include adjusting the total concentration of the exogenous protein chaperones (*e.g.*, the total concentration of one chaperone such as PDI, or the total concentration of the combination of two different chaperones, such as PDI and PPI) in the bacterial extract to be the same. Otherwise, the components of the bacterial extract and the nucleic acid encoding the protein of interest are the same. Exemplary conditions that permit the expression and proper folding of a protein of interest are described in the Examples.

[0052] The terms "peptidyl prolyl isomerase," "peptidyl prolyl cis-trans isomerase" and "prolyl isomerase" (PPI or PPIase) are used interchangeably, and refer to a class of chaperones known as protein folding catalysts. PPI catalyzes the conversion of trans peptidyl prolyl bonds in the amino acid proline to the cis configuration in the native or functional protein. PPIs can have different subunits or modules having different functions, for example, a module having catalytic activity and a module having chaperone or protein binding activity. Three families of PPIs are recognized: cyclophilins (whose isomerase activity is inhibited by cyclosporin A); FKBPs (FK506 binding proteins), which are inhibited by FK506 and rapamycin; and parvulins. Non-limiting examples of cyclophilins include PpiA (RotA). Non-limiting examples of FKBPs include FkpA, SlyD, and trigger factor (TF or tig). Non-limiting examples of parvulins include SurA and PpiD. Additional examples of PPIs include CypA, PpiB, Cpr1, Cpr6, and Fpr1. FkpA, SlyD, and trigger factor are related based on sequence alignments. For FkpA, the chaperone and catalytic activities reside in the N-terminal and C-terminal domains, respectively (Saul F.A., *J. Mol. Biol.* 335:595-608, 2004).

[0053] The term "deaggregase" refers to a protein chaperone that aids in deaggregating and/or solubilizing proteins of interest that are produced, for example, in a bacterial free translation system. Such chaperones are particularly helpful at high concentrations because their mechanism of action is stoichiometric rather than catalytic and is believed to work by stabilizing hydrophobic patches of the newly synthesized protein while the protein is folding. Examples of deaggregases include IbpA, IbpB, and Skp.

[0054] The term "peptide," "protein," and "polypeptide" are used herein interchangeably and refer to a to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. As used herein, the terms encompass amino acid chains of any length, including full-length proteins and truncated proteins, wherein the amino acid residues are linked by covalent peptide bonds.

[0055] The term "properly folded protein" refers to the native conformation of a protein or polypeptide that is biologically active or functional. Thus, the term refers to a protein or polypeptide having a tertiary structure that in the folded state possesses a minimum of free energy. When used in reference to a recombinant protein expressed in bacteria, the term generally refers to proteins that are soluble when overexpressed in the cytosol, such that the

properly folded recombinant protein does not form insoluble aggregates and/or is not denatured or unfolded.

[0056] The term "synergistic" or "synergy" interchangeably refers to the interaction of two or more agents so that their combined effect is greater than the sum of their individual effects. Synergistic drug interactions can be determined using the median effect principle (*see*, Chou and Talalay (1984) *Adv Enzyme Regul* 22:27 and *Synergism and Antagonism in Chemotherapy*, Chou and Rideout, eds., 1996, Academic, pp. 61-102) and quantitatively determined by combination indices using the computer program Calcusyn (Chou and Hayball, 1996, Biosoft, Cambridge, MA). *See also*, Reynolds and Maurer, Chapter 14 in *Methods in Molecular in Medicine*, vol. 110: *Chemosensitivity*, Vol. 1: *In vitro Assays*, Blumenthal, ed., 2005, Humana Press. Combination indices (CI) quantify synergy, summation and antagonism as follows: CI<1 (synergy); CI=1 (summation); CI>1 (antagonism). A CI value of 0.7-0.9 indicates moderate to slight synergism. A CI value of 0.3-0.7 indicates synergism. A CI value of 0.1-0.3 indicates strong synergism. A CI value of <0.1 indicates very strong synergism.

DETAILED DESCRIPTION OF THE INVENTION

INTRODUCTION

[0057] The methods and systems described herein are useful for improving and/or increasing the expression levels of biologically active proteins in a cell free synthesis system, for example a bacterial cell free synthesis system. The increased expression levels of a biologically active protein of interest are achieved by using a bacterial extract having an active oxidative phosphorylation system that comprises an exogenous protein chaperone. The exogenous protein chaperone can be expressed by the bacteria used to prepare the extract. The inventors have surprisingly discovered that by expressing relatively large amounts of an exogenous protein chaperone in the bacteria used to prepare the extract, increased amounts of the biologically active protein of interest are expressed by the cell free synthesis system. Thus, the ability of the extract to express large amounts of protein is surprisingly not adversely affected by the relatively high concentration levels of the protein chaperone, such that the total amount of properly folded and biologically active protein produced in the cell free protein synthesis reaction is substantially higher than the amount of properly folded and biologically active protein expressed by a cell free synthesis system that does not contain an exogenous protein chaperone. Thus, while the total amount of the protein

of interest produced by the cell free protein synthesis system is substantially similar to the total amount of protein produced by a cell free protein synthesis system that does not express an exogenous chaperone, the increased concentration levels of protein chaperone in the extract results in increased amounts of properly folded, assembled, and biologically active protein of interest. The inventors have also surprisingly discovered that by expressing two different classes of protein chaperones (*e.g.*, a protein disulfide isomerase and a peptidyl prolyl cis-trans isomerase), a synergistic improvement in the expression levels of properly folded, biologically active proteins is obtained. The methods and systems will now be described.

[0058] To produce a biologically active protein of interest, the methods and systems described herein use a bacterial extract having an active oxidative phosphorylation system, and other components necessary for cell free protein synthesis, such as biologically functioning tRNA, amino acids and ribosomes. The components of the bacterial extract are described in more detail below. In one aspect, the bacterial extract is prepared from a recombinant bacteria that expresses an exogenous protein chaperone. In some embodiments, the bacteria from which the extract is prepared express the exogenous protein chaperone at a concentration of at least about 1 gram (g)/liter (L) of extract. For example, the bacteria from which the extract is prepared can express the exogenous protein chaperone at a concentration of at least about 1 g/liter, 2 g/liter, 3 g/liter, 4 g/liter, 5 g/liter, 6 g/liter, 7 g/liter, 8 g/liter, 9 g/liter, 10 g/liter or more of extract. In some embodiments, the total concentration of exogenous protein chaperone is between about 1 g/L and 20 g/L, between about 1 g/L and 15 g/L, between about 1 g/L and 10 g/L, or between about 1 g/L and 5 g/L of extract. In some embodiments, the bacteria express the exogenous protein chaperone at an intracellular concentration of at least 1 mg/ml, at least 2 mg/ml, at least 3 mg/ml, at least 4 mg/ml, at least 5 mg/ml, at least 10 mg/ml, at least 15 mg/ml, at least 20 mg/ml, at least 30 mg/ml, or at least 40 mg/ml. In some embodiments, the bacteria express the exogenous protein chaperone at an intracellular concentration in the range of about 1 mg/ml to about 40 mg/ml, about 1 mg/ml to about 20 mg/ml, about 1 mg/ml to about 15 mg/ml, about 1 mg/ml to about 10 mg/ml, or about 1 mg/ml to about 5 mg/ml.

[0059] The exogenous protein chaperone can be any protein chaperone that results in increased production of properly folded and/or biologically functional proteins of interest. As described in more detail herein, the protein chaperone can be a protein that interacts with the target protein of interest to assist in proper folding and/or prevent aggregation of the

protein of interest into non-functional aggregates. While not being bound by theory, molecular chaperones are thought to prevent aggregation by binding exposed hydrophobic moieties in unfolded, partially folded, or misfolded polypetides. Thus, any protein chaperone that binds exposed hydrophobic moieties and prevents aggregation of a protein of interest can be used in the methods described herein.

[0060] The exogenous protein chaperone can also be an enzyme that catalyzes covalent changes important for the formation of native and functional conformations of the protein of interest. For example, in some embodiments, the exogenous protein chaperone is a protein disulfide isomerase (PDI) or a peptidyl-prolyl cis-trans isomerase (PPI). Examples of PDI's include, but are not limited to, a mammalian PDI, a yeast PDI, or a bacterial PDI. In some embodiments, the PDI is a member of the Dsb (disulfide bond formation) family of *E. coli*, for example, DsbA or DsbC. In one embodiment, the exogenous protein chaperone is thioredoxin (Trx). Examples of PPI's include, but are not limited to, cyclophilins (whose isomerase activity is inhibited by cyclosporin A); FKBPs (FK506 binding proteins), which are inhibited by FK506 and rapamycin; and parvulins. The three families of PPIases in E. coli exhibit limited sequence and structural similarity but share a high catalytic activity and a relatively low affinity for nonstructured peptides. As will be understood by those of skill in the art, the PDI and PPI chaperones can have a modular structure that includes both a chaperone (protein binding) and catalytic domains. *See, e.g.,* Kolag, O., et al., *Microbial Cell Factories*, 2009, 8:9; Wang, C-C., *Methods in Enzymology*, 2002, 348:66-75. Other protein chaperones useful in the methods and systems described herein are referred to as deaggregases, including, for example, Skp.

[0061] In another aspect, the disclosure also provides method and systems for expressing properly folded, biologically active proteins in a bacterial cell free synthesis system using a bacterial extract comprising a PDI and a PPIase. The method comprises preparing a bacterial extract comprising components necessary for cell free protein synthesis, such as biologically functioning tRNA, amino acids, ribosomes. The bacterial extract further includes 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 (*e.g.*, increase) the expression of properly folded biologically active proteins. In this embodiment, the expression of a protein disulfide isomerase and a peptidyl-prolyl cis-trans isomerase provides a synergistic improvement in the expression of properly folded biologically active proteins of interest. For example, 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 wherein the incubation conditions are otherwise the same. In embodiments where the expression of a protein disulfide isomerase and a peptidyl-prolyl cis-trans isomerase provides a synergistic improvement in protein expression, the total concentration of the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase is at least about 1 gm/liter (g/L) of extract. For example, in some embodiments, the total concentration of the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase is at least about 1 g/L, 2 g/L, 3 g/L, 4 g/L, 5 g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L, 10 g/L, 11 g/L, 12 g/L, 13 g/L, 14 g/L, 15 g/L or more of extract. In some embodiments, the total concentration of the protein disulfide isomerase and the peptidyl-prolyl cis-trans isomerase is between about 1 g/L and 20 g/L, between about 1 g/L and 15 g/L, between about 1 g/L and 14 g/L, between about 1 g/L and 10 g/L, or between about 1 g/L and 5 g/L of extract. In some embodiments, the PDI is selected from the group consisting of a Dsb family protein, such as DsbA, DsbC, and DsbG, and the PPI is selected from the group consisting of FkpA, SlyD, tig, SurA, and Cpr6.

[0062] The bacterial extracts described herein can be prepared from a bacteria that was co-transformed with genes encoding disulfide isomerases and prolyl isomerases. The bacteria (*e.g.*, *E. coli*) from which the extract is prepared can express the exogenous protein chaperone from a gene operably linked to a constitutive promoter. In some embodiments, the exogenous protein chaperone is DsbA, DsbC, FkpA, SlyD, and/or Skp, or a combination thereof. In some embodiments, the bacterial extract is an S30 extract from *E. coli*.

[0063] The bacterial cell free synthesis systems described herein can have a volume between about 20 microliters and 500 liters, and the incubation time is a time period lasting from about 1 hour to about 36 hours. For example, the incubation time can be between about 1 to 36 hours, about 1 to 24 hours, about 1 to 18 hours, or about 1 to 12 hours.

[0064] In order to produce the protein of interest, the bacterial extract is combined with a nucleic acid that encodes the protein of interest to yield a bacterial cell free synthesis system. The nucleic acid that encodes the protein of interest is typically a DNA or an mRNA. Methods for expressing the protein of interest from a nucleic acid are described in more detail below. The bacterial cell free synthesis system is incubated under conditions that permit the expression and/or proper folding of the protein of interest. In some embodiments, the protein of interest is expressed at a concentration of at least about 100 mg/L, 200 mg/L, 300 mg/L,

400 mg/L, 500 mg/L, 600 mg/L, 700 mg/L, 800 mg/L, 900 mg/L, or 1000 mg or more per L. Conditions for the expression of the protein of interest are described in more detail below.

[0065] In some embodiments, the protein of interest has at least one disulfide bond in its biologically active conformation. In one embodiment, the protein of interest has at least two proline residues. The protein of interest can also be an antibody or antibody fragment. In some embodiments, the protein of interest is expressed as a fusion protein with a chaperon protein described herein.

[0066] In another aspect, the disclosure provides a method for improving the vitality and/or growth rate of an *E. coli* cell culture. The method comprises transforming an *E coli* cell with a Dsb protein operably linked to a constitutive promoter; and culturing the transformed *E coli* cell under conditions that permit the overexpression of the Dsb protein. In some embodiments, the Dsb protein is expressed at an intracellular concentration of at least about 1 mg/ml. For example, in some embodiments, the Dsb protein is expressed at an intracellular concentration of about 1 mg/ml to about 40 mg/ml.

[0067] In some embodiments, the protein chaperone can include a poly-amino acid tag, for example a polyhistidine (*e.g.*, His_6; SEQ ID NO:24) tag or a poly(Ser-Arg) tag, at the N-terminus or C-terminus. In some embodiments, the poly-amino acid tag comprises charged amino acids. In some embodiments, the charged amino acids are positively charged. In some embodiments, the charged amino acids are negatively charged. In some embodiments, the poly-amino acid tag comprises polar amino acids. In some embodiments, the poly-amino acid tag comprises alternating charged and polar amino acids. In some embodiments, the poly-amino acid tag comprises Ser-Arg-Ser-Arg-Ser-Arg-Ser-Arg (SEQ ID NO:25). In some embodiments, the poly-amino acid tag comprises Ser-Lys-Ser-Lys-Ser-Lys-Ser-Lys (SEQ ID NO:26). In some embodiments, the poly-amino acid tag comprises Asp-Asp-Asp-Asp-Asp-Asp (SEQ ID NO:27). In some embodiments, the poly-amino acid tag comprises Glu-Glu-Glu-Glu-Glu-Glu (SEQ ID NO:28). While not being bound by any particular theory or mechanism of action, it is believed that the C-terminal tag increases the solubility of the chaperone, which results in an increase in the amount of the chaperone in extracts prepared from bacteria that express the tagged chaperone. In some embodiments, the presence of a poly-amino acid tag resulted in an increase in the total amount of protein of interest produced. In some embodiments, centrifuging the activated extract containing a poly-amino acid tagged chaperone increases the amount of properly assembled protein of interest.

GENERAL METHODS

[0068] Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by one of ordinary skill in the art to which this invention belongs. Practitioners are particularly directed to Green, M.R. and Sambrook, J., eds., *Molecular Cloning: A Laboratory Manual*, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2012), and Ausubel, F. M., *et al. Current Protocols in Molecular Biology* (Supplement 99), John Wiley & Sons, New York (2012), which are incorporated herein by reference, for definitions and terms of the art. Standard methods also appear in Bindereif, Schón, & Westhof (2005) *Handbook of RNA Biochemistry*, Wiley- VCH, Weinheim, Germany which describes detailed methods for RNA manipulation and analysis, and is incorporated herein by reference. Examples of appropriate molecular techniques for generating recombinant nucleic acids, and instructions sufficient to direct persons of skill through many cloning exercises are found in Green, M.R., and Sambrook, J., (*Id.*); Ausubel, F. M., *et al.* (*Id.*); Berger and Kimmel, *Guide to Molecular Cloning Techniques*, *Methods in Enzymology* (Volume 152 Academic Press, Inc., San Diego, Calif. 1987); and *PCR Protocols: A Guide to Methods and Applications* (Academic Press, San Diego, Calif. 1990), which are incorporated by reference herein.

[0069] Methods for protein purification, chromatography, electrophoresis, centrifugation, and crystallization are described in Coligan *et al.* (2000) *Current Protocols in Protein Science*, Vol. 1, John Wiley and Sons, Inc., New York. Methods for cell-free synthesis are described in Spirin & Swartz (2008) *Cell-free Protein Synthesis*, Wiley- VCH, Weinheim, Germany. Methods for incorporation of non-native amino acids into proteins using cell-free synthesis are described in Shimizu *et al.* (2006) *FEBS Journal*, 273, 4133-4140.

[0070] PCR amplification methods are well known in the art and are described, for example, in Innis *et al. PCR Protocols: A Guide to Methods and Applications*, Academic Press Inc. San Diego, Calif., 1990. An amplification reaction typically includes the DNA that is to be amplified, a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium. Typically a desirable number of thermal cycles is between 1 and 25. Methods for primer design and optimization of PCR conditions are well known in the art and can be found in standard molecular biology texts such as Ausubel *et al. Short Protocols in Molecular Biology*, 5th Edition, Wiley, 2002, and Innis *et al. PCR Protocols*, Academic Press, 1990. Computer programs are useful in the design of primers with the required specificity and optimal

amplification properties (*e.g.*, Oligo Version 5.0 (National Biosciences)). In some embodiments, the PCR primers may additionally contain recognition sites for restriction endonucleases, to facilitate insertion of the amplified DNA fragment into specific restriction enzyme sites in a vector. If restriction sites are to be added to the 5' end of the PCR primers, it is preferable to include a few (*e.g.*, two or three) extra 5' bases to allow more efficient cleavage by the enzyme. In some embodiments, the PCR primers may also contain an RNA polymerase promoter site, such as T7 or SP6, to allow for subsequent *in vitro* transcription. Methods for *in vitro* transcription are well known to those of skill in the art (see, *e.g.*, Van Gelder *et al. Proc. Natl. Acad. Sci. U.S.A.* 87:1663-1667, 1990; Eberwine *et al. Proc. Natl. Acad. Sci. U.S.A.* 89:3010-3014, 1992).

[0071] When the proteins described herein are referred to by name, it is understood that this includes proteins with similar functions and similar amino acid sequences. Thus, the proteins described herein include the wild-type prototype protein, as well as homologs, polymorphic variations and recombinantly created muteins. For example, the name "DsbC protein" includes the wild-type prototype protein from *E. coli* (*e.g.*, SEQ ID NO:1), as well as homologs from other species, polymorphic variations and recombinantly created muteins. Proteins such as DsbC and FkpA are defined as having similar functions if they have substantially the same biological activity or functional capacity as the wild type protein (e.g., at least 80% of either). Proteins such as DsbC and FkpA are defined as having similar amino acid sequences if they have at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to the prototype protein. The sequence identity of a protein is determined using the BLASTP program with the defaults wordlength of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915-10919, 1992).

[0072] A readily conventional test to determine if a protein homolog, polymorphic variant or recombinant mutein is inclusive of a protein chaperone described herein is by specific binding to polyclonal antibodies generated against the prototype protein. For example, a DsbC protein includes proteins that bind to polyclonal antibodies generated against the prototype protein of SEQ ID NO:1, and an FkpA protein includes proteins that bind to polyclonal antibodies generated against the prototype protein of SEQ ID NO:6.

[0073] With regard to the reaction of a protein chaperone described herein to polyclonal antibodies, the test protein will bind under designated immunoassay conditions to the

specified antibodies at least two times the background, and the specified antibodies do not substantially bind in a significant amount to other proteins present in the sample. For example, polyclonal antibodies raised to DsbC, encoded in SEQ ID NO:1, splice variants, or portions thereof, can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with DsbC and not with other proteins, except for polymorphic variants of DsbC. This selection may be achieved by subtracting out antibodies that cross-react with other members of the Dsb family. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (*see, e.g.*, Harlow & Lane, *Antibodies, A Laboratory Manual* (1988) for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity). Typically, a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 to 100 times background.

[0074] It will be understood that at least some of the chaperone proteins described herein are members of large families of related proteins with similar functions and various degrees of sequence homology. Thus, the protein chaperones described herein include homologs of family members having similar function, for example, homologs of PDI and PPIases, homologs of Dsb proteins, homologs of FkpA proteins, *etc*. Thus, in some embodiments, the chaperones can have at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to the chaperones described herein. Further, the data provided in the Examples show that eukaryotic PDI and bacterial DsbC are functionally interchangeable regarding their ability to produce properly assembled IgG, which provides evidence that homologs of the chaperones described herein can be used in the methods and systems described herein.

CELL FREE PROTEIN SYNTHESIS (CFPS) TECHNOLOGY

[0075] In order to express the biologically active proteins of interest described herein, a cell free protein synthesis system can be used. Cell extracts have been developed that support the synthesis of proteins *in vitro* from purified mRNA transcripts or from mRNA transcribed from DNA during the *in vitro* synthesis reaction.

[0076] CFPS of polypeptides in a reaction mix comprises bacterial extracts and/or defined reagents. The reaction mix comprises at least ATP or an energy source; a template for

production of the macromolecule, *e.g.,* DNA, mRNA, *etc.*; amino acids, and such co-factors, enzymes and other reagents that are necessary for polypeptide synthesis, *e.g.*, ribosomes, tRNA, polymerases, transcriptional factors, aminoacyl synthetases, elongation factors, initiation factors, *etc*. In one embodiment of the invention, the energy source is a homeostatic energy source. Also included may be enzyme(s) that catalyze the regeneration of ATP from high-energy phosphate bonds, *e.g.,* acetate kinase, creatine kinase, *etc*. Such enzymes may be present in the extracts used for translation, or may be added to the reaction mix. Such synthetic reaction systems are well-known in the art, and have been described in the literature.

[0077] The term "reaction mix" as used herein, refers to a reaction mixture capable of catalyzing the synthesis of polypeptides from a nucleic acid template. The reaction mixture comprises extracts from bacterial cells, *e.g*, *E. coli* S30 extracts. S30 extracts are well known in the art, and are described in, *e.g.*, Lesley, S.A., *et al.* (1991), *J. Biol. Chem.* **266**, 2632–8. The synthesis can be performed under either aerobic or anaerobic conditions.

[0078] In some embodiments, the bacterial extract is dried. The dried bacterial extract can be reconstituted in milli-Q water (*e.g.*, reverse osmosis water) at 110% of the original solids as determined by measuring the percent solids of the starting material. In one embodiment, an accurately weighed aliquot of dried extract, representing 110% of the original solids of 10 mL of extract, is added to 10 mL of Milli-Q water in a glass beaker with a stir bar on a magnetic stirrer. The resulting mixture is stirred until the powder is dissolved. Once dissolved, the material is transferred to a 15 mL Falcon tube and stored at -80C unless used immediately.

[0079] The volume percent of extract in the reaction mix will vary, where the extract is usually at least about 10% of the total volume; more usually at least about 20%; and in some instances may provide for additional benefit when provided at at least about 50%; or at least about 60%; and usually not more than about 75% of the total volume.

[0080] The general system includes a nucleic acid template that encodes a protein of interest. The nucleic acid template is an RNA molecule (*e.g.*, mRNA) or a nucleic acid that encodes an mRNA (*e.g.*, RNA, DNA) and be in any form (*e.g.*, linear, circular, supercoiled, single stranded, double stranded, *etc*.). Nucleic acid templates guide production of the desired protein.

[0081] To maintain the template, cells that are used to produce the extract can be selected for reduction, substantial reduction or elimination of activities of detrimental enzymes or for enzymes with modified activity. Bacterial cells with modified nuclease or phosphatase activity (*e.g.*, with at least one mutated phosphatase or nuclease gene or combinations thereof) can be used for synthesis of cell extracts to increase synthesis efficiency. For example, an *E. coli* strain used to make an S30 extract for CFPS can be RNase E or RNase A deficient (for example, by mutation).

[0082] CFPS systems can also be engineered to guide the incorporation of detectably labeled amino acids, or unconventional or unnatural amino acids, into a desired protein. The amino acids can be synthetic or derived from another biological source. Various kinds of unnatural amino acids, including without limitation detectably labeled amino acids, can be added to CFPS reactions and efficiently incorporated into proteins for specific purposes. *See*, for example, Albayrak, C. and Swartz, JR., *Biochem. Biophys Res. Commun.*, 431(2):291-5; Yang WC *et al. Biotechnol. Prog.* (2012), 28(2):413-20; Kuechenreuther *et al.*. *PLoS One*, (2012), 7(9):e45850; and Swartz JR., *AIChE Journal*, 58(1):5-13.

[0083] In a generic CFPS reaction, a gene encoding a protein of interest is expressed in a transcription buffer, resulting in mRNA that is translated into the protein of interest in a CFPS extract and a translation buffer. The transcription buffer, cell-free extract and translation buffer can be added separately, or two or more of these solutions can be combined before their addition, or added contemporaneously.

[0084] To synthesize a protein of interest *in vitro*, a CFPS extract at some point comprises a mRNA molecule that encodes the protein of interest. In some CFPS systems, mRNA is added exogenously after being purified from natural sources or prepared synthetically *in vitro* from cloned DNA using RNA polymerases such as RNA polymerase II, SP6 RNA polymerase, T3 RNA polymerase, T7 RNA polymerase, RNA polymerase III and/or phage derived RNA polymerases. In other systems, the mRNA is produced *in vitro* from a template DNA; both transcription and translation occur in this type of CFPS reaction. In some embodiments, the transcription and translation systems are coupled or comprise complementary transcription and translation systems, which carry out the synthesis of both RNA and protein in the same reaction. In such *in vitro* transcription and translation systems, the CFPS extracts contain all the components (exogenous or endogenous) necessary both for transcription (to produce mRNA) and for translation (to synthesize protein) in a single

system. The coupled transcription and translation systems described herein are sometimes referred to as Open-Cell Free Synthesis (OCFS) systems, and are capable of achieving high titers of properly folded proteins of interest, e.g., high titers of antibody expression.

[0085] A cell free protein synthesis reaction mixture comprises the following components: a template nucleic acid, such as DNA, that comprises a gene of interest operably linked to at least one promoter and, optionally, one or more other regulatory sequences (*e.g.*, a cloning or expression vector containing the gene of interest) or a PCR fragment; an RNA polymerase that recognizes the promoter(s) to which the gene of interest is operably linked (*e.g.* T7 RNA polymerase) and, optionally, one or more transcription factors directed to an optional regulatory sequence to which the template nucleic acid is operably linked; ribonucleotide triphosphates (rNTPs); optionally, other transcription factors and co-factors therefor; ribosomes; transfer RNA (tRNA); other or optional translation factors (*e.g.*, translation initiation, elongation and termination factors) and co-factors therefore; one or more energy sources, (*e.g.*, ATP, GTP); optionally, one or more energy regenerating components (*e.g.*, PEP/pyruvate kinase, AP/acetate kinase or creatine phosphate/creatine kinase); optionally factors that enhance yield and/or efficiency (*e.g.*, nucleases, nuclease inhibitors, protein stabilizers, chaperones) and co-factors therefore; and; optionally, solubilizing agents. The reaction mix further comprises amino acids and other materials specifically required for protein synthesis, including salts (*e.g.*, potassium, magnesium, ammonium, and manganese salts of acetic acid, glutamic acid, or sulfuric acids), polymeric compounds (*e.g.*, polyethylene glycol, dextran, diethyl aminoethyl dextran, quaternary aminoethyl and aminoethyl dextran, *etc.*), cyclic AMP, inhibitors of protein or nucleic acid degrading enzymes, inhibitors or regulators of protein synthesis, oxidation/reduction adjuster (*e.g.*, DTT, ascorbic acid, glutathione, and/or their oxides), non-denaturing surfactants (*e.g.*, Triton X-100) , buffer components, spermine, spermidine, putrescine, *etc.* Components of CFPS reactions are discussed in more detail in U.S. Patent Nos. 7,338,789 and 7,351,563, and U.S. App. Pub. Nos. 2010/0184135 and US 2010/0093024, the disclosures of each of which is incorporated by reference in its entirety for all purposes.

[0086] Depending on the specific enzymes present in the extract, for example, one or more of the many known nuclease, polymerase or phosphatase inhibitors can be selected and advantageously used to improve synthesis efficiency.

[0087] Protein and nucleic acid synthesis typically requires an energy source. Energy is required for initiation of transcription to produce mRNA (*e.g.*, when a DNA template is used and for initiation of translation high energy phosphate for example in the form of GTP is used). Each subsequent step of one codon by the ribosome (three nucleotides; one amino acid) requires hydrolysis of an additional GTP to GDP. ATP is also typically required. For an amino acid to be polymerized during protein synthesis, it must first be activated. Significant quantities of energy from high energy phosphate bonds are thus required for protein and/or nucleic acid synthesis to proceed.

[0088] An energy source is a chemical substrate that can be enzymatically processed to provide energy to achieve desired chemical reactions. Energy sources that allow release of energy for synthesis by cleavage of high-energy phosphate bonds such as those found in nucleoside triphosphates, *e.g.*, ATP, are commonly used. Any source convertible to high energy phosphate bonds is especially suitable. ATP, GTP, and other triphosphates can normally be considered as equivalent energy sources for supporting protein synthesis.

[0089] To provide energy for the synthesis reaction, the system can include added energy sources, such as glucose, pyruvate, phosphoenolpyruvate (PEP), carbamoyl phosphate, acetyl phosphate, creatine phosphate, phosphopyruvate, glyceraldehyde-3-phosphate, 3-Phosphoglycerate and glucose-6-phosphate, that can generate or regenerate high-energy triphosphate compounds such as ATP, GTP, other NTPs, *etc*.

[0090] When sufficient energy is not initially present in the synthesis system, an additional source of energy is preferably supplemented. Energy sources can also be added or supplemented during the *in vitro* synthesis reaction.

[0091] In some embodiments, the cell-free protein synthesis reaction is performed using the PANOx-SP system comprising NTPs, *E. coli* tRNA, amino acids, Mg^{2+} acetate, Mg^{2+} glutamate, K^{+} acetate, K^{+} glutamate, folinic acid, Tris pH 8.2, DTT, pyruvate kinase, T7 RNA polymerase, disulfide isomerase, phosphoenol pyruvate (PEP), NAD, CoA, Na^{+} oxalate, putrescine, spermidine, and S30 extract.

[0092] In some embodiments, proteins containing a non-natural amino acid (nnAA) may be synthesized. In such embodiments, the reaction mix may comprise the non-natural amino acid, a tRNA orthogonal to the 20 naturally occurring amino acids, and a tRNA synthetase that can link the nnAA with the orthogonal tRNA. *See*, *e.g.*, US Pat. App. Pub. No. US 2010/0093024. Alternately, the reaction mix may comprise a nnAA conjugated to a tRNA

for which the naturally occurring tRNA synthetase has been depleted. *See*, *e.g.*, PCT Pub. No. WO2010/081111.

[0093] In some instances, the cell-free synthesis reaction does not require the addition of commonly secondary energy sources, yet uses co-activation of oxidative phosphorylation and protein synthesis. In some instances, CFPS is performed in a reaction such as the Cytomim (cytoplasm mimic) system. The Cytomim system is defined as a reaction condition performed in the absence of polyethylene glycol with optimized magnesium concentration. This system does not accumulate phosphate, which is known to inhibit protein synthesis.

[0094] The presence of an active oxidative phosphorylation pathway can be tested using inhibitors that specifically inhibit the steps in the pathway, such as electron transport chain inhibitors. Examples of inhibitors of the oxidative phosphorylation pathway include toxins such as cyanide, carbon monoxide, azide, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), and 2,4-dinitrophenol, antibiotics such as oligomycin, pesticides such as rotenone, and competitive inhibitors of succinate dehydrogenase such as malonate and oxaloacetate.

[0095] In some embodiments, the cell-free protein synthesis reaction is performed using the Cytomim system comprising NTPs, *E. coli* tRNA, amino acids, Mg^{2+} acetate, Mg^{2+} glutamate, K^{+} acetate, K^{+} glutamate, folinic acid, Tris pH 8.2, DTT, pyruvate kinase, T7 RNA polymerase, disulfide isomerase, sodium pyruvate, NAD, CoA, Na^{+} oxalate, putrescine, spermidine, and S30 extract. In some embodiments, the energy surbstrate for the Cytomim system is pyruvate, glutamic acid, and/or glucose. In some embodiments of the system, the nucleoside triphosphates (NTPs) are replaced with nucleoside monophosphates (NMPs).

[0096] The cell extract can be treated with iodoacetamide in order to inactivate enzymes that can reduce disulfide bonds and impair proper protein folding. As further described herein, the cell extract can also be treated with a prokaryotic disulfide bond isomerase, such as, not limited to, *E. coli* DsbC and PDI. The cell extract can be treated with DsbC, FkpA and peptidyl peolyl isomerase. Glutathione disulfide (GSSG) and glutathione (GSH) can also be added to the extract at a ratio that promotes proper protein folding and prevents the formation of aberrant protein disulfides.

[0097] In some embodiments, the CFPS reaction includes inverted membrane vesicles to perform oxidative phosphorylation. These vesicles can be formed during the high pressure homogenization step of the preparation of cell extract process, as described herein, and remain in the extract used in the reaction mix.

[0098] The cell-free extract can be thawed to room temperature before use in the CFPS reaction. The extract can be incubated with 50 μM iodoacetamide for 30 minutes when synthesizing protein with disulfide bonds. In some embodiments, the CFPS reaction includes about 30% (v/v) iodoacetamide-treated extract with about 8 mM magnesium glutamate, about 10 mM ammonium glutamate, about 130 mM potassium glutamate, about 35 mM sodium pyruvate, about 1.2 mM AMP, about 0.86 mM each of GMP, UMP, and CMP, about 2 mM amino acids (about 1 mM for tyrosine), about 4 mM sodium oxalate, about 0.5 mM putrescine, about 1.5 mM spermidine, about 16.7 mM potassium phosphate, about 100 mM T7 RNA polymerase, about 2-10 μg/mL plasmid DNA template, about 1-10 μM E.coli DsbC, and a total concentration of about 2 mM oxidized (GSSG) glutathione. Optionally, the cell free extract can include 1 mM of reduced (GSH).

[0099] The cell free synthesis reaction conditions may be performed as batch, continuous flow, or semi-continuous flow, as known in the art. The reaction conditions are linearly scalable, for example, the 0.3 L scale in a 0.5 L stirred tank reactor, to the 4 L scale in a 10 L fermentor, and to the 100 L scale in a 200 L fermentor.

[0100] The development of a continuous flow *in vitro* protein synthesis system by Spirin et al. (1988) Science 242:1162-1164 proved that the reaction could be extended up to several hours. Since then, numerous groups have reproduced and improved this system (*see, e.g.*, Kigawa *et al.* (1991) *J. Biochem.* 110:166-168; Endo *et al.* (1992) *J. Biotechnol.* 25:221-230). Kim and Choi (*Biotechnol. Prog.* 12: 645-649, 1996) have reported that the merits of batch and continuous flow systems can be combined by adopting a "semicontinuous operation" using a simple dialysis membrane reactor. They were able to reproduce the extended reaction period of the continuous flow system while maintaining the initial rate of a conventional batch system. However, both the continuous and semi-continuous approaches require quantities of expensive reagents, which must be increased by a significantly greater factor than the increase in product yield.

[0101] Several improvements have been made in the conventional batch system (Kim *et al.* (1996) *Eur. J. Biochem.* 239: 881-886; Kuldlicki *et al.* (1992) *Anal. Biochem.* 206:389-393; Kawarasaki *et al.* (1995) *Anal. Biochem.* 226: 320-324). Although the semicontinuous system maintains the initial rate of protein synthesis over extended periods, the conventional batch system still offers several advantages, *e.g.* convenience of operation, easy scale-up,

lower reagent costs and excellent reproducibility. Also, the batch system can be readily conducted in multiplexed formats to express various genetic materials simultaneously.

[0102] Patnaik and Swartz (*Biotechniques* 24:862-868, 1998) have reported that the initial specific rate of protein synthesis could be enhanced to a level similar to that of *in vivo* expression through extensive optimization of reaction conditions. It is notable that they achieved such a high rate of protein synthesis using the conventional cell extract prepared without any condensation steps (Nakano et al. (1996) *J. Biotechnol.* 46:275-282; Kim *et al.* (1996) *Eur. J. Biochem.* 239:881-886). Kigawa *et al.* (1999) *FEBS Lett* 442:15-19 report high levels of protein synthesis using condensed extracts and creatine phosphate as an energy source. These results imply that further improvement of the batch system, especially in terms of the longevity of the protein synthesis reaction, would substantially increase the productivity for batch *in vitro* protein synthesis. However, the reason for the early halt of protein synthesis in the conventional batch system has remained unclear.

[0103] The protein synthesis reactions described herein can utilize a large scale reactor, small scale, or may be multiplexed to perform a plurality of simultaneous syntheses. Continuous reactions can use a feed mechanism to introduce a flow of reagents, and may isolate the end-product as part of the process. Batch systems are also of interest, where additional reagents may be introduced to prolong the period of time for active synthesis. A reactor can be run in any mode such as batch, extended batch, semi-batch, semi-continuous, fed-batch and continuous, and which will be selected in accordance with the application purpose.

GENERATING A LYSATE

[0104] The methods and systems described herein use a cell lysate for *in vitro* translation of a target protein of interest. For convenience, the organism used as a source for the lysate may be referred to as the source organism or host cell. Host cells may be bacteria, yeast, mammalian or plant cells, or any other type of cell capable of protein synthesis. A lysate comprises components that are capable of translating messenger ribonucleic acid (mRNA) encoding a desired protein, and optionally comprises components that are capable of transcribing DNA encoding a desired protein. Such components include, for example, DNA-directed RNA polymerase (RNA polymerase), any transcription activators that are required for initiation of transcription of DNA encoding the desired protein, transfer ribonucleic acids (tRNAs), aminoacyl-tRNA synthetases, 70S ribosomes, N^{10}-formyltetrahydrofolate,

formylmethionine-tRNAf^{Met} synthetase, peptidyl transferase, initiation factors such as IF-1, IF-2, and IF-3, elongation factors such as EF-Tu, EF-Ts, and EF-G, release factors such as RF-1, RF-2, and RF-3, and the like.

[0105] An embodiment uses a bacterial cell from which a lysate is derived. A bacterial lysate derived from any strain of bacteria can be used in the methods of the invention. The bacterial lysate can be obtained as follows. The bacteria of choice are grown to log phase in any of a number of growth media and under growth conditions that are well known in the art and easily optimized by a practitioner for growth of the particular bacteria. For example, a natural environment for synthesis utilizes cell lysates derived from bacterial cells grown in medium containing glucose and phosphate, where the glucose is present at a concentration of at least about 0.25% (weight/volume), more usually at least about 1%; and usually not more than about 4%, more usually not more than about 2%. An example of such media is 2YTPG medium, however one of skill in the art will appreciate that many culture media can be adapted for this purpose, as there are many published media suitable for the growth of bacteria such as E. coli, using both defined and undefined sources of nutrients. Cells that have been harvested overnight can be lysed by suspending the cell pellet in a suitable cell suspension buffer, and disrupting the suspended cells by sonication, breaking the suspended cells in a French press, continuous flow high pressure homogenization, or any other method known in the art useful for efficient cell lysis The cell lysate is then centrifuged or filtered to remove large DNA fragments and cell debris.

[0106] The bacterial strain used to make the cell lysate generally has reduced nuclease and/or phosphatase activity to increase cell free synthesis efficiency. For example, the bacterial strain used to make the cell free extract can have mutations in the genes encoding the nucleases RNase E and RNase A. The strain may also have mutations to stabilize components of the cell synthesis reaction such as deletions in genes such as *tnaA, speA, sdaA or gshA*, which prevent degradation of the amino acids tryptophan, arginine, serine and cysteine, respectively, in a cell-free synthesis reaction. Additionally, the strain may have mutations to stabilize the protein products of cell-free synthesis such as knockouts in the proteases ompT or lonP.

PROTEINS OF INTEREST

[0107] The methods and systems described herein are useful for increasing the expression of properly folded, biologically active proteins of interest. The protein of interest can be any

protein that is capable of being expressed in a bacterial cell free synthesis system. Non-limiting examples include proteins with disulfide bonds and proteins with at least two proline residues. The protein of interest can be, for example, an antibody or fragment thereof, therapeutic proteins, growth factors, receptors, cytokines, enzymes, ligands, *etc*. Additional examples of proteins of interest are described below.

Proteins with disulfide bonds

[0108] The methods provided herein can be used for any protein having at least one disulfide bond in its biologically active confirmation. Disulfide bonds can stabilize tertiary protein structure by locking folding units into stable conformations by linking residues in a covalent manner.

[0109] In prokaryotic cells, disulfide bonds are formed when DsbA protein donates its disulfide bond to a newly synthesized polypeptide that comprises a disulfide bond in its native structure. The integral membrane protein DsbB generates disulfide bonds within itself, which are then transferred to DsbA. In some eukaryotic cells, the major disulfide pathway is composed of the membrane-associated flavoprotein EroI and the soluble thioredoxin-like protein PDI. EroI, using a flavin cofactor to mediate the reoxidation of its cysteine pair by oxygen, generates disulfide bonds within itself, and then transfers the bonds to PDI. In turn, PDI transfers the disulfide bonds directly to newly synthesized polypeptides that have not adopted their native structure.

[0110] Disulfide bonds are present in numerous proteins including, but not limited to secreted proteins, immune proteins, extracellular matrix proteins, glycoproteins, lysosomal proteins and membrane proteins. Detailed descriptions of disulfide bonds and proteins with disulfide bonds can be found in, *e.g.*, Fass, D. *Annu. Rev. Biophys.*, 2012, 41:63-79, Sevier, C.S. and Kaiser, C.A. *Antioxidants & Redox Signaling*, 2006, 8(5):797-811 and de Marco, A., *Microbial Cell Factories*, 2009, 8:26.

Proteins with Prolines

[0111] The methods provided herein can be used for any protein that has at least two proline residues. Proline containing proteins typically favor secondary structure elements such as turns and polyproline helices. A polyproline helix can be an elongated, left-handed helix with torsion angles $\varphi = -78^{\circ}$ and $\psi = +146^{\circ}$ of the peptide backbone. A relatively high proportion of prolines can be found in proteins near the center of transmembrane helices.

Proline residues can also be found in β-turns and α-helical capping motifs, *e.g.*, at the end of an α-helix or even one or two residues from the end. Prolines can also undergo cis-trans isomerization which is important for proper protein folding.

[0112] Proline-rich proteins include proteins with repetitive short proline-rich sequences, with tandemly repeated proline-rich sequences, with non-repetitive proline-rich regions, and with hydroxyproline-rich proteins. Prolines residues can be found in various proteins including, but not limited to integral membrane proteins such as transporters, channels, and receptors, globular proteins, hormones, neuropeptides, mucins, immunoglobulins, and extracellular matrix proteins.

[0113] It has been shown that proline-rich peptides can enhance and/or sustain nitric oxide production in cells, potentiate argininosuccinate synthetase activity in cells, increase intracellular concentration of calcium ions, and serve as ligands for SH3, WW, EVH1 or BHB domain containing proteins. Detailed descriptions of proline-containing proteins can be found in, *e.g.*, Williamson, M. *Biochem. J.* 1994, 297:249-260 and Kay *et al. FASEB J.*, 14:231-241.

CHAPERONES

[0114] To improve the expression of a biologically active protein of interest, the present methods and systems use a bacterial extract comprising an exogenous protein chaperone. Molecular chaperones are proteins that assist the non-covalent folding or unfolding and the assembly or disassembly of other macromolecular structures. One major function of chaperones is to prevent both newly synthesized polypeptide chains and assembled subunits from aggregating into nonfunctional structures. The first protein chaperone identified, nucleoplasmin, assists in nucleosome assembly from DNA and properly folded histones. Such assembly chaperones aid in the assembly of folded subunits into oligomeric structures. Chaperones are concerned with initial protein folding as they are extruded from ribosomes, intracellular trafficking of proteins, as well as protein degradation of misfolded or denatured proteins. Although most newly synthesized proteins can fold in absence of chaperones, a minority strictly requires them. Typically, inner portions of the chaperone are hydrophobic whereas surface structures are hydrophilic. The exact mechanism by which chaperones facilitate folding of substrate proteins is unknown, but it is thought that by lowering the activation barrier between the partially folded structure and the native form, chaperones accelerate the desired folding steps to ensure proper folding. Further, specific chaperones

unfold misfolded or aggregated proteins and rescue the proteins by sequential unfolding and refolding back to native and biologically active forms.

[0115] A subset of chaperones that encapsulate their folding substrates are known as chaperonins (*e.g.*, Group I chaperonin GroEL/GroES complex). Group II chaperonins, for example, the TRiC (TCP-1 Ring Complex, also called CCT for chaperonin containing TCP-1) are thought to fold cytoskeletal proteins actin and tubulin, among other substrates. Chaperonins are characterized by a stacked double-ring structure and are found in prokaryotes, in the cytosol of eukaryotes, and in mitochondria.

[0116] Other types of chaperones are involved in membrane transport in mitochondria and endoplasmic reticulum (ER) in eukaryotes. Bacterial translocation-specific chaperone maintains newly synthesized precursor polypeptide chains in a translocation-competent (generally unfolded) state and guides them to the translocon, commonly known as a translocator or translocation channel. A similar complex of proteins in prokaryotes and eukaryotes most commonly refers to the complex that transports nascent polypeptides with a targeting signal sequence into the interior (cisternal or lumenal) space of the endoplasmic reticulum (ER) from the cytosol, but is also used to integrate nascent proteins into the membrane itself (membrane proteins). In the endoplasmic reticulum (ER) there are general chaperones (BiP, GRP94, GRP170), lectin (calnexin and calreticulin) and non-classical molecular chaperones (HSP47 and ERp29) helping to fold proteins. Folding chaperone proteins include protein disulfide isomerases (PDI, DsbA, DsbC) and peptidyl prolyl cis-trans isomerases (PPI, FkpA, SlyD, TF).

[0117] Many chaperones are also classified as heat shock proteins (Hsp) because they are highly upregulated during cellular stress such as heat shock, and the tendency to aggregate increases as proteins are denatured by elevated temperatures or other cellular stresses. Ubiquitin, which marks proteins for degradation, also has features of a heat shock protein. Some highly specific 'steric chaperones' convey unique structural conformation (steric) information onto proteins, which cannot be folded spontaneously. Other functions for chaperones include assistance in protein degradation, bacterial adhesin activity, and response to prion diseases linked to protein aggregation.

[0118] Enzymes known as foldases catalyze covalent changes essential for the formation of the native and functional conformations of synthesized proteins. Examples of foldases include protein disulfide isomerase (PDI), which acts to catalyze the formation of native

disulfide bonds, and peptidyl prolyl cis-trans isomerase (PPI), which acts to catalyze isomerization of stable trans peptidyl prolyl bonds to the cis configuration necessary for the functional fold of proteins. The formation of native disulfides and the cis-trans isomerization of prolyl imide bonds are both covalent reactions and are frequently rate-limiting steps in the protein folding process. Recently proposed to be chaperone proteins, in stoichiometric concentrations foldases increase the reactivation yield of some denatured proteins. Other examples of chaperone proteins include deaggregases such as Skp, and the redox proteins Trr1 and Glr1.

[0119] In some embodiments, the protein chaperone can be co-expressed with another protein(s) that functions to increase the activity of the desired protein chaperone. For example, the Dsb proteins DsbA and DsbC can be coexpressed with DsbB and DsbD, which oxidize and reduce DsbA and DsbC, respectively.

TRANSFORMING BACTERIA WITH GENES ENCODING THE CHAPERONES

[0120] The bacterial extracts used in the methods and systems described herein contain an exogenous protein chaperone. The exogenous protein chaperones described herein can be added to the extract, or can be expressed by the bacteria used to prepare the cell free extract. In the latter embodiment, the exogenous protein chaperone can be expressed from a gene encoding the exogenous protein chaperone that is operably linked to a promoter that initiates transcription of the gene.

[0121] Promoters that may be used in the present invention include both constitutive promoters and regulated (inducible) promoters. The promoters may be prokaryotic or eukaryotic depending on the host. Among the prokaryotic (including bacteriophage) promoters useful for practice of this invention are lac, T3, T7, lambda Pr'P1' and trp promoters. Among the eukaryotic (including viral) promoters useful for practice of this invention are ubiquitous promoters (*e.g.* HPRT, vimentin, actin, tubulin), intermediate filament promoters (*e.g.* desmin, neurofilaments, keratin, GFAP), therapeutic gene promoters (*e.g.* MDR type, CFTR, factor VIII), tissue-specific promoters (*e.g.* actin promoter in smooth muscle cells), promoters which respond to a stimulus (*e.g.* steroid hormone receptor, retinoic acid receptor), tetracycline-regulated transcriptional modulators, cytomegalovirus immediate-early, retroviral LTR, metallothionein, SV-40, E1a, and MLP promoters. Tetracycline-regulated transcriptional modulators and CMV promoters are described in WO 96/01313,

U.S. Pat. Nos. 5,168,062 and 5,385,839, the entire disclosures of which are incorporated herein by reference.

[0122] In some embodiments, the promoter is a constitutive promoter. Examples of constitutive promoters in bacteria include the *spc* ribosomal protein operon promotor P_{spc}, the β-lactamase gene promotor P_{bla} of plasmid pBR322, the P_L promoter of phage λ, the replication control promoters P_{RNAI} and P_{RNAII} of plasmid pBR322, the P1 and P2 promoters of the *rrnB* ribosomal RNA operon, the *tet* promoter, and the pACYC promoter.

QUANTITATIVELY MEASURING PROTEIN OF INTEREST AND CHAPERONES

[0123] The quantity of the protein of interest produced by the methods and systems described herein can be determined using any method known in the art. For example, the expressed protein of interest can be purified and quantified using gel electrophoresis (*e.g.*, PAGE), Western analysis or capillary electrophoresis (*e.g.*, Caliper LabChip). Protein synthesis in cell-free translation reactions may be monitored by the incorporation of radiolabeled amino acids, typically, ^{35}S-labeled methionine or ^{14}C-labeled leucine. Radiolabeled proteins can be visualized for molecular size and quantitated by autoradiography after electrophoresis or isolated by immunoprecipitation. The incorporation of recombinant His tags affords another means of purification by Ni^{2+} affinity column chromatography. Protein production from expression systems can be measured as soluble protein yield or by using an assay of enzymatic or binding activity.

[0124] The amount of chaperone protein that is added to the cell free synthesis system can be quantified by including a radioactive amino acid, such as ^{14}C-Leucine, in the bacterial cell culture used to prepare the bacterial extract, and quantifying the amount of expressed protein chaperone by, for example, precipitating the radioactive protein using trichloroacetic acid (TCA), and measuring the total amount of radioactivity recovered. The amount of chaperone can also be measured immunologically, for example, by an ELISA in which monoclonal or polyclonal antibodies against the chaperone are used to detect and quantify chaperone protein immobilized in plates or on a Western blot.

QUANTITATIVELY MEASURING BIOLOGICAL ACTIVITY AND PROPER FOLDING OF EXPRESSED PROTEINS

[0125] The biological activity of a protein of interest produced by the methods described herein can be quantified using an *in vitro* or *in vivo* assay specific for the protein of interest. The biological activity of the protein of interest can be expressed as the biological activity per

unit volume of the cell-free protein synthesis reaction mixture. The proper folding of an expressed protein of interest can be quantified by comparing the amount of total protein produced to the amount of soluble protein. For example, the total amount of protein and the soluble fraction of that protein produced can be determined by radioactively labeling the protein of interest with a radiolabeled amino acid such as ^{14}C-leucine, and precipitating the labeled proteins with TCA. The amount of folded and assembled protein can be determined by gel electrophoresis (PAGE) under reducing and non-reducing conditions to measure the fraction of soluble proteins that are migrating at the correct molecular weight. Under non-reducing conditions, protein aggregates can be trapped above the gel matrix or can migrate as higher molecular weight smears that are difficult to characterize as discrete entities, whereas under reducing conditions and upon heating of the sample, proteins containing disulfide bonds are denatured, aggregates are dissociated, and expressed proteins migrate as single bands. Methods for determining the amount of properly folded and assembled antibody proteins are described in the Examples. Functional activity of antibody molecules can be determined using an immunoassay, for example, an ELISA.

EXAMPLES

EXAMPLE 1

[0126] This example demonstrates that chaperone proteins expressed by a bacterial cell free protein synthesis system increase the amount of properly assembled IgG expressed by the cell free protein synthesis system, and that the combination of a bacterial PDI and a PPI acted synergistically to increase the amount of properly assembled IgG.

[0127] Engineering of a bacterial endoplasmic reticulum for the rapid expression of immunoglobulin proteins.

[0128] Materials and Methods:

[0129] Small-scale cell-free expression. 100 µl cell-free protein synthesis reactions were run at 30°C for 12 hr in a 96-well microtiter plate at 650 rpm in a VWR Thermomixer in the presence of 10 µg/mL DNA (2.5 µg/mL trastuzumab light chain DNA, 7.5 µg/mL trastuzumab heavy chain DNA in the expression vector pYD317). Cell-free extracts were treated with 50 µM iodoacetamide for 30 min at RT (20°C) and added to a premix of components. The final concentration in the protein synthesis reaction was 30% cell extract (v/v), 2 mM GSSG, 8 mM magnesium glutamate, 10 mM ammonium glutamate, 130 mM potassium glutamate, 35 mM sodium pyruvate, 1.2 mM AMP, 0.86 mM each of GMP, UMP,

and CMP, 2 mM amino acids (except 1 mM for tyrosine and phenylalanine), 4 mM sodium oxalate, 1 mM putrescine, 1.5 mM spermidine, 15 mM potassium phosphate, 20 ug/mL T7 RNAP, unless otherwise indicated.

[0130] Interchangeability of PDI and DsbC. Cell-free protein synthesis reactions were run at varying concentrations of PDI and DsbC to understand the requirements for disulfide bond isomerases on IgG folding and assembly. 0-5 uM recombinant PDI was added to cell-free reactions in combination with 0-13 uM recombinant DsbC. 100 µl cell-free reactions were run with 30% control extract for 12 hr at 30°C in a 96-well microtiter plate at 650 rpm in a VWR Thermomixer in the presence of 8 µg/mL HC-HIS6 DNA and 2 µg/mL LC DNA. The reactions were subsequently centrifuged at 5000xg for 10 minutes and supernatants were diluted 2-fold with PBS prior to purification on IMAC Phytips (200 µl tips, 5 µl resin bed) using a Biomek robotic system. Samples were eluted in 20 mM Tris pH8, 300 mM NaCl, 500 mM imidazole and the eluted IgG was quantified using capillary electrophoresis on a Caliper LapChip GXII.

[0131] Chaperone sequential expression screen. Candidate chaperones were cloned into the cell-free expression plasmid pYD317. From these plasmids, PCR fragments were generated that contained the chaperone gene sandwiched between T7 promoter and terminator sequences. Chaperones were subsequently expressed from these PCR fragments by cell-free protein synthesis under standard microtiter plate conditions for 16 hr at 30°C. To stabilize the PCR fragments against DNA degradation, 40 ug/mL GamS protein was added to the reactions. Chaperone-expressing extract was subsequently centrifuged at 5000xg for 10 minutes and chaperone-containing supernatants were added into new cell-free reactions at 20% (v/v) for the expression of IgG (8 µg/mL trastuzumab heavy chain DNA and 2 µg/mL trastuzumab light chain DNA) in the presence of ^{14}C-leucine. IgG titers were calculated based on the rate of incorporation of ^{14}C-leucine into the IgG molecule, as previously described (MAbs. 2012 Mar 1;4(2)). Chaperone-related improvements in IgG titer were expressed as a fold improvement over the addition of a GFP-expressing extract. To estimate the amount of chaperone being added to the IgG expression reactions, chaperone cell-free reactions were also run in the presence of ^{14}C-leucine and the expressed protein was quantified.

[0132] 2xDsbC and 2xFkpA extracts. Bicistronic plasmids of the bacterial genes DsbC (2xDsbC) and FkpA (2xFkpA) behind a constitutive promoter (pACYC) were generated and

transformed into bacteria. These strains were grown to log phase and lysed for the production of cell-free extract, as described in Yang W.C. *et al. Biotechnol. Prog.* (2012), 28(2):413-20. FkpA protein was added to an IgG cell-free reaction using 2xDsbC extract to test if FkpA would further improve IgG folding and assembly. The reverse experiment was performed by the addition of 13 μM DsbC protein to a cell-free reaction with 2xFkpA extract.

[0133] Results:

[0134] Interchangeability of PDI and DsbC. To better understand the dependence of IgG folding and assembly on eukaryotic and bacterial disulfide bond isomerases, IgG cell-free protein synthesis reactions were run at varying concentrations of PDI and DsbC. IgG was expressed in cell-free reactions in the presence of 0-5 μM PDI in combination with 0-13 μM DsbC. Expressed IgG-His was purified by Ni^{++} resin and quantified by capillary electrophoresis (**Figure 1**). In the absence of DsbC, IgG was highly dependent on PDI for folding (**Figure 1**, closed circles). However, as the concentration of DsbC in the reaction increased, the dependence on PDI fell such that at 6.4 μM DsbC, there was no additional benefit attributable to PDI in the reaction (**Figure 1**, open triangles). Furthermore, by increasing the concentration of DsbC in the reaction, we saw marked improvements in IgG titers beyond what we had previously observed (**Figure 1**, open circles). In effect, we observed the efficient substitution of a eukaryotic disulfide bond isomerase with a bacterial chaperone of a similar function in the folding of a eukaryotic protein.

[0135] Chaperone sequential expression screen. *In vivo*, eukaryotic chaperones are known to play an important role in the folding and assembly of IgG. Therefore, expression of IgG molecules in bacterial systems which lack these physiological foldases has been challenging (REFS). As such, we undertook a screening approach to identify chaperone proteins that would be positive effectors of IgG folding and/or assembly. Candidate chaperones were expressed in our cell-free system and expressed chaperones were subsequently added into new cell-free reactions for the expression of IgG. Any improvements in IgG folding were expressed as an improvement in titer over the addition of a GFP-expressing control extract, a protein unlikely to interact with IgG. In order to improve the throughput of the screen, chaperones were not purified from the extract before being added to IgG reactions. Because of this, we wanted to ensure that chaperone DNA was not being transcribed and expressed in subsequent IgG reactions. As such, chaperone proteins were expressed from PCR template which is significantly more labile than plasmid DNA. The addition of GamS protein helped

preserve the PCR template, such that sufficient levels of chaperone protein could be synthesized.

[0136] Several families of chaperones were of particular interest given their role in folding IgG *in vivo*. PPIases, foldases, deaggregases, and redox proteins from bacterial, yeast, and human species were tested. Among the redox chaperones, we found that PDI (yeast homologue) and DsbC significantly aided IgG formation, consistent with our previous findings (**Figure 2B**). Interestingly, human PDI (hPDI) did not significantly impact IgG folding, probably due to its poor expression in cell extract which did not allow it to be added in sufficient quantities to aid IgG folding. By contrast, the bacterial protein DsbC expressed very well in the extract, allowing the addition of ~5 uM DsbC to the IgG reaction (DsbC was expressed at ~25 uM and it was added at 20% to an IgG reaction). Among the PPIases tested, several proved to be beneficial to IgG expression (**Figure 2B**). From these, we decided to follow-up on Skp, SlyD, and FkpA.

[0137] Purified Skp, SlyD, and FkpA can improve IgG titers. To confirm our hits from the chaperone screen, we expressed and purified Skp, SlyD, and FkpA and added them back into IgG cell-free protein synthesis reactions (**Figure 3**). For the chaperone Skp, we saw that Skp aided the solubility of HC and LC, but did not increase the amount of assembled IgG significantly. However, for the prolyl isomerases, SlyD and FkpA, we observed that the more of these chaperones we added, the amount of soluble proteins and assembled IgG increased proportionately. We reasoned that prolyl isomerization was a function that was previously limiting for IgG formation in our cell-free protein synthesis system and the addition of these exogenous proteins improved IgG folding and assembly dramatically. Because of the vast improvements observed with DsbC and FkpA, we decided to further characterize their roles in IgG folding.

[0138] FkpA and DsbC work synergistically to fold and assemble IgG. To better understand the roles that FkpA and DsbC play in IgG formation, we independently evaluated their contributions to IgG folding (**Figure 4**). Interestingly, the addition of FkpA significantly reduced the degree of higher molecular weight aggregates formed during HC and LC synthesis. With increasing amounts of FkpA, we also observe the formation of IgG, as well as a number of partially assembled products. These proteins migrated as fuzzy bands, suggesting that they may represent mixed populations of cross-disulfide bonded proteins. The addition of DsbC, on the other hand, generated clear sharp bands of IgG. However,

without FkpA, a significant proportion of the expressed proteins formed higher order aggregates that could not completely enter the SDS-PAGE gel.

[0139] When the two chaperones were combined into the same IgG reaction, they acted synergistically to fold IgG (**Figure 5**). HC and LC were expressed in a DsbC-containing extract (2xDsbC) and different amounts of exogenous FkpA protein were added. At 50 uM FkpA, on the order of 900 ug/mL of assembled IgG could be expressed. To follow-up on this, a bacterial strain overexpressing FkpA was engineered from which cell extract was generated. IgG was synthesized from FkpA extract with the addition of exogenous DsbC protein (**Figure 6**). IgG was produced at ~600 ug/mL with reduced aggregation under our standard conditions of 30% extract (v/v). To further increase the concentration of FkpA in each reaction, we titrated up the FkpA-containing extract in the reaction which brought the IgG titers to > 900 ug/mL (**Figure 6**).

[0140] The above example demonstrates that the combination of two different classes of protein chaperones, a PDI and a PPI, provides a synergistic effect on proper protein folding and assembly in a cell free expression system.

EXAMPLE 2

[0141] This example demonstrates that overexpression of exogenous protein chaperones in bacterial strains used to prepare cell extracts does not inhibit the production of a protein of interest such as GMCSF.

[0142] Strain Descriptions:

[0143] SBDG028: SBJY001 + pACYC 2x DsbC + ΔRF1

[0144] SBDG031: SBJY001 + pACYC 2x DsbC

[0145] SBDG044: SBJY001 + pACYC 2x FkpA

[0146] SBDG049: SBJY001 + pACYC 2x FkpA-6xHis

[0147] Cell Extract Preparation:

[0148] Extracts from *E. coli* strains SBDG028, SBDG031, SBDG044 and SBDG049 were prepared essentially as described in Zawada *et al.*, *Biotechnology and Bioengineering* Vol. 108, No. 7, July 2011.

[0149] GMCSF CFPS Reaction

[0150] The cell-free reaction procedure for GMCSF protein production was performed as described in Zawada *et al. Biotechnology and Bioengineering* Vol. 108, No. 7, July 2011, which is incorporated by reference herein in its entirety.

[0151] **Figure 7** shows the amount of GMCSF protein produced by the CFPS in extracts from the indicated strains that overexpress DsbC or FkpA. In control extracts prepared from bacteria that do not express an exogenous DsbC or FkpA, very little GMCSF is produced (data not shown).

EXAMPLE 3

[0152] This example demonstrates that bacterial cells overexpressing protein chaperones have similar growth rates as bacteria that do not overexpress protein chaperones.

Methods: Bacterial strains were transformed with recombinant plasmids that express one (1X) or two (2X) copies DsbC and FkpA, as described in Example 1. These strains were grown to log phase lysed for the production of cell-free extract. The growth rates (doubling times) for the strains were determined, and the amount of protein chaperone produced by the bacteria strains was quantified using Western analysis and/or ELISA.

[0153] To determine the intracellular concentration of the expressed protein chaperones, the periplasm of shake flask grown cells was lysed using osmotic shock. The periplasmic lysate was separated by gel electrophoresis with standards of known DsbC concentration. Densitometry was used to compare the intensity of the standard DsbC bands to the intensity of the bands in the periplasmic lysate. The intensity of the bands was used to determine the DsbC concentration in the lysate, which was used to back calculate the concentration of DsbC in the cells.

[0154] The amount of chaperone protein in the cell-free extracts was determined by ELISA. The ELISA to determine DsbC and FkpA titers in cell-free extract is the Direct ELISA format. The assay consists of coating an assay plate with standards and samples, then allowing an antibody that recognizes DsbC or FkpA to bind, washing away excess DsbC and FkpA antibody, introducing an HRP conjugated secondary antibody to rabbit IgG (the DsbC and FkpA antibodies were produced in rabbit), washing away excess conjugated secondary antibody, and then using an ABTS substrate to detect the HRP present on the conjugated secondary antibody. Purified DsbC and FkpA with known concentrations were used to create a 7 point standard curve to use in the determination of sample concentrations.

[0155] DsbC: MSD (Minimum Sample Dilution): 1/120,000; LLOQ (Lower Limit of Quantitation) at MSD: 187.5 ug/ml.

[0156] FkpA: MSD (Minimum Sample Dilution): 1/75,000; LLOQ (Lower Limit of Quantitation) at MSD: 390 ug/ml

[0157] Results:

[0158] **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. The growth rates of strains expressing 1X and 2X copies of DsbC were similar to a control strain that was not transformed with the expression plasmids. The lower panel of **Figure 8** shows the amount of DsbC protein present in the periplasmic lysate, as described above.

[0159] **Figure 9** shows the amount of DsbC protein produced by the bacterial strains overexpressing 1X or 2X copies of DsbC. The upper panel shows the intracellular concentration, determined as described above. The lower panel shows the extract concentration, determined by ELISA.

[0160] **Figure 10** shows growth rate of bacterial strains transformed with plasmids that express 1X or 2X copies of FkpA under the control of a constitutive promoter. The growth rates of strains expressing 1X and 2X copies of FkpA were similar to a control strain that was not transformed with the expression plasmids. The lower left panel of Figure 10 shows the amount of FkpA protein present in total extracts prepared from the bacteria expressing 1X and 2X copies of FkpA. **Figure 11** shows the quantitation of FkpA concentration in extracts from bacteria expressing 1X and 2X copies of FkpA.

[0161] The results of representative ELISA experiments are shown in the Tables below. The ELISA data for FkpA is from a different extract preparation than that shown in **Figure 9**, which accounts for the different DsbC concentrations.

Table 1. DsbC concentrations determined in extracts by ELISA.

Strain	Description	DsbC Titer (mg/ml)	Standard Deviation (mg/ml)
SBJY-001	WT Control Extract	< 0.188	N/A
SBDG-026	1x DsbC Extract	1.084	0.016
SBDG-	2x DsbC Extract	3.155	0.351

028			
SBDG-031	2x DsbC Extract (No RF1 Deletion)	3.267	0.353
SBDG-033	3x DsbC Extract	2.854	0.272

Table 2. FkpA concentrations determined in extracts by ELISA.

Strain	Description	FkpA Titer (mg/ml)	Standard Deviation (mg/ml)
SBJY-001	WT Control Extract	< 0.390	N/A
SBDG-034	1x FkpA	3.029	0.305
SDDG-044	2x FkpA	5.121	0.076
SBDG-048	2x FkpA with leader sequences removed for cytoplasmic expression	1.960	0.252
SBDG-049	2x FkpA w/6xHis (SEQ ID NO:24) tag	5.415	0.147
SBDG-052	1x FkpA Pc7 Promoter	2.786	0.059

[0162] This example demonstrates that recombinant bacterial strains that overexpress chaperone proteins are capable of rapid growth and are useful for preparing high quality extracts for cell free protein synthesis.

EXAMPLE 4

[0163] This example shows that including a poly-charged amino acid tag on the C-terminal of the chaperone FkpA increased the amount of FkpA in the extract, and increased the amount of total protein produced by the cell free protein synthesis system.

[0164] The gene encoding FkpA was cloned with either a His_6 (SEQ ID NO:24)or $(Ser-Arg)_4$ (SEQ ID NO:25) tag on the C-terminus in vector pACYC-Pc. These vectors were transformed into strain SBJY001 and extract was produced as described above. An FkpA ELISA showed that extract levels of the His-tagged FkpA variants were increased by a final centrifugal spin of the extract, post-activation (**Figure 12a**). Compared to extract containing wt FkpA, extracts containing these solubility-tagged FkpA proteins produced more total protein. In addition, assembled IgG levels were enhanced by a final spin of the extract after activation (**Figure 12b**).

[0165] This example demonstrates that adding a poly-charged amino acid tag on the C-terminus of FkpA increased the amount of FkpA expressed by bacteria used to make the extract and increased the amount of total protein produced. Further, for extracts containing the C-terminal His-tagged FkpA, spinning the extract down after activation resulted in an increase in the amount of correctly assembled IgG.

EXAMPLE 5

[0166] This example demonstrates that genomic integration of the chaperones *dsbC* and FkpA in two independent bacterial strains resulted in cells with a high growth rate that produced high chaperone levels, and cell-free extracts derived from these strains contained high levels of both chaperones and supported cell-free synthesis of high levels recombinant IgG and GMC-SF.

Strain 108

[0167] Strain SBDG108 is a derivative of SBMT095. This strain has 2 copies of *dsbC* integrated onto the chromosome into the galK locus behind a medium strength constitutive promoter prepared using homologous recombination. SBMT095 was made competent and then transformed with pACYC-Pc0-2xFkpA, a medium copy plasmid with two copies of FkpA behind a constitutive promoter. Both copies coded for wild type *E. coli* FkpA, but one gene had been synthesized to reduce nucleotide homology to the WT gene, enabling each to be propagated stably in the same plasmid.

[0168] In a standard extract fermentation using DM80-80 in batch mode, strain SBDG108 was capable of achieving a high growth rate while still producing very high chaperone levels (See Table 3).

Table 3. Properties of 108 in extract fermentation.

Intracellular DsbC titer	4.1 mg/ml
Intracellular FkpA titer	13.9 mg/ml
Specific Growth Rate	0.49 /h

[0169] The extract made from strain 108 contained high levels of both chaperones and supported cell-free synthesis of very high levels of recombinant IgGs and other proteins (see Table 4).

Table 4. Cell-Free protein titers.

GMC-SF	0.44 mg/ml
Trastuzumab	1.1 mg/ml

Strain 150

[0170] Strain SBMT150 is a derivative of SBHS016, a KGK10 derivative with ompT sensitive RF1. To produce SBMT150, 2 copies of DsbC were integrated onto the chromosome into the xylA locus. Two copies of FkpA were integrated into the galK locus. Both chromosomal integrations were introduced with homologous recombination.

[0171] In a standard extract fermentation using DM80-80 in batch mode, strain SBMT150 was capable of achieving a high growth rate while still producing high chaperone levels (see Table 5). Because the chaperones are overexpressed from the genome, no antibiotics are required during the fermentation of this strain.

Table 5. Properties of 150 in extract fermentation.

Intracellular DsbC titer	2.5 mg/ml
Intracellular FkpA titer	3.4 mg/ml
Specific Growth Rate	.071 /h

[0172] The extract made from strain 108 contained high levels of both chaperones and supported cell-free synthesis of high levels of recombinant IgGs and other proteins, as shown in the Table 6 below.

Table 6. Cell Free Protein Titers.

GMC-SF	0.46 mg/ml
Trastuzumab	0.49 mg/ml

[0173] In summary, this example demonstrates that bacterial strains can be engineered to stably incorporate chaperone expression cassettes that express high levels of chaperone

proteins without compromising growth rates, and that cell free extracts derived from these strains yield high levels of recombinant proteins of interest.

EXAMPLE 6

[0174] This example shows that extracts derived from bacterial cells that overexpress the DsbC and FkpA chaperones can improve the expression and assembly of multiple different IgG's.

[0175] Methods:

[0176] 2xDsbC and 2xFkpA extracts. The *E. coli* strain SBJY001 (Yin G, et al., Aglycosylated antibodies and antibody fragments produced in a scalable in vitro transcription-translation system. *mAbs* 2012; 4) was transformed with pACYC-based chaperone overexpression plasmids and harvested in log phase to make cellular extracts. Plasmids carrying one copy (1xDsbC) or two tandem copies (2xDsbC) of *dsbC* behind the E. coli promoter Mt-cons-10 (Thouvenot B. *et al.* The strong efficiency of the Escherichia coli gapA P1 promoter depends on a complex combination of functional determinants. *Biochem J* 2004; 383:371–82) were generated and transformed into bacteria, as were one copy (1xFkpA) or two copies (2xFkpA) of *fkpA*. These strains were grown to log phase and lysed for the production of cell-free extract, as described (Zawada J.F. *et al.* Microscale to manufacturing scale-up of cell-free cytokine production--a new approach for shortening protein production development timelines. *Biotechnol Bioeng* 2011; 108:1570–8). The IgG-producing activities of each of these extracts were tested, either alone or in combination with exogenously added purified protein. A bacterial strain SBHS016 (derived from bacterial strain SBJY001) optimized for OCFS extracts was further modified to enhance the production of DsbC protein. This strain has dual tandem copies of *dsbC* integrated into the bacterial *galK* locus, constitutively expressed using a modified MT-cons-10 promoter (Thouvenot B. *et al.* *Biochem J* 2004; 383:371–82). This is in addition to the wild type gene at the normal *dsbC* locus. The dual tandem gene cassette contains one copy of the parental *dsbC* gene, and one copy of a synthetic version of the *dsbC* gene designed to encode the wild type protein, but with altered codons to suppress unwanted sequence recombination with other versions of *dsbC* gene elsewhere in the genome. This DsbC overexpressing strain was transformed with the 2xFkpA plasmid to produce strain '2xD + 2xF'.

[0177] Results:

[0178] A panel of different IgG's were translated in a bacterial *in vitro* transcription/translation system described herein. The IgG's were translated in a control extract (SBJY001), a DsbC extract (2xDsbC extract), and a DsbC + FkpA extract (2xD + 2xF). The panel included the therapeutic antibodies trastuzumab (an anti-Her2 IgG1) and brentuximab (an anti-CD30 IgG1), in addition to two germline Heavy Chains VH3-7 and VH3-23 in combination with the Light Chain Vk3-20. As shown in **Figure 13**, expression of the IgG's in the 2xDsbC extract dramatically improved the yield of all four IgG's. Further improvements were observed in the DsbC + FkpA extract, bringing expression levels to 1 g/L for both trastuzumab and brentuximab and nearly 1.5 g/L for the germline IgGs.

[0179] This example demonstrates that extracts from engineered bacteria that overexpress the chaperones DsbC and FkpA can increase the expression of a wide-range of immunoglobulin proteins in a OCFS coupled transcription-translation system.

[0180] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, sequence accession numbers, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

Informal Sequence Listing:

SEQ ID NO:1 NP_417369 protein disulfide isomerase II [Escherichia coli str. K-12 substr. MG1655] (DsbC; xprA) (UniProt P0AEG6)

```
        1 MKKGFMLFTL LAAFSGFAQA DDAAIQQTLA KMGIKSSDIQ PAPVAGMKTV LTNSGVLYIT
       61 DDGKHIIQGP MYDVSGTAPV NVTNKMLLKQ LNALEKEMIV YKAPQEKHVI TVFTDITCGY
      121 CHKLHEQMAD YNALGITVRY LAFPRQGLDS DAEKEMKAIW CAKDKNKAFD DVMAGKSVAP
      181 ASCDVDIADH YALGVQLGVS GTPAVVLSNG TLVPGYQPPK EMKEFLDEHQ KMTSGK
```

SEQ ID NO:2 NP_418297 periplasmic protein disulfide isomerase I [Escherichia coli str. K-12 substr. MG1655] (DsbA; dsf; ppfA) (UniProt P0AEG4)

```
        1 MKKIWLALAG LVLAFSASAA QYEDGKQYTT LEKPVAGAPQ VLEFFSFFCP HCYQFEEVLH
       61 ISDNVKKKLP EGVKMTKYHV NFMGGDLGKD LTQAWAVAMA LGVEDKVTVP LFEGVQKTQT
      121 IRSASDIRDV FINAGIKGEE YDAAWNSFVV KSLVAQQEKA AADVQLRGVP AMFVNGKYQL
      181 NPQGMDTSNM DVFVQQYADT VKYLSEKK
```

SEQ ID NO:3 NP_415703 oxidoreductase that catalyzes reoxidation of DsbA protein disulfide isomerase I [Escherichia coli str. K-12 substr. MG1655] (DsbB; roxB; ycgA) (UniProt P0A6M2)

```
        1 MLRFLNQCSQ GRGAWLLMAF TALALELTAL WFQHVMLLKP CVLCIYERCA LFGVLGAALI
       61 GAIAPKTPLR YVAMVIWLYS AFRGVQLTYE HTMLQLYPSP FATCDFMVRF PEWLPLDKWV
      121 PQVFVASGDC AERQWDFLGL EMPQWLLGIF IAYLIVAVLV VISQPFKAKK RDLFGR
```

SEQ ID NO:4 NP_418559 fused thiol:disulfide interchange protein: activator of DsbC/conserved protein [Escherichia coli str. K-12 substr. MG1655] (DsbD; C-type cytochrome biogenesis protein CycZ; inner membrane copper tolerance protein; protein-disulfide reductase) (UniProt P36655)

```
        1 MAQRIFTLIL LLCSTSVFAG LFDAPGRSQF VPADQAFAFD FQQNQHDLNL TWQIKDGYYL
       61 YRKQIRITPE HAKIADVQLP QGVWHEDEFY GKSEIYRDRL TLPVTINQAS AGATLTVTYQ
      121 GCADAGFCYP PETKTVPLSE VVANNAAPQP VSVPQQEQPT AQLPFSALWA LLIGIGIAFT
      181 PCVLPMYPLI SGIVLGGKQR LSTARALLLT FIYVQGMALT YTALGLVVAA AGLQFQAALQ
      241 HPYVLIGLAI VFTLLAMSMF GLFTLQLPSS LQTRLTLMSN RQQGGSPGGV FVMGAIAGLI
      301 CSPCTTAPLS AILLYIAQSG NMWLGGGTLY LYALGMGLPL MLITVFGNRL LPKSGPWMEQ
      361 VKTAFGFVIL ALPVFLLERV IGDVWGLRLW SALGVAFFGW AFITSLQAKR GWMRIVQIIL
      421 LAAALVSVRP LQDWAFGATH TAQTQTHLNF TQIKTVDELN QALVEAKGKP VMLDLYADWC
      481 VACKEFEKYT FSDPQVQKAL ADTVLLQANV TANDAQDVAL LKHLNVLGLP TILFFDGQGQ
      541 EHPQARVTGF MDAETFSAHL RDRQP
```

SEQ ID NO:5 NP_415137 thiol:disulfide interchange protein, periplasmic [Escherichia coli str. K-12 substr. MG1655] (DsbG; ybdP) (UniProt P77202)

```
        1 MLKKILLLAL LPAIAFAEEL PAPVKAIEKQ GITIIKTFDA PGGMKGYLGK YQDMGVTIYL
       61 TPDGKHAISG YMYNEKGENL SNTLIEKEIY APAGREMWQR MEQSHWLLDG KKDAPVIVYV
      121 FADPFCPYCK QFWQQARPWV DSGKVQLRTL LVGVIKPESP ATAAAILASK DPAKTWQQYE
      181 ASGGKLKLNV PANVSTEQMK VLSDNEKLMD DLGANVTPAI YYMSKENTLQ QAVGLPDQKT
      241 LNIIMGNK
```

SEQ ID NO:6 NP_417806 FKBP-type peptidyl-prolyl cis-trans isomerase (rotamase) [Escherichia coli str. K-12 substr. MG1655] (FkpA; PPIase) (UniProt P45523)

```
        1 MKSLFKVTLL ATTMAVALHA PITFAAEAAK PATAADSKAA FKNDDQKSAY ALGASLGRYM
       61 ENSLKEQEKL GIKLDKDQLI AGVQDAFADK SKLSDQEIEQ TLQAFEARVK SSAQAKMEKD
      121 AADNEAKGKE YREKFAKEKG VKTSSTGLVY QVVEAGKGEA PKDSDTVVVN YKGTLIDGKE
      181 FDNSYTRGEP LSFRLDGVIP GWTEGLKNIK KGGKIKLVIP PELAYGKAGV PGIPPNSTLV
      241 FDVELLDVKP APKADAKPEA DAKAADSAKK
```

SEQ ID NO:7 NP_417808 FKBP-type peptidyl prolyl cis-trans isomerase (rotamase) [Escherichia coli str. K-12 substr. MG1655] (SlyD; histidine-

rich protein; metallochaperone SlyD; sensitivity to lysis protein D; WHP; PPIase) (UniProt P0A9K9)

```
        1 MKVAKDLVVS LAYQVRTEDG VLVDESPVSA PLDYLHGHGS LISGLETALE GHEVGDKFDV
       61 AVGANDAYGQ YDENLVQRVP KDVFMGVDEL QVGMRFLAET DQGPVPVEIT AVEDDHVVVD
      121 GNHMLAGQNL KFNVEVVAIR EATEEELAHG HVHGAHDHHH DHDHDGCCGG HGHDHGHEHG
      181 GEGCCGGKGN GGCGCH
```

SEQ ID NO:8 NP_414595 peptidyl-prolyl cis-trans isomerase (PPIase) [Escherichia coli str. K-12 substr. MG1655] (SurA; peptidyl-prolyl cis-trans isomerase SurA; rotamase SurA; survival protein A; PPIase SurA) (UniProt P0ABZ6)

```
        1 MKNWKTLLLG IAMIANTSFA APQVVDKVAA VVNNGVVLES DVDGLMQSVK LNAAQARQQL
       61 PDDATLRHQI MERLIMDQII LQMGQKMGVK ISDEQLDQAI ANIAKQNNMT LDQMRSRLAY
      121 DGLNYNTYRN QIRKEMIISE VRNNEVRRRI TILPQEVESL AQQVGNQNDA STELNLSHIL
      181 IPLPENPTSD QVNEAESQAR AIVDQARNGA DFGKLAIAHS ADQQALNGGQ MGWGRIQELP
      241 GIFAQALSTA KKGDIVGPIR SGVGFHILKV NDLRGESKNI SVTEVHARHI LLKPSPIMTD
      301 EQARVKLEQI AADIKSGKTT FAAAAKEFSQ DPGSANQGGD LGWATPDIFD PAFRDALTRL
      361 NKGQMSAPVH SSFGWHLIEL LDTRNVDKTD AAQKDRAYRM LMNRKFSEEA ASWMQEQRAS
      421 AYVKILSN
```

SEQ ID NO:9 NP_414720 periplasmic chaperone [Escherichia coli str. K-12 substr. MG1655] (Skp; chaperone protein skp; DNA-binding 17 kDa protein; histone-like protein HLP-1; hlpA) (UniProt P0AEU7)

```
        1 MKKWLLAAGL GLALATSAQA ADKIAIVNMG SLFQQVAQKT GVSNTLENEF KGRASELQRM
       61 ETDLQAKMKK LQSMKAGSDR TKLEKDVMAQ RQTFAQKAQA FEQDRARRSN EERGKLVTRI
      121 QTAVKSVANS QDIDLVVDAN AVAYNSSDVK DITADVLKQV K
```

SEQ ID NO:10 NP_009887 protein disulfide isomerase PDI1 [Saccharomyces cerevisiae S288c] (yPDI; thioredoxin-related glycoprotein 1; TRG1; MFP1) (UniProt P17967)

```
        1 MKFSAGAVLS WSSLLLASSV FAQQEAVAPE DSAVVKLATD SFNEYIQSHD LVLAEFFAPW
       61 CGHCKNMAPE YVKAAETLVE KNITLAQIDC TENQDLCMEH NIPGFPSLKI FKNSDVNNSI
      121 DYEGPRTAEA IVQFMIKQSQ PAVAVVADLP AYLANETFVT PVIVQSGKID ADFNATFYSM
      181 ANKHFNDYDF VSAENADDDF KLSIYLPSAM DEPVVYNGKK ADIADADVFE KWLQVEALPY
      241 FGEIDGSVFA QYVESGLPLG YLFYNDEEEL EEYKPLFTEL AKKNRGLMNF VSIDARKFGR
      301 HAGNLNMKEQ FPLFAIHDMT EDLKYGLPQL SEEAFDELSD KIVLESKAIE SLVKDFLKGD
      361 ASPIVKSQEI FENQDSSVFQ LVGKNHDEIV NDPKKDVLVL YYAPWCGHCK RLAPTYQELA
      421 DTYANATSDV LIAKLDHTEN DVRGVVIEGY PTIVLYPGGK KSESVVYQGS RSLDSLFDFI
      481 KENGHFDVDG KALYEEAQEK AAEEADADAE LADEEDAIHD EL
```

SEQ ID NO:11 NP_000909 protein disulfide-isomerase precursor [Homo sapiens] (hPDI; PDI; protein disulfide isomerase-associated 1; DSI; protocollagen hydroxylase; collagen prolyl 4-hydroxylase beta, procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide; prolyl 4-hydroxylase subunit beta; P4HB; PHDB; PO4DB; PO4HB; PROHB; P4Hbeta; protein disulfide isomerase family A, member 1; PDIA1; protein disulfide isomerase/oxidoreductase; thyroid hormone-binding protein p55; glutathione-insulin transhydrogenase; protein disulfide-isomerase; prolyl 4-hydroxylase subunit beta; cellular thyroid hormone-binding protein; glutathione-insulin transhydrogenase; GIT; ERBA2L) (UniProt P07237)

```
        1 MLRRALLCLA VAALVRADAP EEEDHVLVLR KSNFAEALAA HKYLLVEFYA PWCGHCKALA
       61 PEYAKAAGKL KAEGSEIRLA KVDATEESDL AQQYGVRGYP TIKFFRNGDT ASPKEYTAGR
      121 EADDIVNWLK KRTGPAATTL PDGAAAESLV ESSEVAVIGF FKDVESDSAK QFLQAAEAID
      181 DIPFGITSNS DVFSKYQLDK DGVVLFKKFD EGRNNFEGEV TKENLLDFIK HNQLPLVIEF
      241 TEQTAPKIFG GEIKTHILLF LPKSVSDYDG KLSNFKTAAE SFKGKILFIF IDSDHTDNQR
      301 ILEFFGLKKE ECPAVRLITL EEEMTKYKPE SEELTAERIT EFCHRFLEGK IKPHLMSQEL
      361 PEDWDKQPVK VLVGKNFEDV AFDEKKNVFV EFYAPWCGHC KQLAPIWDKL GETYKDHENI
      421 VIAKMDSTAN EVEAVKVHSF PTLKFFPASA DRTVIDYNGE RTLDGFKKFL ESGGQDGAGD
      481 DDDLEDLEEA EEPDMEEDDD QKAVKDEL
```

SEQ ID NO:12 NP_010640 thioredoxin-disulfide reductase TRR1 [Saccharomyces cerevisiae S288c] (yTrr1; cytoplasmic thioredoxin reductase) (UniProt P29509)

```
   1 MVHNKVTIIG SGPAAHTAAI YLARAEIKPI LYEGMMANGI AAGGQLTTTT EIENFPGFPD
  61 GLTGSELMDR MREQSTKFGT EIITETVSKV DLSSKPFKLW TEFNEDAEPV TTDAIILATG
 121 ASAKRMHLPG EETYWQKGIS ACAVCDGAVP IFRNKPLAVI GGGDSACEEA QFLTKYGSKV
 181 FMLVRKDHLR ASTIMQKRAE KNEKIEILYN TVALEAKGDG KLLNALRIKN TKKNEETDLP
 241 VSGLFYAIGH TPATKIVAGQ VDTDEAGYIK TVPGSSLTSV PGFFAAGDVQ DSKYRQAITS
 301 AGSGCMAALD AEKYLTSLE
```

SEQ ID NO:13 NP_015234 glutathione-disulfide reductase GLR1 [Saccharomyces cerevisiae S288c] (yGlr1; glutathione reductase; GR; GRase; LPG17) (UniProt P41921)

```
   1 MLSATKQTFR SLQIRTMSTN TKHYDYLVIG GGSGGVASAR RAASYGAKTL LVEAKALGGT
  61 CVNVGCVPKK VMWYASDLAT RVSHANEYGL YQNLPLDKEH LTFNWPEFKQ KRDAYVHRLN
 121 GIYQKNLEKE KVDVVFGWAR FNKDGNVEVQ KRDNTTEVYS ANHILVATGG KAIFPENIPG
 181 FELGTDSDGF FRLEEQPKKV VVVGAGYIGI ELAGVFHGLG SETHLVIRGE TVLRKFDECI
 241 QNTITDHYVK EGINVHKLSK IVKVEKNVET DKLKIHMNDS KSIDDVDELI WTIGRKSHLG
 301 MGSENVGIKL NSHDQIIADE YQNTNVPNIY SLGDVVGKVE LTPVAIAAGR KLSNRLFGPE
 361 KFRNDKLDYE NVPSVIFSHP EAGSIGISEK EAIEKYGKEN IKVYNSKFTA MYYAMLSEKS
 421 PTRYKIVCAG PNEKVVGLHI VGDSSAEILQ GFGVAIKMGA TKADFDNCVA IHPTSAEELV
 481 TMR
```

SEQ ID NO:14 NP_414970 peptidyl-prolyl cis/trans isomerase (trigger factor) [Escherichia coli str. K-12 substr. MG1655](tig; TF; ECK0430; JW0426; PPIase) (UniProt P0A850)

```
   1 MQVSVETTQG LGRRVTITIA ADSIETAVKS ELVNVAKKVR IDGFRKGKVP MNIVAQRYGA
  61 SVRQDVLGDL MSRNFIDAII KEKINPAGAP TYVPGEYKLG EDFTYSVEFE VYPEVELQGL
 121 EAIEVEKPIV EVTDADVDGM LDTLRKQQAT WKEKDGAVEA EDRVTIDFTG SVDGEEFEGG
 181 KASDFVLAMG QGRMIPGFED GIKGHKAGEE FTIDVTFPEE YHAENLKGKA AKFAINLKKV
 241 EERELPELTA EFIKRFGVED GSVEGLRAEV RKNMERELKS AIRNRVKSQA IEGLVKANDI
 301 DVPAALIDSE IDVLRRQAAQ RFGGNEKQAL ELPRELFEEQ AKRRVVVGLL LGEVIRTNEL
 361 KADEERVKGL IEEMASAYED PKEVIEFYSK NKELMDNMRN VALEEQAVEA VLAKAKVTEK
 421 ETTFNELMNQ QA
```

SEQ ID NO:15 NP_000933 peptidyl-prolyl cis-trans isomerase B precursor [Homo sapiens] (hPPIB; PPIase B; PPIB; rotamase B; cyclophilin B; cyclophilin-like protein; S-cyclophilin; SCYLP; CYP-S1; CYPB) (UniProt P23284)

```
   1 MLRLSERNMK VLLAAALIAG SVFFLLLPGP SAADEKKKGP KVTVKVYFDL RIGDEDVGRV
  61 IFGLFGKTVP KTVDNFVALA TGEKGFGYKN SKFHRVIKDF MIQGGDFTRG DGTGGKSIYG
 121 ERFPDENFKL KHYGPGWVSM ANAGKDTNGS QFFITTVKTA WLDGKHVVFG KVLEGMEVVR
 181 KVESTKTDSR DKPLKDVIIA DCGKIEVEKP FAIAKE
```

SEQ ID NO:16 NP_010439 peptidylprolyl isomerase CPR1 [Saccharomyces cerevisiae S288c] (Cpr1, peptidyl-prolyl cis-trans isomerase, cyclophilin, CPH1, CYP1, cyclosporin A-binding protein, rotamase, PPIase, PPI-II) (UniProt P14832)

```
   1 MSQVYFDVEA DGQPIGRVVF KLYNDIVPKT AENFRALCTG EKGFGYAGSP FHRVIPDFML
  61 QGGDFTAGNG TGGKSIYGGK FPDENFKKHH DRPGLLSMAN AGPNTNGSQF FITTVPCPWL
 121 DGKHVVFGEV VDGYDIVKKV ESLGSPSGAT KARIVVAKSG EL
```

SEQ ID NO:17 NP_013317 peptidylprolyl isomerase CPR6 [Saccharomyces cerevisiae S288c] (Cpr6, cyclophilin, CYP40, rotamase CPR6, PPIase CPR6) (UniProt P53691)

```
   1 MTRPKTFFDI SIGGKPQGRI VFELYNDIVP KTAENFLKLC EGNAGMAKTK PDVPLSYKGS
  61 IFHRVIKDFM CQFGDFTNFN GTGGESIYDE KFEDENFTVK HDKPFLLSMA NAGPNTNGSQ
 121 AFITCVPTPH LDGKHVVFGE VIQGKRIVRL IENQQCDQEN NKPLRDVKID DCGVLPDDYQ
 181 VPENAEATPT DEYGDNYEDV LKQDEKVDLK NFDTVLKAIE TVKNIGTEQF KKQNYSVALE
 241 KYVKCDKFLK EYFPEDLEKE QIEKINQLKV SIPLNIAICA LKLKDYKQVL VASSEVLYAE
 301 AADEKAKAKA LYRRGLAYYH VNDTDMALND LEMATTFQPN DAAILKAIHN TKLKRKQQNE
```

361 KAKKSLSKMF S

SEQ ID NO:18 NP_014264 peptidylprolyl isomerase FPR1 [Saccharomyces cerevisiae S288c] (Fpr1, FK506-binding protein 1, FKBP, FKB1, rapamycin-binding protein, RBP1, PPIase) (UniProt P20081)

1 MSEVIEGNVK IDRISPGDGA TFPKTGDLVT IHYTGTLENG QKFDSSVDRG SPFQCNIGVG
61 QVIKGWDVGI PKLSVGEKAR LTIPGPYAYG PRGFPGLIPP NSTLVFDVEL LKVN

SEQ ID NO:19 NP_057390 dnaJ homolog subfamily B member 11 precursor [Homo sapiens] (hERdj3; DnaJ (Hsp40) homolog, subfamily B, member 11; ER-associated DNAJ; ER-associated Hsp40 co-chaperone; ER-associated dnaJ protein 3; ERdj3; ERj3p; EDJ; ERJ3; ERj3; HEDJ; human DnaJ protein 9; DnaJ protein homolog 9HDJ9; DJ9; Dj-9; hDj-9; PWP1-interacting protein 4; APOBEC1-binding protein 2; ABBP-2; ABBP2; DNAJB11; PRO1080; UNQ537) (UniProt Q9UBS4)

1 MAPQNLSTFC LLLLYLIGAV IAGRDFYKIL GVPRSASIKD IKKAYRKLAL QLHPDRNPDD
61 PQAQEKFQDL GAAYEVLSDS EKRKQYDTYG EEGLKDGHQS SHGDIFSHFF GDFGFMFGGT
121 PRQQDRNIPR GSDIIVDLEV TLEEVYAGNF VEVVRNKPVA RQAPGKRKCN CRQEMRTTQL
181 GPGRFQMTQE VVCDECPNVK LVNEERTLEV EIEPGVRDGM EYPFIGEGEP HVDGEPGDLR
241 FRIKVVKHPI FERRGDDLYT NVTISLVESL VGFEMDITHL DGHKVHISRD KITRPGAKLW
301 KKGEGLPNFD NNNIKGSLII TFDVDFPKEQ LTEEAREGIK QLLKQGSVQK VYNGLQGY

SEQ ID NO:20 NP_005338 78 kDa glucose-regulated protein precursor [Homo sapiens] (BiP; endoplasmic reticulum lumenal Ca(2+)-binding protein grp78; GRP-78; heat shock 70 kDa protein 5; HSPA5; immunoglobulin heavy chain-binding protein; MIF2) (UniProt P11021)

1 MKLSLVAAML LLLSAARAEE EDKKEDVGTV VGIDLGTTYS CVGVFKNGRV EIIANDQGNR
61 ITPSYVAFTP EGERLIGDAA KNQLTSNPEN TVFDAKRLIG RTWNDPSVQQ DIKFLPFKVV
121 EKKTKPYIQV DIGGGQTKTF APEEISAMVL TKMKETAEAY LGKKVTHAVV TVPAYFNDAQ
181 RQATKDAGTI AGLNVMRIIN EPTAAAIAYG LDKREGEKNI LVFDLGGGTF DVSLLTIDNG
241 VFEVVATNGD THLGGEDFDQ RVMEHFIKLY KKKTGKDVRK DNRAVQKLRR EVEKAKRALS
301 SQHQARIEIE SFYEGEDFSE TLTRAKFEEL NMDLFRSTMK PVQKVLEDSD LKKSDIDEIV
361 LVGGSTRIPK IQQLVKEFFN GKEPSRGINP DEAVAYGAAV QAGVLSGDQD TGDLVLLDVC
421 PLTLGIETVG GVMTKLIPRN TVVPTKKSQI FSTASDNQPT VTIKVYEGER PLTKDNHLLG
481 TFDLTGIPPA PRGVPQIEVT FEIDVNGILR VTAEDKGTGN KNKITITNDQ NRLTPEEIER
541 MVNDAEKFAE EDKKLKERID TRNELESYAY SLKNQIGDKE KLGGKLSSED KETMEKAVEE
601 KIEWLESHQD ADIEDFKAKK KELEEIVQPI ISKLYGSAGP PPTGEEDTAE KDEL

SEQ ID NO:21 NP_013911 Hsp90 family chaperone HSC82 [Saccharomyces cerevisiae S288c] (yHsc82; HSC82; ATP-dependent molecular chaperone HSC82; 82 kDa heat shock cognate protein; heat shock protein Hsp90 constitutive isoform; HSP90; cytoplasmic chaperone of the Hsp90 family) (UniProt P15108)

1 MAGETFEFQA EITQLMSLII NTVYSNKEIF LRELISNASD ALDKIRYQAL SDPKQLETEP
61 DLFIRITPKP EEKVLEIRDS GIGMTKAELI NNLGTIAKSG TKAFMEALSA GADVSMIGQF
121 GVGFYSLFLV ADRVQVISKN NEDEQYIWES NAGGSFTVTL DEVNERIGRG TVLRLFLKDD
181 QLEYLEEKRI KEVIKRHSEF VAYPIQLLVT KEVEKEVPIP EEEKKDEEKK DEDDKKPKLE
241 EVDEEEEEKK PKTKKVKEEV QELEELNKTK PLWTRNPSDI TQEEYNAFYK SISNDWEDPL
301 YVKHFSVEGQ LEFRAILFIP KRAPFDLFES KKKKNNIKLY VRRVFITDEA EDLIPEWLSF
361 VKGVVDSEDL PLNLSREMLQ QNKIMKVIRK NIVKKLIEAF NEIAEDSEQF DKFYSAFAKN
421 IKLGVHEDTQ NRAALAKLLR YNSTKSVDEL TSLTDYVTRM PEHQKNIYYI TGESLKAVEK
481 SPFLDALKAK NFEVLFLTDP IDEYAFTQLK EFEGKTLVDI TKDFELEETD EEKAEREKEI
541 KEYEPLTKAL KDILGDQVEK VVVSYKLLDA PAAIRTGQFG WSANMERIMK AQALRDSSMS
601 SYMSSKKTFE ISPKSPIIKE LKKRVDEGGA QDKTVKDLTN LLFETALLTS GFSLEEPTSF
661 ASRINRLISL GLNIDEDEET ETAPEASTEA PVEEVPADTE MEEVD

SEQ ID NO:22 NP_418142 heat shock chaperone [Escherichia coli str. K-12 substr. MG1655] (IbpA; small heat shock protein IbpA; 16 kDa heat shock protein A; hslT; htpN; ECK3679; JW3664) (UniProt P0C054)

1 MRNFDLSPLY RSAIGFDRLF NHLENNQSQS NGGYPPYNVE LVDENHYRIA IAVAGFAESE
61 LEITAQDNLL VVKGAHADEQ KERTYLYQGI AERNFERKFQ LAENIHVRGA NLVNGLLYID
121 LERVIPEAKK PRRIEIN

SEQ ID NO:23 NP_418141 heat shock chaperone [Escherichia coli str. K-12 substr. MG1655] (IbpB; small heat shock protein IbpB; 16 kDa heat shock protein B; hslS; htpE; ECK3678; JW3663) (UniProt P0C058)

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        1 MRNFDLSPLM RQWIGFDKLA NALQNAGESQ SFPPYNIEKS DDNHYRITLA LAGFRQEDLE
       61 IQLEGTRLSV KGTPEQPKEE KKWLHQGLMN QPFSLSFTLA ENMEVSGATF VNGLLHIDLI
      121 RNEPEPIAAQ RIAISERPAL NS
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WHAT IS CLAIMED IS:

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

i) preparing a bacterial extract having an active oxidative phosphorylation system and comprising biologically functioning tRNA, amino acids and ribosomes necessary for cell free protein synthesis, wherein the bacteria from which the extract is prepared expresses an exogenous protein chaperone at a concentration of at least about 1 gm/liter of extract;

ii) combining the bacterial extract with a nucleic acid encoding a protein of interest to yield a bacterial cell free synthesis system; and,

iii) 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.

2. The method of claim 1, wherein the protein chaperone is selected from the group consisting of disulfide isomerases, prolyl isomerases and deaggregases.

3. The method of claim 1, wherein the bacteria from which the extract is prepared was co-transformed with genes encoding disulfide isomerases and prolyl isomerases.

4. The method of claim 1, wherein the exogenous protein chaperone is selected from the group consisting of DsbC, FkpA, and SlyD.

5. The method of claim 1, wherein the bacteria is *Escherichia coli.*

6. The method of claim 1 wherein the bacteria from which the extract is prepared expresses the exogenous protein chaperone 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 and ribosomes necessary for cell free protein synthesis and wherein an exogenous protein chaperone was expressed in the bacteria at a level of at least 1 gm/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 about 100 mg/L.

12. The system of claim 11, wherein the protein chaperone is selected from the group consisting of disulfide isomerases and prolyl isomerases.

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

14. The system of claim 11, wherein the exogenous protein chaperone is selected from the group consisting of DsbC, FkpA, and SlyD.

15. The system of claim 11, wherein the bacteria is *Escherichia coli.*

16. The system of claim 11, wherein the bacteria from which the extract is prepared expresses the exogenous protein chaperone from a gene operably linked to a constitutive promoter.

17. The system of claim 11, wherein the extract is an S30 extract of *E. coli.*

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

i) preparing a bacterial extract comprising 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;

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

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

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

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

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

22. The method of claim 18, wherein the protein disulfide isomerase is selected from the group consisting of DsbA, DsbB, DsbC, and DsbD, and the peptidyl-prolyl cis/trans isomerase is selected from the group consisting of FkpA and SlyD.

23. The method of claim 18, wherein the bacteria from which the extract is prepared expresses at least one of the exogenous protein chaperones from a gene operably linked to a constitutive promoter.

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

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

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

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

28. A bacterial cell free synthesis system for expressing biologically active proteins in a 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 about 100 mg/L.

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

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

31. 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.

32. The system of claim 28, wherein the protein disulfide isomerase is selected from the group consisting of DsbA, DsbB, DsbC, and DsbD, and the peptidyl-prolyl cis-trans isomerase is selected from the group consisting of FkpA and SlyD.

33. The system of claim 28, wherein the bacteria from which the extract is prepared expresses at least one of the exogenous protein chaperones from a gene operably linked to a constitutive promoter.

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

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

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

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