PROCESS FOR IMPROVED PROTEIN EXPRESSION BY STRAIN ENGINEERING

CROSS REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Application No. 60/591,489, filed July 26, 2004.

FIELD OF THE INVENTION

This invention is in the field of protein production, and in particular is a process for improving the production levels of recombinant proteins or peptides or improving the level of active recombinant proteins or peptides expressed in host cells.

BACKGROUND

More than 155 recombinantly produced proteins and peptides have been approved by the U.S. Food and Drug Administration (FDA) for use as biotechnology drugs and vaccines, with another 370 in clinical trials. Unlike small molecule therapeutics that are produced through chemical synthesis, proteins and peptides are most efficiently produced in living cells. In many cases, the cell or organism has been genetically modified to produce or increase the production of the protein.

When a cell is modified to produce large quantities of a target protein, the cell is placed under stress and often reacts by inducing or suppressing other proteins. The stress that a host cell undergoes during production of recombinant proteins can increase expression of, for example, specific proteins or cofactors to cause degradation of the overexpressed recombinant protein. The increased expression of compensatory proteins can be counterproductive to the goal of expressing high levels of active, full-length recombinant protein. Decreased expression or lack of adequate expression of other proteins can cause misfolding and aggregation of the recombinant protein. While it is known that a cell under stress will change its profile of protein expression, it is not known in any given example which specific proteins will be upregulated or downregulated.

Microarrays

Microarray technology can be used to identify the presence and level of expression of a large number of polynucleotides in a single assay. See for eg. U.S. Patent No. 6,040,138, filed September 15, 1995, U.S. Patent No. 6,344,316, filed June 25, 1997, U.S. Patent No. 6,261,776, filed April 15, 1999, U.S. Patent No. 6,403,957, filed October 16, 2000, U.S.

Patent No. 6,451,536, filed September 27, 2000, U.S. Patent No. 6,532,462, filed August 27, 2001, U.S. Patent No. 6,551,784, filed May 9, 2001, U.S. Patent No. 6,420,108, filed February 9, 1998, U.S. Patent No. 6,410,229, filed December 14, 1998, U.S. Patent No. 6,576,424, filed January 25, 2001, U.S. Patent No. 6,687,692, filed November 2, 2000, U.S. Patent No. 6,600,031, filed April 21, 1998, and U.S. Patent No. 6,567,540, filed April 16, 2001, all assigned to Affymetrix, Inc.

U.S. Patent No. 6,607,885 to E. I. duPont de Nemours and Co. describes methods to profile and identify gene expression changes after subjecting a bacterial cell to expression altering conditions by comparing a first and second microarray measurement.

Wei et al. used a microarray analysis to investigate gene expression profiles of *E. coli* with lac gene induction (Wei Y., et al. (2001) High-density microarray-mediated gene expression profiling of *Escherichia coli*. *J Bacteriol.* 183(2):545-56). Other groups have also investigated transcriptional profiles regulated after mutation of endogenous genes or deletion of regulatory genes (Sabina, J. et al (2003) Interfering with Different Steps of Protein Synthesis Explored by Transcriptional Profiling of *Escherichia coli* K-12 *J Bacteriol.* 185:6158-6170; Lee JH (2003) Global analyses of transcriptomes and proteomes of a parent strain and an L-threonine-overproducing mutant strain. *J Bacteriol.* 185(18):5442-51; Kabir MM, et al. (2003) Gene expression patterns for metabolic pathway in pgi knockout *Escherichia coli* with and without phb genes based on RT-PCR *J Biotechnol.* 105(1-2):11-31; Eymann C., et al. (2002) *Bacillus subtilis* functional genomics: global characterization of the stringent response by proteome and transcriptome analysis. *J Bacteriol.* 184(9):2500-20).

Gill et al. disclose the use of microarray technology to identify changes in the expression of stress related genes in *E. coli* after expression of recombinant chloramphenicol acetyltransferase fusion proteins (Gill et al. (2001) Genomic Analysis of High-Cell-Density Recombinant *Escherichia coli* Fermentation and "Cell Conditioning" for Improved Recombinant Protein Yield *Biotech. Bioengin.* 72:85-95). The stress gene transcription profile, comprising only 16% of the total genome, at high cell density was used to evaluate "cell conditioning" strategies to alter the levels of chaperones, proteases, and other intracellular proteins prior to recombinant protein overexpression. The strategies for "conditioning" involved pharmacological manipulation of the cells, including through dithiothreitol and ethanol treatments.

Asai et al. described the use of microarray analysis to identify target genes activated by over-expression of certain sigma factors that are typically induced after cell stresses (Asai K., et al. (2003) DNA microarray analysis of *Bacillus subtilis* sigma factors of

extracytoplasmic function family. *FEMS Microbiol. Lett.* 220(1):155-60). Cells overexpressing sigma factors as well as reporter genes linked to sigma factor promoters were used to show stress regulated gene induction.

Choi et al. described the analysis and up-regulation of metabolic genes that are down-regulated in high-density batch cultures of *E. coli* expressing human insulin-like growth factor fusion protein (IGF-I_f) (Choi et al. (2003) Enhanced Production of Insulin-Like Growth Factor I Fusion Protein in *Escherichia coli* by Coexpression of the Down-Regulated Genes Identified by Transcriptome Profiling *App. Envir. Microbio.* 69:4737-4742). The focus of this work was on the metabolic changes that occur during high-density conditions after protein induction. Genes that were down regulated after induction of recombinant protein production during high density growth conditions were identified and specific metabolic genes that had been down-regulated were expressed in cells producing recombinant IGF-I_f. The work showed that increasing metabolic production of certain nucleotide bases and amino acids could increase protein production and that growth rates could be modified by increasing expression of a down-regulated metabolic transporter molecule. These strategies were designed to alter the cellular environment to reduce metabolic stresses associated with the protein production generally or with high density culture.

Protein Degradation

Unwanted degradation of recombinant protein presents an obstacle to the efficient use of certain expression systems. The expression of exogenous proteins often induces stress responses in host cells, which can be, for example, natural defenses to a limited carbon source. All cells contain a large number of genes capable of producing degradative proteins. It is not possible to predict which proteases will be regulated by a given host in response to expression of a particular recombinant protein. For example, the bacteria *P. fluorescens* contains up to 200 proteases and protease related proteins.

In the cytoplasm of *E. coli*, proteolysis is generally carried out by a group of proteases and cofactor molecules. Most early degradation steps are carried out by five ATP-dependent Hsps: Lon/La FtsH/HflB, ClpAP, ClpXP, and ClpYQ/HslUV (Gottesman S (1996) Proteases and their targets in *Escherichia coli*. *Annu. Rev. Genet.* 30:465-506). Along with FtsH (an inner membrane-associated protease the active site of which faces the cytoplasm), ClpAP and ClpXP are responsible for the degradation of proteins modified at their carboxyl termini by addition of the non-polar destabilizing tail AANDENYALAA (Gottesman S, et al. (1998) The ClpXP and ClpAP proteases degrade proteins with carboxyl-terminal peptide tails added

by the SsrA-tagging system. *Genes Dev.* 12:1338-1347; Herman C, et al. (1998) Degradation of carboxy-terminal-tagged cytoplasmic proteins by the *Escherichia coli* protease HflB (FtsH). *Genes Dev.* 12:1348-1355).

Several approaches have been taken to avoid degradation during recombinant protein production. One approach is to produce host strains bearing mutations in a protease gene. Baneyx and Georgiou, for example, utilized a protease-deficient strain to improve the yield of a protein A–β-lactamase fusion protein (Baneyx F, Georgiou G. (1991) Construction and characterization of *Escherichia coli* strains deficient in multiple secreted proteases: protease III degrades high-molecular-weight substrates in vivo. *J Bacteriol* 173: 2696–2703). Park et al. used a similar mutational approach to improve recombinant protein activity 30% compared with the parent strain of *E. coli* (Park S. et al. (1999) Secretory production of recombinant protein by a high cell density culture of a protease negative mutant *Escherichia coli* strain. *Biotechnol. Progr.* 15:164–167). U.S. Patent No. 5,264,365 and 5,264,365 describe the construction of protease-deficient *E. coli*, particularly multiply protease deficient strains, to produce proteolytically sensitive polypeptides. PCT Publication No. WO 90/03438 describes the production of strains of *E. coli* that include protease deficient strains or strains including a protease inhibitor. Similarly, PCT Publication No. WO 02/48376 describes *E. coli* strains deficient in proteases DegP and Prc.

Protein Folding

Another major obstacle in the production of recombinant proteins in host cells is that the cell often is not adequately equipped to produce either soluble or active protein. While the primary structure of a protein is defined by its amino acid sequence, the secondary structure is defined by the presence of alpha helixes or beta sheets, and the ternary structure by covalent bonds between adjacent protein stretches, such as disulfide bonds. When expressing recombinant proteins, particularly in large-scale production, the secondary and tertiary structure of the protein itself is of critical importance. Any significant change in protein structure can yield a functionally inactive molecule, or a protein with significantly reduced biological activity. In many cases, a host cell expresses folding modulators (FMs) that are necessary for proper production of active recombinant protein. However, at the high levels of expression generally required to produce usable, economically satisfactory biotechnology products, a cell often can not produce enough native folding modulator or modulators to process the recombinant protein.

In certain expression systems, overproduction of exogenous proteins can be accompanied by their misfolding and segregation into insoluble aggregates. In bacterial cells

these aggregates are known as inclusion bodies. In *E. coli,* the network of folding modulators/chaperones includes the Hsp70 family. The major Hsp70 chaperone, DnaK, efficiently prevents protein aggregation and supports the refolding of damaged proteins. The incorporation of heat shock proteins into protein aggregates can facilitate disaggregation. However, proteins processed to inclusion bodies can, in certain cases, be recovered through additional processing of the insoluble fraction. Proteins found in inclusion bodies typically have to be purified through multiple steps, including denaturation and renaturation. Typical renaturation processes for inclusion body targeted proteins involve attempts to dissolve the aggregate in concentrated denaturant and subsequent removal of the denaturant by dilution. Aggregates are frequently formed again in this stage. The additional processing adds cost, there is no guarantee that the *in vitro* refolding will yield biologically active product, and the recovered proteins can include large amounts of fragment impurities.

One approach to reduce protein aggregation is through fermentation engineering, most commonly by reducing the cultivation temperature (see Baneyx F (1999) *In vivo* folding of recombinant proteins in *Escherichia coli.* In *Manual of Industrial Microbiology and Biotechnology,* Ed. Davies *et al.* Washington, DC: American Society for Microbiology ed. 2:551-565 and references therein). The more recent realization that *in vivo* protein folding is assisted by molecular chaperones, which promote the proper isomerization and cellular targeting of other polypeptides by transiently interacting with folding intermediates, and by foldases, which accelerate rate-limiting steps along the folding pathway, has provided additional approaches combat the problem of inclusion body formation (see for e.g. Thomas JG et al. (1997). Molecular chaperones, folding catalysts and the recovery of active recombinant proteins from *E. coli*: to fold or to refold. *Appl Biochem Biotechnol,* 66:197-238).

In certain cases, the overexpression of chaperones has been found to increase the soluble yields of aggregation-prone proteins (see Baneyx, F. (1999) Recombinant Protein Expression in *E. coli Curr. Opin. Biotech.* 10:411-421 and references therein). The process does not appear to involve dissolution of preformed recombinant inclusion bodies but is related to improved folding of newly synthesized protein chains. For example, Nishihara et al. coexpressed *groESL* and *dnaJK/grpE* in the cytoplasm to improve the stability and accumulation of recombinant Cryj2 (an allergen of Japanese cedar pollen) (Nishihara K, Kanemori M, Kitagawa M, Yanagi H, Yura T. 1998. Chaperone coexpression plasmids: differential and synergistic roles of DnaK-DnaJ-GrpE and GroEL-GroES in assisting folding of an allergen of Japanese cedar pollen, Cryj2, in *Escherichia coli. Appl. Environ. Microbiol.*

64:1694). Lee and Olins also coexpressed GroESL and DnaK and increased the accumulation of human procollagenase by tenfold (Lee S, Olins P. 1992. Effect of overproduction of heat shock chaperones GroESL and DnaK on human procollagenase production in *Escherichia coli*. *JBC* 267:2849–2852). The beneficial effect associated with an increase in the intracellular concentration of these chaperones appears highly dependent on the nature of the overproduced protein, and success is by no means guaranteed.

A need exists for processes for development of host strains that show improved recombinant protein or peptide production, activity or solubility in order to reduce manufacturing costs and increase the yield of active products.

It is therefore an object of the invention to provide processes for improving recombinant protein expression in a host.

It is a further object of the invention to provide processes that increase expression levels in host cells expressing recombinant proteins or peptides.

It is another object of the invention to provide processes to increase the levels of soluble protein made in recombinant expression systems.

It is yet another object of the invention to provide processes to increase the levels of active protein made in recombinant expression systems.

SUMMARY

A process is provided for improving the expression of a recombinant protein or peptide comprising:

i) expressing the recombinant protein or peptide in a host cell;

ii) analyzing a genetic profile of the cell and identifying one or more endogenous gene products that are up-regulated upon expression or overexpression of the recombinant protein or peptide; and

iii) changing expression of one or more identified endogenous gene products by genetically modifying the cell.

The process can provide improved expression as measured by improved yields of protein, or can improve the recovery of active protein, for example by increasing solubility of the expressed recombinant protein, or a related protein or peptide.

Using this process, it can be determined which of the many cellular proteins are "chosen" by the cell to compensate for the expression of the foreign recombinant protein, and this information can lead to development of more effective protein expression systems. For example, it is known that, typically, a cell will selectively upregulate one or more proteases to

degrade an overexpressed recombinant protein. However, it cannot be predicted in advance which protease(s) the cell will upregulate to compensate for the stress caused by any given recombinant protein. Analysis of the cell's genetic profile by microarray or equivalent technology can identify which proteases are upregulated in a given cell in response to exogenous protein production. This information is then used to genetically modify the cell to decrease the expression of these particular proteases, while sparing other proteins that are useful or even necessary for cell homeostasis.

As another example, a cell may selectively upregulate one or more folding modulators or cofactors to increase the folding capability or solubility of the recombinant protein. Again, it cannot be predicted in advance which folding modulators or cofactors will be selected in a given system to assist in the processing of a specific recombinant protein. Analyzing the genetic profile by microarray or equivalent technology allows identification of the folding modulators or cofactors that have been upregulated. Based on this information, the cell is genetically modified to increase the expression of the selected folding modulators or cofactors preferred by the cell for the given recombinant protein. This modification can increase the percent of active protein recovered, while minimizing the detrimental impact on cell homeostasis.

Therefore, the yield and/or activity and/or solubility of the recombinant protein can be increased by modifying the host organism via either increasing or decreasing the expression of a compensatory protein (i.e. a protein that is upregulated in response to given cell stress) in a manner that is selective and that leaves whole other beneficial mechanisms of the cell.

The process can be used iteratively until the expression of active recombinant protein is optimized. For example, using the process described above, the host cell or organism is genetically modified to upregulate, down regulate, knock-in or knock-out one or more identified compensatory proteins. The host cell or organism so modified can then be cultured to express the recombinant protein, or a related protein or peptide, and additional compensatory proteins identified via microarray or equivalent analysis. The modified host cell or organism is then again genetically modified to upregulate, down regulate, knock-in or knock-out the additional selected compensatory proteins. This process can be iterated until a host cell or organism is obtained that exhibits maximum expression of active and/or soluble protein without undue weakening of the host organism or cell. These steps for example can be repeated for example, one, two, three, four, five, six, seven, eight, nine, or ten or more times.

In another embodiment, the process further comprises: iv) expressing the recombinant protein or peptide in a genetically modified cell. In yet another embodiment, the process further comprises: v) analyzing a second genetic profile of the genetically modified cell expressing recombinant protein or peptide and identifying one or more additional gene products that are differentially expressed in the modified cell expressing recombinant protein or peptide. In a further embodiment, the process additionally comprises: vi) changing the expression of one or more identified additional gene products to provide a double modified cell. Optionally, the recombinant protein or peptide, or a related protein or peptide, can be expressed in the double modified cell. The differentially regulated gene products identified in the modified cell can be up- or down-regulated when compared to the host cell or when compared to the modified cell not expressing recombinant protein or peptide.

In yet another embodiment, the process further comprises: iv) analyzing a second genetic profile of a genetically modified cell expressing recombinant protein or peptide and identifying one or more additional gene products that are differentially expressed in the modified cell that is not expressing recombinant protein or peptide. In a further embodiment, the process additionally comprises: v) changing the expression of one or more additional identified gene products in the modified cell to provide a double modified cell. The differentially regulated gene products identified in the modified cell can be up- or down-regulated when compared to the host cell or organism or when compared to the modified cell not expressing recombinant protein or peptide.

In one specific embodiment, a process is provided for improving the expression of a recombinant protein or peptide comprising: i) expressing the recombinant protein or peptide in a host cell; ii) analyzing a genetic profile of the cell and identifying at least one protease that is up-regulated when the recombinant protein or peptide is expressed; and iii) changing expression of an identified protease by genetically modifying the host cell or organism to reduce the expression of the upregulated protease. In a further embodiment, the process comprises changing the expression of at least a second identified protease in the modified cell to provide a double protease modified cell. In another embodiment, the process further comprises: iv) expressing the recombinant protein or peptide, or a related protein or peptide, in a protease modified cell. In another embodiment, the process further comprises analyzing a second genetic profile of the protease modified cell to identify one or more additional gene products that are differentially expressed in the modified cell.

In another embodiment, a process is provided for improving the expression of a recombinant protein or peptide comprising: i) expressing the recombinant protein or peptide

in a host cell; ii) analyzing a genetic profile of the cell and identifying at least one up-regulated folding modulator (FM) that is up-regulated after overexpression of the recombinant protein or peptide; and iii) changing expression of at least one identified folding modulator by genetically modifying the cell to provide a FM modified cell. In a further embodiment, the process comprises changing the expression of at least a second identified folding modulator in the modified cell to provide a double FM modified cell. In another embodiment, the process further comprises: iv) expressing the recombinant protein or peptide, or a related protein or peptide, in a FM modified cell. In another embodiment, the process further comprises analyzing a second genetic profile of the FM modified cell to identify one or more additional gene products that are differentially expressed in the modified cell.

The term "genetic profile" as used herein is meant to include an analysis of genes in a genome, mRNA transcribed from genes in the genome (or the equivalent cDNA), transcription products that have been modified by a cell such as splice variants of genes in eukaryotic systems, or proteins or peptides translated from genes in a genome, including proteins that are modified by the cell or translated from splice variants of mRNA translated from the genome. A genetic profile is meant to include more than one gene or gene product, and typically includes a group of at least 5, 10, 50, 100 or more genes or gene products that are analyzed.

In one embodiment, the genetic profile analyzed can be a transcriptome profile, i.e. a profile of the transcription products of genes from the genome. The process can include analyzing the transcriptome profile using a microarray or equivalent technology. In this embodiment, the microarray can include binding partners to at least a portion of the transcriptome of the host cell, and typically includes samples from binding partners to gene products of at least 50% of the genome of the organism. More typically, the microarray includes samples from at least 80%, 90%, 95%, 98%, 99% or 100% of the binding partners to gene products in the genome of the host cell.

In a separate embodiment, the microarray can include a selected subset of binding partners to genes or gene products which represent classes of products that are affected by the recombinant protein expression. Nonlimiting examples include putative or known proteases, co-factors of proteases or protease-like proteins; folding modulators, co-factors of folding modulators or proteins that may improve protein folding or solubility; transcription factors; proteins involved in nucleic acid stability or translational initiation; kinases; extracellular or intracellular receptors; metabolic enzymes; metabolic cofactors; envelope proteins; sigma

factors; membrane bound proteins; transmembrane proteins; membrane associated proteins and housekeeping genes. The genetic profile can be analyzed by measuring the binding of the expressed genes of the host cell expressing the recombinant protein or peptide to the microarray. The transcriptome profile can also be analyzed using non-microarray assays such as blot assays, including northern blot assays, or columns coated with binding partners.

In another embodiment, the genetic profile analyzed can be a proteome profile, i.e. a profile of the proteins produced from genes in a given organism. The process can include analyzing the proteome profile using, for example, two-dimensional electrophoresis. Techniques like mass spectrometry in combination with separation tools such as two-dimensional gel electrophoresis or multidimensional liquid chromatography, can also be used in the process. In two dimensional electrophoresis, the proteins separated can include proteins from at least 10% of the proteome of the organism. More typically, proteins from at least 20%, 30%, 40%, 60%, 80% or 90% of the proteins in the proteome of the host cell are separated and analysed by techniques such as staining of proteins and/or mass spectrometry.

In additional embodiment, the proteome profile is analyzed using mass spectrometry. There are several related techniques that use liquid chromatography (LC) coupled to mass spectrometry (MS) and tandem mass spectrometry (MS/MS) to identify proteins and measure their relative abundance. Often, one sample is labeled with a heavy-isotope tag that allows for comparison to another sample without changing the chemical properties. For example, in one sample the amino acid cysteine can be labeled with a tag containing eight hydrogen atoms. The other sample is labeled with a tag that contains eight deuterium ("heavy") atoms instead (+8 Daltons). MS data can be used to find pairs of peptides 8 Daltons apart and quantitate the difference. MS/MS data from the same peptides provides an approximation of primary sequence, and the protein ID. Other experiments label the proteins in vivo by growing cells with "heavy" amino acids. These types of techniques can be used to identify thousands of proteins in a single experiment and estimate relative abundance if present in both samples (see Goodlett DR and Aebersold RH (2001). Mass Spectrometry in Proteomics. Chem Rev101:269-295). ICAT is a type of MS/MS, it stands for Isotope Coded Affinity Tags (see Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, and Aebersold RH (1999). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotech 17:994-999).

In another embodiment, the process can include analyzing the proteome profile using, for example, a microarray. In this embodiment, the array can include binding partners to at least a portion of the proteins expressed by the host cell under appropriate growth conditions,

and typically includes binding partners to proteins from at least 10% of the proteome of the organism. More typically, the microarray includes binding partners to proteins from at least 20%, 30%, 40%, 60%, 80% or 90% of the proteins in the proteome of the host cell. The binding partners can be antibodies, which can be antibody fragments such as single chain antibody fragments. In a separate embodiment, the microarray can include binding partners for a selected subset of proteins from the proteome, including, for example, putative protease proteins or putative folding modulators. The microarray can typically also include a set of binding partners to proteins that are used as controls. The genetic profile can be analyzed by measuring the binding of the proteins of the host cell expressing the recombinant protein or peptide to the binding partners on the microarray. The proteome profile can also be analyzed in a standard assay format, such as an Elisa assay or a standard western blot assay.

The samples in the genetic profile can be analyzed individually or grouped into clusters. The clusters can typically be grouped by similarity in gene expression. In particular embodiments, the clusters can be grouped as genes that are upregulated to a similar extent or genes that are down-regulated to a similar extent.

The identified up-regulated gene is typically identified by comparing a genetic profile of the host cell expressing the recombinant protein or peptide to a genetic profile of the host cell not expressing the recombinant protein or peptide. In a further embodiment, a host cell expressing a protein homologous to the first recombinant protein is analyzed.

The genome of the host cell expressing the recombinant protein or peptide can be modified by recombination, for example homologous recombination or heterologous recombination. The genome can also be modified by mutation of one or more nucleotides in an open reading frame encoding a gene, particularly an identified protease. In another embodiment, the host cell is modified by including one or more vectors that encode an inhibitor of an identified gene or gene product, such as a protease inhibitor. In another embodiment, the host cell is modified by inhibition of a promoter, which can be a native promoter. In a separate embodiment, the host cell is modified by including one or more vectors that encode a gene, typically a folding modulator or a cofactor of a folding modulator. In another embodiment, the host cell is modified by enhancing a promoter for an identified folding modulator or a cofactor for a folding modulator, including by adding an exogenous promoter to the host cell genome.

The host cell can be any cell capable of producing recombinant protein or peptide. In one embodiment, the host cell is a prokaryote, such as a bacterial cell including, but not limited to an *Escherichia* or a *Pseudomonas* species. The host cell may be a Pseudomonad

cell such as a *P. fluorescens* cell. In other embodiments, the host cell is an *E. coli* cell. In another embodiment the host cell is a eukaryotic cell, for example an insect cell, including but not limited to a cell from a *Spodoptera, Trichoplusia Drosophila* or an *Estigmene* species, or a mammalian cell, including but not limited to a murine cell, a hamster cell, a monkey, a primate or a human cell. In another embodiment, the host cell is a plant cell, including, but not limited to, a tobacco cell, corn, a cell from an *Arabidopsis* species, potato or rice cell. In another embodiment, a whole organism is analyzed in the process, including but not limited to a transgenic organism.

In one embodiment, the identified upregulated compensatory genes or gene products are one or more proteases and/or one or more folding modulators. In certain embodiments, an identified gene or gene product can also be a subunit of a protease or a folding modulator or a cofactor of a protease or a cofactor of a folding modulator. In one embodiment, the identified gene can be selected from a serine, threonine, cysteine, aspartic or metallo peptidase. In certain other embodiments, the identified gene or gene product can be selected from *hsIV*, *hsIU*, *clpA*, *clpB* and *clpX*. The identified gene or gene product can also be a cofactor of a protease. In another embodiment, the identified gene or gene product is a folding modulator. In certain embodiments, the identified gene or gene product can be selected from a chaperone protein, a foldase, a peptidyl prolyl isomerase and a disulfide bond isomerase. In one embodiment, the identified gene or gene product can be selected from *htpG, cbpA, dnaJ, dnaK* and *fkbP*. In one embodiment, a gene or gene product homologous to the identified up-regulated gene is modified in the genome of the host.

The process can lead to increased production of recombinant protein or peptide in a host cell, by for example, increasing the amount of protein per gram of host protein (total cell protein) in a given amount of time, or increasing the amount of length of time during which the cell or organism is producing the recombinant protein. The increased production may optimize the efficiency of the cell or organism by for example, decreasing the energy expenditure, increasing the use of available resources, or decreasing the requirements for growth supplements in growth media. The increased production may also result in an increased level of recoverable protein or peptide, such as soluble protein, produced per gram of recombinant or per gram of host cell protein.

The invention also includes an improved recombinant host cell that is produced by the claimed process.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph of a growth comparison (optical density over time) of different strains of *P. fluorescens*. The cells were induced with 0.3 M of IPTG at 24 hr after inoculation. The strains are: DC280 harboring the empty vector pDOW1339, DC240 that produces the soluble cytoplasmic nitrilase enzyme, and DC271 that produces the partially insoluble periplasmic hGH. DC206, the parental strain of DC280, DC240, and DC271 was included as a control. Samples were taken at 0 and 4 hrs post-IPTG induction for RNA isolation and gene expression profiling, as indicated by arrows.

Figure 2 is an graph of hierarchical clustering of all genes from *P. fluorescens* strains DC280, DC240 and DC271 into 12 clusters at 4 hr after IPTG when compared to 0 hr IPTG (indicated at the bottom of the figure). Based on the value and trend, genes were clustered and grouped using the hierarchical clustering algorithm from Spotfire DecisionSite. Broken lines indicate data points that were filtered out due to poor spots quality or low level of expression. The x-axis represents the comparison of each strain; the y-axis represents the relative expression value 4 hrs after to before IPTG induction. All the identified FMs are highlighted. Cluster 7 shows 2 FM and 2 protease subunit genes that are highly expressed in strain DC271, which overproduces the periplasmic hGH protein. The remaining FM genes are grouped in cluster 6.

Figure 3 is a hierarchical cluster analysis of cluster 6 from Figure 2. In the new cluster 8, two folding modulators, DnaK and DnaJ, were identified both of which showed higher expression levels for periplasmic recombinant protein production similar to the previously identified HslVU, CbpA, and HtpG. Cluster 6 shows where the rest of the FMs are grouped.

Figure 4 is a Venn diagram showing the up-regulated protease and FMs from the three sets of experiments in Table 5, 6 and 7. As summarized in Table 5, 6 and 7, the list of genes were organized in Venn diagram to highlight the overlap of the gene list among the three sets of experiments indicated at the corner. For each gene, the ratio of each experiment was shown with 2 as a cut off.

Figure 5 is a graph of the sequence analysis of the *hslV* (RXF01961) and *hslU* (RXF01957) genes from *P. fluorescens* generated by Artemis. The codon usage plot (top panel) indicates that the gene boundary are correct. This is corroborated by the best homologues of HslV and HslU protein sequences to *P. aeruginosa* as indicated beneath the genes of RXF01961 and RXF01957. The Phrap quality score plot shows that the sequence quality is good, *i.e.* the score line is above the horizontal line indicating a better quality than 1

error in 10kb (middle panel). The open white pointed boxes below the genes show the location of the probes generated for use in the DNA microarray experiments.

Figure 6 is a schematic illustration of an *hslU* mutant construction where an approximately 550 bp PCR product of *hslU* (light blue box) was ligated into TOPO TA2.1 cloning vector (circle). The resulting plasmid was transformed into competent *P. fluorescens* cells and kanamycin (kan)-resistant colonies were analyzed in diagnostic PCR to confirm the construction of an insertion mutation in the *hslU* gene.

Figure 7 is a graph of a growth curve assays comparing wild type with *hslU* mutant strain overproducing hGH or pbp::hGH in shake flask production medium. The arrows indicate time points where samples were taken.

Figure 8 is an image of SDS-PAGE analysis of strains DC271 and DC373 expressing pbp::hGH. Samples were taken from DC271 (wild-type, W) and DC373 (*hslU* mutant, M) just before protein induction (0 hr) and then 4 hr, 8 hr, 24 hr, and 30 hr after IPTG addition. Soluble (S) and insoluble (I) fractions were prepared for each sample analyzed. The production of unprocessed and processed hGH is indicated by arrows. The molecular weight (MW) marker (Ma) is shown on the right hand side of the gels.

Figure 9 is an image of the SDS-PAGE analysis of strains DC369 and DC372 expressing hGH in the cytoplasm. Samples were taken from DC369 (wild-type, W) and DC372 (*hslU* mutant, M) just before protein induction (0 hr) and then 4 hrs, 8 hrs, 24 hrs, 30 hrs, and 50 hrs after IPTG addition. Soluble (S) and insoluble (I) fractions were prepared for each sample analyzed. The production of hGH is indicated by an arrow. The molecular weight (MW) marker (Ma) is shown on the right hand side of the gels.

Figure 10 is a graph of growth curves of strains expressing the hGH::COP fusion protein. The strains include: DC369 expressing hGH only (not fused to COP) as a negative control; HJ104, the wild type expressing hGH::COP; HJ105, the *hslU* mutant expressing hGH::COP.

Figure 11 is a graph of the green fluorescence activity measurements for strains expressing the hGH::COP fusion protein using a fluorimeter. Five OD600 of cell culture were sampled for each strain harboring hGH or hGH::COP at different time points after IPTG induction. The strains tested include: DC369 expressing hGH only (not fused to COP) as a negative control; HJ104, the wild type expressing hGH::COP; HJ105, the *hslU* mutant expressing hGH::COP. The inserted table shows percent increase of relative fluorescence in the *hslU* mutant compared to the wild type at different time points after IPTG induction.

Figure 12 is a pictoral representation of the process of measuring relative abundance of mRNA between two samples.

Figure 13 is a representation of the construction of chromosomal deletion of *hslUV* gene in *pyrF*-negative strain. A. Plasmid pDOW2050 contains 505bp and 634 bp DNA fragments flanking the *hslUV* gene. Since suicide plasmid pDOW2050 can not replicate in *P. fluorescens*, tetracycline-resistant cells will only be generated after a single recombination event at one of the homologous regions that integrates the entire plasmid into the genome. B. Tetracycline-resistant cells contains the entire plasmid integrated into the genome. These cells also contain the *pyrF* gene encoded from the plasmid. Selection for cells that has the second recombinant event occurred by plating cells on agar plates supplemented with FOA, which in *pyrF*-positive strains, is converted into a toxic compound. C. The chromosomal deletion strain was confirmed by sequencing analysis

Figure 14 is a graph of relative fluorescence over time for green fluorescence activity measurements for the strains expressing the hGH::COP fusion protein using a fluorimeter. Duplicates were used for both the wild type (HJ104) and *hslUV* deletion strain (HJ117).

Figure 15 is images of SDS-PAGE gels of strains expressing hGH with or without folding modulators GrpE-DnakJ. Samples were removed at various times after induction by IPTG (0, 4, 8, 24 and 48 hr), normalized to OD600 of 20 and lysed using EasyLyse. The soluble (S) insoluble (I) fractions were separated on a BioRad Criterion 15% Tris HCl SDS-PAGE gel and stained with Coomassie.

DETAILED DESCRIPTION

A process is provided for improving the expression of a recombinant protein or peptide comprising i) expressing the recombinant protein or peptide in a host cell; ii) analyzing a genetic profile of the cell and identifying one or more endogenous up-regulated gene products, including one or more proteases or folding modulators that are up-regulated upon expression of the recombinant protein or peptide; and iii) changing expression of one or more identified gene products by genetically modifying the cell. In another embodiment, the process further comprises expressing the recombinant protein or peptide in a genetically modified cell. In another embodiment, the process further comprises analyzing a second genetic profile of the genetically modified cell to identify one or more additional gene products that are differentially expressed in the modified cell. In a further embodiment, the process comprises changing the expression of at least a second identified gene product in the modified cell to provide a double modified cell. The process can provide improved

expression as measured by improved yields of protein, or can improve the recovery of active protein, for example by increasing solubility of the expressed recombinant protein.

More generally, the invention includes a process for improving the expression of a recombinant protein or peptide in a host cell or organism comprising:

i) expressing the recombinant protein or peptide in the recombinant host cell or organism;

ii) analyzing a genetic profile of the recombinant cell to identify a compensatory gene or gene product that is expressed at a higher level in the recombinant cell than in one of either a host cell that has not been modified to express the recombinant protein or a recombinant cell that is not expressing the recombinant protein; and

iii) changing expression of the identified compensatory gene or gene product in the recombinant cell by genetic modification to provide a modified recombinant cell that achieves an increase in recombinant protein expression, activity or solubility.

Throughout the specification, when a range is provided, it should be understood that the components are meant to be independent. For example, a range of 1-6 means independently 1, 2, 3, 4, 5 or 6.

The steps of the process are described in more detail below.

Step I: Genetic modification of host cell or organism to express a recombinant protein or peptide in a host cell

In the first step of the process, a host cell is modified to have the capacity to express a recombinant protein or peptide. The host cell can be modified using any techniques known in the art. For example, the recombinant protein can be expressed from an expression vector that is exogenous to the genome of the cell and that is transfected or transformed into the cell. The construction of expression vectors as well as techniques for transfection or transformation are described below. The host cell can also be modified to express a recombinant protein or peptide from a genomic insert as described below. A gene encoding the recombinant protein or peptide can be inserted into the genome of the host cell or organism by techniques such as homologous or heterologous recombination. These techniques are described below.

The recombinant protein or peptide can be expressed under the control of an element that requires further manipulation of the cell. For example, chemical treatment of the cell may be required to initiate or enhance protein or peptide expression. Promoter and repressor

elements that govern the expression of recombinant proteins or peptides in host cells are described below and are well known in the art. These can include promoter elements based on the "tac" promoter, responsive to IPTG.

Selection of a host cell or organism

The process of the invention can be used in any given host system, including of either eukaryotic or prokaryotic origin. The process is generally limited only by the availability of enough genetic information for analysis of a genetic profile to identify a identified gene. Although it is generally typical that representative sequences from a large percentage of the genome is available, for example at least 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99% or 100% of the sequences expressed or found in the genome, transcriptome, or proteome, the invention can be practiced using only a portion of the sequences in the genome, transcriptome, or proteome. In particular, in instances when the information available includes information on a group of related sequences, such as a metabolically linked group, only a small portion of representative sequences from the genome can be used for the process of the invention. The process is also not limited to particular recombinant proteins being expressed, as a key aspect of the process is the capacity to rationally and iteratively design expression systems based on techniques for identifying cellular changes that occur in a host cell upon expression of recombinant proteins or peptides and modulating the host cell using procedures known in the art.

The host cell can be any cell capable of producing recombinant protein or peptide. In one embodiment, the host cell is a microbial cell, ie. a cell from a bacteria, fungus, yeast, or other unicellular eukaryotes, prokaryotes and viruses. The most commonly used systems to produce recombinant proteins or peptides include certain bacterial cells, particularly *E. coli*, because of their relatively inexpensive growth requirements and potential capacity to produce protein in large batch cultures. Yeast are also used to express biologically relevant proteins and peptides, particularly for research purposes. Systems include *Saccharomyces cerevisiae* or *Pichia pastoris*. These systems are well characterized, provide generally acceptable levels of total protein expression and are comparatively fast and inexpensive. Insect cell expression systems have also emerged as an alternative for expressing recombinant proteins in biologically active form. In some cases, correctly folded proteins that are post-translationally modified can be produced. Mammalian cell expression systems, such as Chinese hamster ovary cells, have also been used for the expression of recombinant proteins. On a small scale, these expression systems are often effective. Certain biologics can be derived from

mammalian proteins, particularly in animal or human health applications. In another embodiment, the host cell is a plant cell, including, but not limited to, a tobacco cell, corn, a cell from an *Arabidopsis* species, potato or rice cell. In another embodiment, a multicellular organism is analyzed or is modified in the process, including but not limited to a transgenic organism. Techniques for analyzing and/or modifying a multicellular organism are generally based on techniques described for modifying cells described below.

In one embodiment, the host cell can be a prokaryote such as a bacterial cell including, but not limited to an *Escherichia* or a *Pseudomonas* species. Typical bacterial cells are described, for example, in "Biological Diversity: Bacteria and Archaeans", a chapter of the On-Line Biology Book, provided by Dr MJ Farabee of the Estrella Mountain Community College, Arizona, USA at URL: http://www.emc.maricopa.edu/faculty/farabee/BIOBK/BioBookDiversity_2.html. In certain embodiments, the host cell can be a Pseudomonad cell, and can typically be a *P. fluorescens* cell. In other embodiments, the host cell can also be an *E. coli* cell. In another embodiment the host cell can be a eukaryotic cell, for example an insect cell, including but not limited to a cell from a *Spodoptera, Trichoplusia Drosophila* or an *Estigmene* species, or a mammalian cell, including but not limited to a murine cell, a hamster cell, a monkey, a primate or a human cell.

In certain embodiments, the host cell is a Pseudomonad cell, and can be for example a *P. fluorescens* organism.

In one embodiment, the host cell can be a member of any of the bacterial taxa. The cell can, for example, be a member of any species of eubacteria. The host can be a member any one of the taxa: *Acidobacteria, Actinobacteira, Aquificae, Bacteroidetes, Chlorobi, Chlamydiae, Choroflexi, Chrysiogenetes, Cyanobacteria, Deferribacteres, Deinococcus, Dictyoglomi, Fibrobacteres, Firmicutes, Fusobacteria, Gemmatimonadetes, Lentisphaerae, Nitrospirae, Planctomycetes, Proteobacteria, Spirochaetes, Thermodesulfobacteria, Thermomicrobia, Thermotogae, Thermus (Thermales),* or *Verrucomicrobia.* In one embodiment of a eubacterial host cell, the cell can be a member of any species of eubacteria, excluding Cyanobacteria.

The bacterial host can also be a member of any species of Proteobacteria. A proteobacterial host cell can be a member of any one of the taxa *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria,* or *Epsilonproteobacteria.* In addition, the host can be a member of any one of the taxa *Alphaproteobacteria,*

Betaproteobacteria, or *Gammaproteobacteria,* and a member of any species of *Gammaproteobacteria.*

In one embodiment of a Gammaproteobacterial host, the host will be member of any one of the taxa *Aeromonadales, Alteromonadales, Enterobacteriales, Pseudomonadales,* or *Xanthomonadales*; or a member of any species of the *Enterobacteriales* or *Pseudomonadales.* In one embodiment, the host cell can be of the order *Enterobacteriales,* the host cell will be a member of the family *Enterobacteriaceae,* or a member of any one of the genera *Erwinia, Escherichia,* or *Serratia*; or a member of the genus *Escherichia.* In one embodiment of a host cell of the order *Pseudomonadales,* the host cell will be a member of the family *Pseudomonadaceae,* even of the genus *Pseudomonas.* Gamma Proteobacterial hosts include members of the species *Escherichia coli* and members of the species *Pseudomonas fluorescens.*

Other *Pseudomonas* organisms may also be useful. Pseudomonads and closely related species include Gram(-) Proteobacteria Subgroup 1, which include the group of Proteobacteria belonging to the families and/or genera described as "Gram-Negative Aerobic Rods and Cocci" by R.E. Buchanan and N.E. Gibbons (eds.), Bergey's Manual of Determinative Bacteriology, pp. 217-289 (8th ed., 1974) (The Williams & Wilkins Co., Baltimore, MD, USA) (hereinafter "Bergey (1974)"). The following table presents these families and genera of organisms.

Families and Genera Listed in the Part, "Gram-Negative Aerobic Rods and Cocci" (in Bergey (1974))	
Family I. Pseudomonadaceae	*Gluconobacter* *Pseudomonas* *Xanthomonas* *Zoogloea*
Family II. Azotobacteraceae	*Azomonas* *Azotobacter* *Beijerinckia* *Derxia*
Family III. Rhizobiaceae	*Agrobacterium* *Rhizobium*
Family IV. Methylomonadaceae	*Methylococcus* *Methylomonas*

Family V. Halobacteriaceae	*Halobacterium* *Halococcus*
Other Genera	*Acetobacter* *Alcaligenes* *Bordetella* *Brucella* *Francisella* *Thermus*

"Gram(-) Proteobacteria Subgroup 1" also includes Proteobacteria that would be classified in this heading according to the criteria used in the classification. The heading also includes groups that were previously classified in this section but are no longer, such as the genera *Acidovorax, Brevundimonas, Burkholderia, Hydrogenophaga, Oceanimonas, Ralstonia, and Stenotrophomonas*, the genus *Sphingomonas* (and the genus *Blastomonas*, derived therefrom), which was created by regrouping organisms belonging to (and previously called species of) the genus *Xanthomonas,* the genus *Acidomonas,* which was created by regrouping organisms belonging to the genus *Acetobacter* as defined in Bergey (1974). In addition hosts can include cells from the genus *Pseudomonas, Pseudomonas enalia* (ATCC 14393), *Pseudomonas nigrifaciens* (ATCC 19375), and *Pseudomonas putrefaciens* (ATCC 8071), which have been reclassified respectively as *Alteromonas haloplanktis*, *Alteromonas nigrifaciens*, and *Alteromonas putrefaciens*. Similarly, e.g., *Pseudomonas acidovorans* (ATCC 15668) and *Pseudomonas testosteroni* (ATCC 11996) have since been reclassified as *Comamonas acidovorans* and *Comamonas testosteroni*, respectively; and *Pseudomonas nigrifaciens* (ATCC 19375) and *Pseudomonas piscicida* (ATCC 15057) have been reclassified respectively as *Pseudoalteromonas nigrifaciens* and *Pseudoalteromonas piscicida*. "Gram(-) Proteobacteria Subgroup 1" also includes Proteobacteria classified as belonging to any of the families: Pseudomonadaceae, Azotobacteraceae (now often called by the synonym, the "Azotobacter group" of Pseudomonadaceae), Rhizobiaceae, and Methylomonadaceae (now often called by the synonym, " Methylococcaceae"). Consequently, in addition to those genera otherwise described herein, further Proteobacterial genera falling within "Gram(-) Proteobacteria Subgroup 1" include: 1) Azotobacter group bacteria of the genus *Azorhizophilus;* 2) Pseudomonadaceae family bacteria of the genera *Cellvibrio, Oligella, and Teredinibacter*; 3) Rhizobiaceae family bacteria of the genera

Chelatobacter, Ensifer, Liberibacter (also called "*Candidatus Liberibacter*"), and *Sinorhizobium*; and 4) Methylococcaceae family bacteria of the genera *Methylobacter, Methylocaldum, Methylomicrobium, Methylosarcina, and Methylosphaera.*

In another embodiment, the host cell is selected from "Gram(-) Proteobacteria Subgroup 2." "Gram(-) Proteobacteria Subgroup 2" is defined as the group of Proteobacteria of the following genera (with the total numbers of catalog-listed, publicly-available, deposited strains thereof indicated in parenthesis, all deposited at ATCC, except as otherwise indicated): *Acidomonas* (2); *Acetobacter* (93); *Gluconobacter* (37); *Brevundimonas* (23); *Beijerinckia* (13); *Derxia* (2); *Brucella* (4); *Agrobacterium* (79); *Chelatobacter* (2); *Ensifer* (3); *Rhizobium* (144); *Sinorhizobium* (24); *Blastomonas* (1); *Sphingomonas* (27); *Alcaligenes* (88); *Bordetella* (43); *Burkholderia* (73); *Ralstonia* (33); *Acidovorax* (20); *Hydrogenophaga* (9); *Zoogloea* (9); *Methylobacter* (2); *Methylocaldum* (1 at NCIMB); *Methylococcus* (2); *Methylomicrobium* (2); *Methylomonas* (9); *Methylosarcina* (1); *Methylosphaera*; *Azomonas* (9); *Azorhizophilus* (5); *Azotobacter* (64); *Cellvibrio* (3); *Oligella* (5); *Pseudomonas* (1139); *Francisella* (4); *Xanthomonas* (229); *Stenotrophomonas* (50); and *Oceanimonas* (4).

Exemplary host cell species of "Gram(-) Proteobacteria Subgroup 2" include, but are not limited to the following bacteria (with the ATCC or other deposit numbers of exemplary strain(s) thereof shown in parenthesis): *Acidomonas methanolica* (ATCC 43581); *Acetobacter aceti* (ATCC 15973); *Gluconobacter oxydans* (ATCC 19357); *Brevundimonas diminuta* (ATCC 11568); *Beijerinckia indica* (ATCC 9039 and ATCC 19361); *Derxia gummosa* (ATCC 15994); *Brucella melitensis* (ATCC 23456), *Brucella abortus* (ATCC 23448); *Agrobacterium tumefaciens* (ATCC 23308), *Agrobacterium radiobacter* (ATCC 19358), *Agrobacterium rhizogenes* (ATCC 11325); *Chelatobacter heintzii* (ATCC 29600); *Ensifer adhaerens* (ATCC 33212); *Rhizobium leguminosarum* (ATCC 10004); *Sinorhizobium fredii* (ATCC 35423); *Blastomonas natatoria* (ATCC 35951); *Sphingomonas paucimobilis* (ATCC 29837); *Alcaligenes faecalis* (ATCC 8750); *Bordetella pertussis* (ATCC 9797); *Burkholderia cepacia* (ATCC 25416); *Ralstonia pickettii* (ATCC 27511); *Acidovorax facilis* (ATCC 11228); *Hydrogenophaga flava* (ATCC 33667); *Zoogloea ramigera* (ATCC 19544); *Methylobacter luteus* (ATCC 49878); *Methylocaldum gracile* (NCIMB 11912); *Methylococcus capsulatus* (ATCC 19069); *Methylomicrobium agile* (ATCC 35068); *Methylomonas methanica* (ATCC 35067); *Methylosarcina fibrata* (ATCC 700909); *Methylosphaera hansonii* (ACAM 549); *Azomonas agilis* (ATCC 7494); *Azorhizophilus paspali* (ATCC 23833); *Azotobacter chroococcum* (ATCC 9043); *Cellvibrio mixtus* (UQM 2601); *Oligella urethralis* (ATCC 17960); *Pseudomonas aeruginosa* (ATCC 10145),

Pseudomonas fluorescens (ATCC 35858); *Francisella tularensis* (ATCC 6223); *Stenotrophomonas maltophilia* (ATCC 13637); *Xanthomonas campestris* (ATCC 33913); and *Oceanimonas doudoroffii* (ATCC 27123).

In another embodiment, the host cell is selected from "Gram(-) Proteobacteria Subgroup 3." "Gram(-) Proteobacteria Subgroup 3" is defined as the group of Proteobacteria of the following genera: *Brevundimonas; Agrobacterium; Rhizobium; Sinorhizobium; Blastomonas; Sphingomonas; Alcaligenes; Burkholderia; Ralstonia ; Acidovorax; Hydrogenophaga; Methylobacter; Methylocaldum; Methylococcus; Methylomicrobium; Methylomonas; Methylosarcina ; Methylosphaera; Azomonas; Azorhizophilus; Azotobacter; Cellvibrio; Oligella; Pseudomonas ; Teredinibacter; Francisella; Stenotrophomonas; Xanthomonas*; and *Oceanimonas*.

In another embodiment, the host cell is selected from "Gram(-) Proteobacteria Subgroup 4." "Gram(-) Proteobacteria Subgroup 4" is defined as the group of Proteobacteria of the following genera: *Brevundimonas; Blastomonas; Sphingomonas ; Burkholderia; Ralstonia; Acidovorax; Hydrogenophaga; Methylobacter; Methylocaldum; Methylococcus; Methylomicrobium; Methylomonas; Methylosarcina; Methylosphaera; Azomonas; Azorhizophilus; Azotobacter; Cellvibrio; Oligella; Pseudomonas; Teredinibacter; Francisella ; Stenotrophomonas; Xanthomonas*; and *Oceanimonas*.

In an embodiment, the host cell is selected from "Gram(-) Proteobacteria Subgroup 5." "Gram(-) Proteobacteria Subgroup 5" is defined as the group of Proteobacteria of the following genera: *Methylobacter; Methylocaldum; Methylococcus; Methylomicrobium; Methylomonas; Methylosarcina; Methylosphaera; Azomonas; Azorhizophilus; Azotobacter; Cellvibrio; Oligella; Pseudomonas ; Teredinibacter; Francisella; Stenotrophomonas; Xanthomonas*; and *Oceanimonas*.

The host cell can be selected from "Gram(-) Proteobacteria Subgroup 6." "Gram(-) Proteobacteria Subgroup 6" is defined as the group of Proteobacteria of the following genera: *Brevundimonas; Blastomonas; Sphingomonas; Burkholderia; Ralstonia; Acidovorax; Hydrogenophaga; Azomonas; Azorhizophilus; Azotobacter; Cellvibrio; Oligella; Pseudomonas; Teredinibacter; Stenotrophomonas; Xanthomonas*; and *Oceanimonas*.

The host cell can be selected from "Gram(-) Proteobacteria Subgroup 7." "Gram(-) Proteobacteria Subgroup 7" is defined as the group of Proteobacteria of the following genera: Azomonas; *Azorhizophilus; Azotobacter; Cellvibrio; Oligella; Pseudomonas; Teredinibacter; Stenotrophomonas; Xanthomonas*; and *Oceanimonas*.

The host cell can be selected from "Gram(-) Proteobacteria Subgroup 8." "Gram(-) Proteobacteria Subgroup 8" is defined as the group of Proteobacteria of the following genera: *Brevundimonas; Blastomonas; Sphingomonas; Burkholderia; Ralstonia; Acidovorax; Hydrogenophaga; Pseudomonas; Stenotrophomonas; Xanthomonas;* and *Oceanimonas.*

The host cell can be selected from "Gram(-) Proteobacteria Subgroup 9." "Gram(-) Proteobacteria Subgroup 9" is defined as the group of Proteobacteria of the following genera: *Brevundimonas; Burkholderia; Ralstonia; Acidovorax; Hydrogenophaga; Pseudomonas; Stenotrophomonas*; and *Oceanimonas.*

The host cell can be selected from "Gram(-) Proteobacteria Subgroup 10." "Gram(-) Proteobacteria Subgroup 10" is defined as the group of Proteobacteria of the following genera: *Burkholderia; Ralstonia; Pseudomonas; Stenotrophomonas*; and *Xanthomonas.*

The host cell can be selected from "Gram(-) Proteobacteria Subgroup 11." "Gram(-) Proteobacteria Subgroup 11" is defined as the group of Proteobacteria of the genera: *Pseudomonas; Stenotrophomonas*; and *Xanthomonas.* The host cell can be selected from "Gram(-) Proteobacteria Subgroup 12." "Gram(-) Proteobacteria Subgroup 12" is defined as the group of Proteobacteria of the following genera: *Burkholderia; Ralstonia; Pseudomonas.* The host cell can be selected from "Gram(-) Proteobacteria Subgroup 13." "Gram(-) Proteobacteria Subgroup 13" is defined as the group of Proteobacteria of the following genera: *Burkholderia; Ralstonia; Pseudomonas*; and *Xanthomonas.* The host cell can be selected from "Gram(-) Proteobacteria Subgroup 14." "Gram(-) Proteobacteria Subgroup 14" is defined as the group of Proteobacteria of the following genera: *Pseudomonas* and *Xanthomonas.* The host cell can be selected from "Gram(-) Proteobacteria Subgroup 15." "Gram(-) Proteobacteria Subgroup 15" is defined as the group of Proteobacteria of the genus *Pseudomonas.*

The host cell can be selected from "Gram(-) Proteobacteria Subgroup 16." "Gram(-) Proteobacteria Subgroup 16" is defined as the group of Proteobacteria of the following *Pseudomonas* species (with the ATCC or other deposit numbers of exemplary strain(s) shown in parenthesis): *Pseudomonas abietaniphila* (ATCC 700689); *Pseudomonas aeruginosa* (ATCC 10145); *Pseudomonas alcaligenes* (ATCC 14909); *Pseudomonas anguilliseptica* (ATCC 33660); *Pseudomonas citronellolis* (ATCC 13674); *Pseudomonas flavescens* (ATCC 51555); *Pseudomonas mendocina* (ATCC 25411); *Pseudomonas nitroreducens* (ATCC 33634); *Pseudomonas oleovorans* (ATCC 8062); *Pseudomonas pseudoalcaligenes* (ATCC 17440); *Pseudomonas resinovorans* (ATCC 14235); *Pseudomonas straminea* (ATCC 33636); *Pseudomonas agarici* (ATCC 25941); *Pseudomonas alcaliphila; Pseudomonas*

alginovora; Pseudomonas andersonii; Pseudomonas asplenii (ATCC 23835); *Pseudomonas azelaica* (ATCC 27162); *Pseudomonas beijerinckii* (ATCC 19372); *Pseudomonas borealis*; *Pseudomonas boreopolis* (ATCC 33662); *Pseudomonas brassicacearum; Pseudomonas butanovora* (ATCC 43655); *Pseudomonas cellulosa* (ATCC 55703); *Pseudomonas aurantiaca* (ATCC 33663); *Pseudomonas chlororaphis* (ATCC 9446, ATCC 13985, ATCC 17418, ATCC 17461); *Pseudomonas fragi* (ATCC 4973); *Pseudomonas lundensis* (ATCC 49968); *Pseudomonas taetrolens* (ATCC 4683); *Pseudomonas cissicola* (ATCC 33616); *Pseudomonas coronafaciens; Pseudomonas diterpeniphila; Pseudomonas elongata* (ATCC 10144); *Pseudomonas flectens* (ATCC 12775); *Pseudomonas azotoformans; Pseudomonas brenneri; Pseudomonas cedrella; Pseudomonas corrugata* (ATCC 29736); *Pseudomonas extremorientalis; Pseudomonas fluorescens* (ATCC 35858); *Pseudomonas gessardii; Pseudomonas libanensis; Pseudomonas mandelii* (ATCC 700871); *Pseudomonas marginalis* (ATCC 10844); *Pseudomonas migulae; Pseudomonas mucidolens* (ATCC 4685); *Pseudomonas orientalis; Pseudomonas rhodesiae; Pseudomonas synxantha* (ATCC 9890); *Pseudomonas tolaasii* (ATCC 33618); *Pseudomonas veronii* (ATCC 700474); *Pseudomonas frederiksbergensis; Pseudomonas geniculata* (ATCC 19374); *Pseudomonas gingeri; Pseudomonas graminis; Pseudomonas grimontii; Pseudomonas halodenitrificans; Pseudomonas halophila; Pseudomonas hibiscicola* (ATCC 19867); *Pseudomonas huttiensis* (ATCC 14670); *Pseudomonas hydrogenovora; Pseudomonas jessenii* (ATCC 700870); *Pseudomonas kilonensis; Pseudomonas lanceolata* (ATCC 14669); *Pseudomonas lini; Pseudomonas marginata* (ATCC 25417); *Pseudomonas mephitica* (ATCC 33665); *Pseudomonas denitrificans* (ATCC 19244); *Pseudomonas pertucinogena* (ATCC 190); *Pseudomonas pictorum* (ATCC 23328); *Pseudomonas* psychrophila; *Pseudomonas* fulva (ATCC 31418); *Pseudomonas monteilii* (ATCC 700476); *Pseudomonas mosselii; Pseudomonas oryzihabitans* (ATCC 43272); *Pseudomonas plecoglossicida* (ATCC 700383); *Pseudomonas putida* (ATCC 12633); *Pseudomonas reactans; Pseudomonas spinosa* (ATCC 14606); *Pseudomonas balearica; Pseudomonas luteola* (ATCC 43273); *Pseudomonas stutzeri* (ATCC 17588); *Pseudomonas amygdali* (ATCC 33614); *Pseudomonas avellanae* (ATCC 700331); *Pseudomonas caricapapayae* (ATCC 33615); *Pseudomonas cichorii* (ATCC 10857); *Pseudomonas ficuserectae* (ATCC 35104); *Pseudomonas fuscovaginae*; *Pseudomonas meliae* (ATCC 33050); *Pseudomonas syringae* (ATCC 19310); *Pseudomonas viridiflava* (ATCC 13223); *Pseudomonas thermocarboxydovorans* (ATCC 35961); *Pseudomonas thermotolerans*; *Pseudomonas*

thivervalensis; *Pseudomonas vancouverensis* (ATCC 700688); *Pseudomonas wisconsinensis*; and *Pseudomonas xiamenensis*.

The host cell can be selected from "Gram(-) Proteobacteria Subgroup 17." "Gram(-) Proteobacteria Subgroup 17" is defined as the group of Proteobacteria known in the art as the "fluorescent Pseudomonads" including those belonging, e.g., to the following *Pseudomonas* species: *Pseudomonas azotoformans; Pseudomonas brenneri; Pseudomonas cedrella; Pseudomonas corrugata; Pseudomonas extremorientalis; Pseudomonas fluorescens; Pseudomonas gessardii; Pseudomonas libanensis; Pseudomonas mandelii; Pseudomonas marginalis; Pseudomonas migulae; Pseudomonas mucidolens; Pseudomonas orientalis; Pseudomonas rhodesiae; Pseudomonas synxantha; Pseudomonas tolaasii*; and *Pseudomonas veronii*.

The host cell can be selected from "Gram(-) Proteobacteria Subgroup 18." "Gram(-) Proteobacteria Subgroup 18" is defined as the group of all subspecies, varieties, strains, and other sub-special units of the species *Pseudomonas fluorescens*, including those belonging, e.g., to the following (with the ATCC or other deposit numbers of exemplary strain(s) shown in parenthesis): *Pseudomonas fluorescens* biotype A, also called biovar 1 or biovar I (ATCC 13525); *Pseudomonas fluorescens* biotype B, also called biovar 2 or biovar II (ATCC 17816); *Pseudomonas fluorescens* biotype C, also called biovar 3 or biovar III (ATCC 17400); *Pseudomonas fluorescens* biotype F, also called biovar 4 or biovar IV (ATCC 12983); *Pseudomonas fluorescens* biotype G, also called biovar 5 or biovar V (ATCC 17518); *Pseudomonas fluorescens* biovar VI; *Pseudomonas fluorescens* Pf0-1; *Pseudomonas fluorescens* Pf-5 (ATCC BAA-477); *Pseudomonas fluorescens* SBW25; and *Pseudomonas fluorescens subsp. cellulosa* (NCIMB 10462).

The host cell can be selected from "Gram(-) Proteobacteria Subgroup 19." "Gram(-) Proteobacteria Subgroup 19" is defined as the group of all strains of *Pseudomonas fluorescens* biotype A. A typical strain of this biotype is *P. fluorescens* strain MB101 (see US Patent No. 5,169,760 to Wilcox), and derivatives thereof. An example of a derivative thereof is *P. fluorescens* strain MB214, constructed by inserting into the MB101 chromosomal asd (aspartate dehydrogenase gene) locus, a native *E. coli* PlacI-lacI-lacZYA construct (i.e. in which PlacZ was deleted).

Additional *P. fluorescens* strains that can be used in the present invention include *Pseudomonas fluorescens* Migula and *Pseudomonas fluorescens* Loitokitok, having the following ATCC designations: [NCIB 8286]; NRRL B-1244; NCIB 8865 strain CO1; NCIB 8866 strain CO2; 1291 [ATCC 17458; IFO 15837; NCIB 8917; LA; NRRL B-1864;

pyrrolidine; PW2 [ICMP 3966; NCPPB 967; NRRL B-899]; 13475; NCTC 10038; NRRL B-1603 [6; IFO 15840]; 52-1C; CCEB 488-A [BU 140]; CCEB 553 [IEM 15/47]; IAM 1008 [AHH-27]; IAM 1055 [AHH-23]; 1 [IFO 15842]; 12 [ATCC 25323; NIH 11; den Dooren de Jong 216]; 18 [IFO 15833; WRRL P-7]; 93 [TR-10]; 108 [52-22; IFO 15832]; 143 [IFO 15836; PL]; 149 [2-40-40; IFO 15838]; 182 [IFO 3081; PJ 73]; 184 [IFO 15830]; 185 [W2 L-1]; 186 [IFO 15829; PJ 79]; 187 [NCPPB 263]; 188 [NCPPB 316]; 189 [PJ227; 1208]; 191 [IFO 15834; PJ 236; 22/1]; 194 [Klinge R-60; PJ 253]; 196 [PJ 288]; 197 [PJ 290]; 198 [PJ 302]; 201 [PJ 368]; 202 [PJ 372]; 203 [PJ 376]; 204 [IFO 15835; PJ 682]; 205 [PJ 686]; 206 [PJ 692]; 207 [PJ 693]; 208 [PJ 722]; 212 [PJ 832]; 215 [PJ 849]; 216 [PJ 885]; 267 [B-9]; 271 [B-1612]; 401 [C71A; IFO 15831; PJ 187]; NRRL B-3178 [4; IFO 15841]; KY 8521; 3081; 30-21; [IFO 3081]; N; PYR; PW; D946-B83 [BU 2183; FERM-P 3328]; P-2563 [FERM-P 2894; IFO 13658]; IAM-1126 [43F]; M-1; A506 [A5-06]; A505 [A5-05-1]; A526 [A5-26]; B69; 72; NRRL B-4290; PMW6 [NCIB 11615]; SC 12936; A1 [IFO 15839]; F 1847 [CDC-EB]; F 1848 [CDC 93]; NCIB 10586; P17; F-12; AmMS 257; PRA25; 6133D02; 6519E01; N1; SC15208; BNL-WVC; NCTC 2583 [NCIB 8194]; H13; 1013 [ATCC 11251; CCEB 295]; IFO 3903; 1062; or Pf-5.

Other suitable hosts include those classified in other parts of the reference, such as Gram (+) Proteobacteria. In one embodiment, the host cell is an *E. coli*. The genome sequence for *E. coli* has been established for *E. coli* MG1655 (Blattner, et al. (1997) The complete genome sequence of *Escherichia coli* K-12 *Science* 277(5331): 1453-74) and DNA microarrays are available commercially for *E. coli* K12 (MWG Inc, High Point, NC). *E. coli* can be cultured in either a rich medium such as Luria-Bertani (LB) (10 g/L tryptone, 5 g/L NaCl, 5 g/L yeast extract) or a defined minimal medium such as M9 (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl, pH 7.4) with an appropriate carbon source such as 1% glucose. Routinely, an over night culture of *E. coli* cells is diluted and inoculated into fresh rich or minimal medium in either a shake flask or a fermentor and grown at 37°C.

A host can also be of mammalian origin, such as a cell derived from a mammal including any human or non-human mammal. Mammals can include, but are not limited to primates, monkeys, porcine, ovine, bovine, rodents, ungulates, pigs, swine, sheep, lambs, goats, cattle, deer, mules, horses, monkeys, apes, dogs, cats, rats, and mice.

A host cell may also be of plant origin. Any plant can be selected for the identification of genes and regulatory sequences. Examples of suitable plant targets for the isolation of genes and regulatory sequences would include but are not limited to alfalfa, apple, apricot, Arabidopsis, artichoke, arugula, asparagus, avocado, banana, barley, beans,

beet, blackberry, blueberry, broccoli, brussels sprouts, cabbage, canola, cantaloupe, carrot, cassava, castorbean, cauliflower, celery, cherry, chicory, cilantro, citrus, clementines, clover, coconut, coffee, corn, cotton, cranberry, cucumber, Douglas fir, eggplant, endive, escarole, eucalyptus, fennel, figs, garlic, gourd, grape, grapefruit, honey dew, jicama, kiwifruit, lettuce, leeks, lemon, lime, Loblolly pine, linseed, mango, melon, mushroom, nectarine, nut, oat, oil palm, oil seed rape, okra, olive, onion, orange, an ornamental plant, palm, papaya, parsley, parsnip, pea, peach, peanut, pear, pepper, persimmon, pine, pineapple, plantain, plum, pomegranate, poplar, potato, pumpkin, quince, radiata pine, radiscchio, radish, rapeseed, raspberry, rice, rye, sorghum, Southern pine, soybean, spinach, squash, strawberry, sugarbeet, sugarcane, sunflower, sweet potato, sweetgum, tangerine, tea, tobacco, tomato, triticale, turf, turnip, a vine, watermelon, wheat, yams, and zucchini. In some embodiments, plants useful in the process are Arabidopsis, corn, wheat, soybean, and cotton.

For expression of a recombinant protein or peptide, or for modulation of an identified compensatory gene, any plant promoter can be used. A promoter may be a plant RNA polymerase II promoter. Elements included in plant promoters can be a TATA box or Goldberg-Hogness box, typically positioned approximately 25 to 35 basepairs upstream (5') of the transcription initiation site, and the CCAAT box, located between 70 and 100 basepairs upstream. In plants, the CCAAT box may have a different consensus sequence than the functionally analogous sequence of mammalian promoters (Messing et al., In: Genetic Engineering of Plants, Kosuge et al., eds., pp. 211-227, 1983). In addition, virtually all promoters include additional upstream activating sequences or enhancers (Benoist and Chambon, Nature 290:304-310, 1981; Gruss et al., Proc. Nat. Acad. Sci. USA 78:943-947, 1981; and Khoury and Gruss, Cell 27:313-314, 1983) extending from around -100 bp to -1,000 bp or more upstream of the transcription initiation site.

Expression of recombinant protein or peptide

As described below, a host cell or organism can be engineered to express recombinant protein or peptide using standard techniques. For example, recombinant protein can be expressed from a vector or from an exogenous gene inserted into the genome of the host. Vectors that can be used to express exogenous proteins are well known in the art and are described below. Genes for expressing recombinant protein or peptide can also be inserted into the genome using techniques such as homologous or heterologous recombination, as described below.

The recombinant protein or peptide can be expressed after induction with a chemical compound or upon expression of an endogenous gene or gene product. The recombinant protein can also be expressed when the host cell is placed in a particular environment. Specific promoter elements are described below. These include, but are not limited to, promoters that can be induced upon treatment of the cell with chemicals such as IPTG, benzoate or anthranilate.

Recombinant proteins/peptides

The host cell has been designed to express a recombinant protein or peptide. These can be of any species and of any size. However, in certain embodiments, the recombinant protein or peptide is a therapeutically useful protein or peptide. In some embodiments, the protein can be a mammalian protein, for example a human protein, and can be, for example, a growth factor, a cytokine, a chemokine or a blood protein. The recombinant protein or peptide can be expressed primarily in an inactive form in the host cell. In certain embodiments, the recombinant protein or peptide is less than 100kD, less than 50kD, or less than 30kD in size. In ceratin embodiments, the recombinant protein or peptide is a peptide of at least 5, 10, 15, 20, 30, 40, 50 or 100 amino acids.

Expression vectors exist that enable recombinant protein production in *E. coli*. For all these protein expression systems routine cloning procedures as described earlier can be followed (Sambrook, et al. (2000) *Molecular cloning: A laboratory manual, third edition* Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press).

The Champion™ pET expression system provides a high level of protein production. Expression is induced from the strong T7*lac* promoter. This system takes advantage of the high activity and specificity of the bacteriophage T7 RNA polymerase for high level transcription of the gene of interest. The *lac* operator located in the promoter region provides tighter regulation than traditional T7-based vectors, improving plasmid stability and cell viability (Studier, F. W. and B. A. Moffatt (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes *Journal of Molecular Biology* 189(1): 113-30; Rosenberg, et al. (1987) Vectors for selective expression of cloned DNAs by T7 RNA polymerase *Gene* 56(1): 125-35). The T7 expression system uses the T7 promoter and T7 RNA polymerase (T7 RNAP) for high-level transcription of the gene of interest. High-level expression is achieved in T7 expression systems because the T7 RNAP is more processive than native *E. coli* RNAP and is dedicated to the transcription of the gene of interest. Expression of the identified gene is induced by providing a source of T7 RNAP in

the host cell. This is accomplished by using a BL21 *E. coli* host containing a chromosomal copy of the T7 RNAP gene. The T7 RNAP gene is under the control of the *lac*UV5 promoter which can be induced by IPTG. T7 RNAP is expressed upon induction and transcribes the gene of interest.

The pBAD expression system allows tightly controlled, titratable expression of recombinant protein through the presence of specific carbon sources such as glucose, glycerol and arabinose (Guzman, et al. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promote" *Journal of Bacteriology* 177(14): 4121-30). The pBAD vectors are uniquely designed to give precise control over expression levels. Heterologous gene expression from the pBAD vectors is initiated at the *araBAD* promoter. The promoter is both positively and negatively regulated by the product of the *araC* gene. AraC is a transcriptional regulator that forms a complex with L-arabinose. In the absence of L-arabinose, the AraC dimer blocks transcription. For maximum transcriptional activation two events are required: (i.) L-arabinose binds to AraC allowing transcription to begin. (ii.) The cAMP activator protein (CAP)-cAMP complex binds to the DNA and stimulates binding of AraC to the correct location of the promoter region.

The *trc* expression system allows high-level, regulated expression in *E. coli* from the *trc* promoter. The *trc* expression vectors have been optimized for expression of eukaryotic genes in *E. coli.* The *trc* promoter is a strong hybrid promoter derived from the tryptophane (*trp*) and lactose (*lac*) promoters. It is regulated by the *lacO* operator and the product of the $lacI^{Q}$ gene (Brosius, J. (1984) Toxicity of an overproduced foreign gene product in *Escherichia coli* and its use in plasmid vectors for the selection of transcription terminators *Gene* 27(2): 161-72).

The invention also includes the improved recombinant host cell that is produced by the claimed process. In one embodiment, the invention includes a cell produced by the described process. In another embodiment, the invention includes a host cell or organism that expresses a recombinant protein that has been genetically modified to reduce the expression of at least two proteases. In other embodiments, the invention includes a host cell or organism that expresses a recombinant protein that has been genetically modified to reduce the expression of at least one protease selected from the group consisting of products of *hslV, hslU, clpX, clpA* and *clpB* genes, and in certain subembodiments, the cell or organism has been modified to reduce the expression of HslV or HslU. In certain embodiments, the modified host cell or organism expresses a recombinant mammalian derived protein, and may express a recombinant human derived protein, which may be human growth hormone. The

cell can be modified by any techniques known in the art, for example by a technique wherein at least one protease gene is knocked out of the genome, or by mutating at least one protease gene to reduce expression of a protease, or by altering at least one promoter of at least one protease gene to reduce expression of a protease.

In another embodiment, a host or organism that expresses a recombinant protein that is presented that has been genetically modified to increase the expression of at least one, at least two folding modulators, or at least three folding modulators. In certain subembodiments, the folding modulators that are not folding modulator subunits. The folding modulator may be selected from the group consisting of products of *cbpA, htpG, dnaK, dnaJ, fkbP2,groES* and *groEL* genes, and, in certain subembodiments, can be *htpG* or *cbpA*. The host cell or organism can in a non-limiting example, express a mammalian protein, such as a human protein. The protein may be human growth hormone. The folding modulator or modulators can be increased by, for example, including an expression vector as described herein in the cell. The folding modulator expression can also be increased by, for example, mutating a promoter of a folding modulator or folding modulator subunit. A host cell or organism that expresses a recombinant protein can also be genetically modified to increase the expression of at least one folding modulators and decrease the expression of at least one protease or protease protein. Organisms comprising one or more cells produced by the described process are also included in the invention.

Step II: Analyzing a genetic profile to identify a compensatory gene or gene product that is expressed at a higher level in the recombinant cell

The process of the invention includes analyzing a genetic profile of the recombinant cell to identify a compensatory gene or gene product that is expressed at a higher level in the recombinant cell than in either a host cell that has not been modified to express the recombinant protein or a recombinant cell that is not expressing the recombinant protein.

A "genetic profile" as used herein can include genes in a genome, mRNA transcribed from genes in the genome or cDNA derived from mRNA transcribed from genes in the genome. A gentic profile can also include transcription products that have been modified by a cell such as splice variants of genes in eukaryotic systems, or proteins translated from genes in a genome, including proteins that are modified by the cell or translated from splice variants of mRNA translated from the genome. A genetic profile is meant to refer solely to the simultaneous analysis of multiple entitities, such as in an array or other multiplex system, including multiple simultaneous blot analysis or column chromatography with multiple

binding partners attached to the packing. According to the invention, at least 5, 10, 25, 50, 70, 80, 90 or 100 or more genes or gene products that are analyzed simultaneously.

Transcriptome

In one embodiment, the genetic profile analyzed is a transcriptome profile. A complete transcriptome refers to the complete set of mRNA transcripts produced by the genome at any one time. Unlike the genome, the transcriptome is dynamic and varies considerably in differing circumstances due to different patterns of gene expression. Transcriptomics, the study of the transcriptome, is a comprehensive means of identifying gene expression patterns. The transcriptome analyzed can include the complete known set of genes transcribed, i.e. the mRNA content or corresponding cDNA of a host cell or host organism. The cDNA can be a chain of nucleotides, an isolated polynucleotide, nucleotide, nucleic acid molecule, or any fragment or complement thereof that originated recombinantly or synthetically and be double-stranded or single-stranded, coding and/or noncoding, an exon or an intron of a genomic DNA molecule, or combined with carbohydrate, lipids, protein or inorganic elements or substances. The nucleotide chain can be at least 5, 10, 15, 30, 40, 50, 60, 70, 80, 90 or 100 nucleotides in length. The transcriptome can also include only a portion of the known set of genetic transcripts. For example, the transcriptome can include less than 98%, 95, 90, 85, 80, 70, 60, or 50% of the known transcripts in a host. The transcriptome can also be targeted to a specific set of genes.

In one embodiment, the screening process can include screening using an array or a microarray to identify a genetic profile. In another embodiment, the transcriptome profile can be analyzed by using known processes such as hybridization in blot assays such as northern blots. In another embodiment, the process can include PCR-based processes such as RT-PCR that can quantify expression of a particular set of genes. In one embodiment of the invention, an identified gene, for example a folding modulator protein (FM) or protease protein, i.e. a protease, peptidase, or associated polypeptide or cofactor, is identified by a high throughput screening process .

The process can include analyzing the transcriptome profile using a microarray or equivalent technique. In this embodiment, the microarray can include at least a portion of the transcribed genome of the host cell, and typically includes binding partners to samples from genes of at least 50% of the transcribed genes of the organism. More typically, the microarray or equivalent technique includes binding partners for samples from at least 80%, 90%, 95%, 98%, 99% or 100% of the transcribed genes in the genome of the host cell.

However, in a separate embodiment, the microarray can include binding partners to a selected subset of genes from the genome, including but not limited to putative protease genes or putative folding modulator genes. A microarray or equivalent technique can typically also include binding partners to a set of genes that are used as controls, such as housekeeper genes. A microarray or equivalent technique can also include genes clustered into groups such as genes coding for degradative proteins, folding modulators and cofactors, metabolic proteins such as proteins involved in glucose metabolism or amino acid or nucleobase synthesis, transcription factors, nucleic acid stabilizing factors, extracellular signal regulated genes such as kinases and receptors or scaffolding proteins.

A microarray is generally formed by linking a large number of discrete binding partners, which can include polynucleotides, aptamers, chemicals, antibodies or other proteins or peptides, to a solid support such as a microchip, glass slide, or the like, in a defined pattern. By contacting the microarray with a sample obtained from a cell of interest and detecting binding of the binding partners expressed in the cell that hybridize to sequences on the chip, the pattern formed by the hybridizing polynucleotides allows the identification of genes or clusters of genes that are expressed in the cell. Furthermore, where each member linked to the solid support is known, the identity of the hybridizing partners from the nucleic acid sample can be identified. One strength of microarray technology is that it allows the identification of differential gene expression simply by comparing patterns of hybridization.

Examples of high throughput screening processes include hybridization of host cell mRNA or substantially corresponding cDNA, to a hybridizable array(s) or microarray(s). The array or microarray can be one or more array(s) of nucleic acid or nucleic acid analog oligomers or polymers. In one embodiment, the array(s) or microarray(s) will be independently or collectively a host-cell-genome-wide array(s) or microarray(s), containing a population of nucleic acid or nucleic acid analog oligomers or polymers whose nucleotide sequences are hybridizable to representative portions of all genes known to encode or predicted as encoding FMs in the host cell strain or all genes known to encode or predicted to encode proteases or protease proteins in the host cell strain. A genome-wide microarray includes sequences that bind to a representative portion of all of the known or predicted open reading frame (ORF) sequences, such as from mRNA or corresponding cDNA of the host.

The oligonucleotide sequences or analogs in the array typically hybridize to the mRNA or corresponding cDNA sequences from the host cell and typically comprise a nucleotide sequence complimentary to at least a portion of a host mRNA or cDNA sequence, or a sequence homologous to the host mRNA or cDNA sequence. Single DNA strands with

complementary sequences can pair with each other and form double-stranded molecules. Microarrays generally apply the hybridization principle in a highly parallel format. Instead of one identified, thousands of different potential identifieds can be arrayed on a miniature solid support. Instead of a unique labeled DNA probe, a complex mixture of labeled DNA molecules is used, prepared from the RNA of a particular cell type or tissue. The abundances of individual labeled DNA molecules in this complex probe typically reflect the expression levels of the corresponding genes. In a simplified process, when hybridized to the array, abundant sequences will generate strong signals and rare sequences will generate weak signals. The strength of the signal can represent the level of gene expression in the original sample.

In one embodiment, a genome-wide array or microarray will be used. In one embodiment, the array represents more than 50% of the open reading frames in the genome of the host, or more than 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the known open reading frames in the genome. The array can also represent at least a portion of at least 50% of the sequences known to encode protein in the host cell. In separate embodiments, the array represents more than 50% of the genes or putative genes of the host cell, or more than 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the known genes or putative genes. In one embodiment, more than one oligonucleotide or analog can be used for each gene or putative gene sequence or open reading frame. In one embodiment, these multiple oligonucleotide or analogs represent different portions of a known gene or putative gene sequence. For each gene or putative gene sequence, from about 1 to about 10000 or from 1 to about 100 or from 1 to about 50, 45, 40, 35, 30, 25, 20, 15, 10 or less oligonucleotides or analogs can be present on the array.

A microarray or a complete genome-wide array or microarray may be prepared according to any process known in the art, based on knowledge of the sequence(s) of the host cell genome, or the proposed coding sequences in the genome, or based on the knowledge of expressed mRNA sequences in the host cell or host organism.

For different types of host cells, the same type of microarray can be applied. The types of microarrays include complementary DNA (cDNA) microarrays (Schena, M. et al. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270:467-70) and oligonucleotide microarrays (Lockhart, et al. (1996) Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat Biotechnol* 14:1675-80). For cDNA microarray, the DNA fragment of a partial or entire open

reading frame is printed on the slides. The hybridization characteristics can be different throughout the slide because different portions of the molecules can be printed in different locations. For the oligonucleotide arrays, 20~80-mer oligos can be synthesized either *in situ* (on-chip) or by conventional synthesis followed by on-chip immobilization, however in general all probes are designed to be similar with regard to hybridization temperature and binding affinity (Butte, A. (2002) The use and analysis of microarray data. *Nat Rev Drug Discov* 1:951-60).

In analyzing the transcriptome profile, the nucleic acid or nucleic acid analog oligomers or polymers can be RNA, DNA, or an analog of RNA or DNA. Such nucleic acid analogs are known in the art and include, e.g.: peptide nucleic acids (PNA); arabinose nucleic acids; altritol nucleic acids; bridged nucleic acids (BNA), e.g., 2'-O,4'-C-ethylene bridged nucleic acids, and 2'-O,4'-C-methylene bridged nucleic acids; cyclohexenyl nucleic acids; 2',5'-linked nucleotide-based nucleic acids; morpholino nucleic acids (nucleobase-substituted morpholino units connected, e.g., by phosphorodiamidate linkages); backbone-substituted nucleic acid analogs, e.g., 2'-substituted nucleic acids, wherein at least one of the 2' carbon atoms of an oligo- or poly-saccharide-type nucleic acid or analog is independently substituted with, e.g., any one of a halo, thio, amino, aliphatic, oxyaliphatic, thioaliphatic, or aminoaliphatic group (wherein aliphatic is typically C_1-C_{10} aliphatic).

Oligonucleotides or oligonucleotide analogs in the array can be of uniform size and, in one embodiment, can be about 10 to about 1000 nucleotides, about 20 to about 1000, 20 to about 500, 20 to about 100, about 20, about 25, about 30, about 40, about 50, about 60, about 70, about 80, about 90 or about 100 nucleotides long.

The array of oligonucleotide probes can be a high density array comprising greater than about 100, or greater than about 1,000 or more different oligonucleotide probes. Such high density arrays can comprise a probe density of greater than about 60, more generally greater than about 100, most generally greater than about 600, often greater than about 1000, more often greater than about 5,000, most often greater than about 10,000, typically greater than about 40,000 more typically greater than about 100,000, and in certain instances is greater than about 400,000 different oligonucleotide probes per cm^2 (where different oligonucleotides refers to oligonucleotides having different sequences). The oligonucleotide probes range from about 5 to about 500, or about 5 to 50, or from about 5 to about 45 nucleotides, or from about 10 to about 40 nucleotides and most typically from about 15 to about 40 nucleotides in length. Particular arrays contain probes ranging from about 20 to about 25 oligonucleotides in length. The array may comprise more than 10, or more than 50,

or more than 100, and typically more than 1000 oligonucleotide probes specific for each identified gene. In one embodiment, the array comprises at least 10 different oligonucleotide probes for each gene. In another embodiment, the array has 20 or fewer oligonucleotides complementary each gene. Although a planar array surface is typical, the array may be fabricated on a surface of virtually any shape or even on multiple surfaces.

The array may further comprise mismatch control probes. Where such mismatch controls are present, the quantifying step may comprise calculating the difference in hybridization signal intensity between each of the oligonucleotide probes and its corresponding mismatch control probe. The quantifying may further comprise calculating the average difference in hybridization signal intensity between each of the oligonucleotide probes and its corresponding mismatch control probe for each gene.

In some assay formats, the oligonucleotide probe can be tethered, i.e., by covalent attachment, to a solid support. Oligonucleotide arrays can be chemically synthesized by parallel immobilized polymer synthesis processes or by light directed polymer synthesis processes, for example on poly-L-lysine substrates such as slides. Chemically synthesized arrays are advantageous in that probe preparation does not require cloning, a nucleic acid amplification step, or enzymatic synthesis. The array includes test probes which are oligonucleotide probes each of which has a sequence that is complementary to a subsequence of one of the genes (or the mRNA or the corresponding antisense cRNA) whose expression is to be detected. In addition, the array can contain normalization controls, mismatch controls and expression level controls as described herein.

An array may be designed to include one hybridizing oligonucleotide per known gene in a genome. The oligonucleotides or equivalent binding partners can be 5'-amino modified to support covalent binding to epoxy-coated slides. The oligonucleotides can be designed to reduce cross-hybridization, for example by reducing sequence identity to less than 25% between oligonucleotides. Generally, melting temperature of oligonucleotides is analyzed before design of the array to ensure consistent GC content and Tm, and secondary structure of oligonucleotide binding partners is optimized. For transcriptome profiling, secondary structure is typically minimized. In one embodiment, each oligonucleotide is printed at at least two different locations on the slide to increase accuracy. Control oligonucleotides can also be designed based on sequences from different species than the host cell or organism to show background binding.

The samples in the genetic profile can be analyzed individually or grouped into clusters. The clusters can typically be grouped by similarity in gene expression. In one

embodiment, the clusters may be grouped individually as genes that are regulated to a similar extent in a host cell. The clusters may also include groups of genes that are regulated to a similar extent in a recombinant host cell, for example genes that are up-regulated or down-regulated to a similar extent compared to a host cell or a modified or an unmodified cell. The clusters can also include groups related by gene or protein structure, function or, in the case of a transcriptome array, by placement or grouping of binding partners to genes in the genome of the host. Groups of binding partners or groups of genes or proteins analyzed can include genes selected from, but not limited to: genes coding for putative or known proteases, co-factors of proteases or protease-like proteins; folding modulators, co-factors of folding modulators or proteins that could improve protein folding or solubility; transcription factors; proteins involved in nucleic acid stability or translational initiation; kinases; extracellular or intracellular receptors; metabolic enzymes; metabolic cofactors; envelope proteins; sigma factors; membrane bound proteins; transmembrane proteins; membrane associated proteins and housekeeping genes.

Proteome

In another embodiment, the genetic profile analyzed is a proteome profile. The proteome of a host is the complete set of proteins produced by the genome at any one time. The proteome is generally much more complex than either the genome or the transcriptome because each protein can be chemically modified after synthesis. Many proteins are cleaved during production, are phosphorylated, acetylated, methylated, or have carbohydrate groups added to them, depending on the host cell. The proteome is also very dynamic. Proteomics, the study of the proteome, can cover a number of different aspects of protein structure, protein expression, and function. The techniques for proteome analysis are not as straightforward as those used in transcriptomics. However, an advantage of proteomics is that the functional molecules of the cell are being studied.

The process can include techniques that measure protein expression levels, protein-protein interactions, protein-small molecule interactions or enzymatic activities. In one embodiment, the proteome is analyzed using a screening process that includes measurement of size of certain proteins, typically using mass spectrometry. In one embodiment, the technique to analyze the proteome profile includes hybridization of an antibody to a protein

of interest. For example, the process can include Western blot processes as known in the art or can include column chromatography. The process can also include standard processes such as Elisa screening known in the art. The process can also include binding of nucleic acid modified binding partners, which can be aptamers or can be protein or chemical binding partners for proteins or peptide fragments in the proteome and a screening process can include amplification of the nucleic acids. The process can also include chemical compounds that bind to the proteins or fragments of proteins in a proteome and the process can include measurement of the binding by chemical means. The measurement can also include measurement of reaction products in a chemical reaction, or by activation of a fluorophore. Techniques like mass spectrometry in combination with separation tools such as two-dimensional gel electrophoresis or multidimensional liquid chromatography, can also be used in the process. Typically, the process includes a high throughput screening technique.

The process of the invention can include analyzing the proteome profile using, for example, two-dimensional electrophoresis. This is a method for the separation and identification of proteins in a sample by displacement in two dimensions oriented at right angles to one another. This allows the sample to separate over a larger area, increasing the resolution of each component. The first dimension is typically based on the charge of a particular molecule while the second dimension may be based on the size of a molecule. In the first dimension, proteins are resolved in according to their isoelectric points using immobilized pH gradient electrophoresis (IPGE), isoelectric focusing (IEF), or non-equilibrium pH gradient electrophoresis. Under standard conditions of temperature and urea concentration, the observed focusing points of the great majority of proteins closely approximate the predicted isoelectric points calculated from the proteins' amino acid compositions. Generally, the first step after preparation of a host sample includes running the sample against a pH gradient, a process known as isoelectric focusing. The pH gradients can be generated by adding ampholytes to an acrylamide gel. These are a mixture of amphoteric species with a range of pI values. The pH gradients can also be generated by adding Immobilines, which are similar to ampholytes but have been immobilised within the polyacrylamide gel producing an immobilised pH gradient that does not need to be pre-focused.

The second dimension in two-dimensional electrophoresis may be separation by size of proteins. Proteins may be separated according to their approximate molecular weight using sodium dodecyl sulfate poly-acrylamide-electrophoresis (SDS-PAGE). The technique is widely used and known in the art. The basic idea is to coat proteins with a detergent

(SDS), which coats all proteins in a sample and negatively charges them. The proteins are then subjected to gel electrophoresis. The gels can typically be acrylamide gels and can be in a gradient of density. The charge placed on the gel pushes the proteins through the gel based on size. In two dimensional electrophoresis, the proteins separated can include proteins from at least 10% of the proteome of the organism. More typically, proteins from at least 20%, 30%, 40%, 60%, 80% or 90% of the proteins in the proteome of the host cell are separated and analysed by techniques such as staining of proteins and/or mass spectrometry.

The process of the invention can also include analyzing the proteome profile using a microarray. In this embodiment, the microarray can include binding partners to at least a portion of the proteins expressed by the host cell under appropriate growth conditions, and typically includes binding partners to proteins from at least 5 % of the proteome of the organism. More typically, the microarray includes binding partners to proteins from at least 10%, 20%, 30%, 40%, 60%, 80% or 90% of the proteins in the proteome of the host cell. The binding partners can be antibodies, which can be antibody fragments such as single chain antibody fragments. The binding partners can also include aptamers, which are molecules including nucleic acids that bind to specific proteins or portions of proteins. In a separate embodiment, the microarray can include binding partners for a selected subset of proteins from the proteome, including, for example, putative protease proteins or putative folding modulators. The microarray can typically also include a set of binding partners to proteins that are used as controls. The genetic profile can be analyzed by measuring the binding of the proteins of the host cell expressing the recombinant protein or peptide to the binding partners on the microarray.

The simplest protein array format generally consists of a large number of protein capture reagents bound to defined spots on a planar support material. This array is then exposed to a complex protein sample. The binding of the specific analyte proteins to the individual spots can then be monitored using different approaches. In cases where the analytes have been pre-labeled with a fluorescent dye, the binding can be monitored directly using a fluorescence scanner. Often the classical antibody sandwich type format is used, in which two protein binding reagents simultaneously bind to the same antigen: one antibody is immobilized onto the surface, and the other one is fluorescently labeled or conjugated to an enzyme that can produce a fluorescent, luminescent or colored product when supplied with the appropriate substrate.

Monoclonal antibodies or their antigen-binding fragments are currently one choice for capture agents due to their high specificity, affinity and stability. They have been used in a

variety of classical single analyte protein profiling assays such as enzyme-linked immunosorbent assays (ELISA) since the seventies. Additionally, phage-display libraries of antibody fragments offer the potential for antibody production at proteomic scales. These libraries can be used to isolate high-affinity binding agents against protein identified in a significantly shorter time frame than it is possible with immunization-based processes. Ribosome display and mRNA display are additional, completely *in vitro,* processes that rely on physically linking the library proteins to their encoding mRNA sequences. Such processes have successfully been used to select high-affinity binding reagents to identified proteins (Wilson, DS, et al. (2001) The use of mRNA display to select high-affinity protein-binding peptides *Proc Natl Acad Sci U S A* 98:3750-3755). Several groups have taken a different approach to develop high affinity protein capture reagents for protein biochips. For example, aptamers have been used, which are single stranded RNA or DNA molecules originating from *in vitro* selection experiments (termed SELEX: systematic evolution of ligands by exponential enrichment) with high affinities to proteins. A further development in aptamer technologies are so called photoaptamers. These molecules have an additional attribute that enhances their utility as protein capture reagents. They carry the photoactivatible crosslinking group 5'-bromodeoxyuridine, which, when activated by UV light, can cause covalent crosslinking with bound identified proteins (Petach, H & Gold, L (2002) Dimensionality is the issue: use of photoaptamers in protein microarrays *Curr Opin Biotechnol* 13:309-314). The photo-crosslinking event provides a second dimension of specificity similar to the binding of a secondary detection antibody in a sandwich immunoassay.

A wide variety of surface substrates and attachment chemistries have been evaluated for the immobilization of capture agents on protein microarrays. One way to immobilize proteins on a solid support relies on non-covalent interactions based on hydrophobic or van der Waals interactions, hydrogen bonding or electrostatic forces. Examples of electrostatic immobilization include the use of materials such as nitrocellulose and poly-lysine- or aminopropyl silane-coated glass slides. Protein microarrays were also fabricated by means of physical adsorption onto plastic surfaces of 96-well plates. An example of covalent attachment of proteins to the surface has been described by MacBeath and Schreiber (*MacBeath, G & Schreiber, SL*(2000) Printing proteins as microarrays for high-throughput function determination *Science* 289:1760-1763). Due to the very high affinity of streptavidin to biotin, the immobilization of biotinylated proteins onto streptavidin surfaces can be considered quasi covalent (*Peluso, P et al. (2003)* Optimizing antibody immobilization strategies for the construction of protein microarrays *Anal Biochem* 312:113-124). Further

strategies have been described (Ruiz-Taylor, LA, et al (2001) X-ray photoelectron spectroscopy and radiometry studies of biotin-derivatized poly(L-lysine)-grafted-poly(ethylene glycol) monolayers on metal oxides (*Langmuir*) 7313-7322; Ruiz-Taylor, LA et al. (2001) Monolayers of derivatized poly(L-lysine)-grafted poly(ethylene glycol) on metal oxides as a class of biomolecular interfaces *Proc Natl Acad Sci U S A* 2001, 98:852-857; Espejo A, Bedford MT. (2004) Protein-domain microarrays Processes Mol Biol. 264:173-81; Zhu, H. et al. (2001) Global analysis of protein activities using proteome chips. Science Express).

The samples in the genetic profile can be analyzed individually or grouped into clusters. The clusters can typically be grouped by similarity in gene expression. In one embodiment, the clusters may be grouped individually as proteins that are regulated to a similar extent in a host cell. The clusters may also include groups of proteins that are regulated to a similar extent in a recombinant host cell, for example, that are up-regulated or down-regulated to a similar extent compared to a host cell or a modified or an unmodified cell. The clusters can also include groups related by protein structure, function, or processing. Groups of protein binding partners in an array, or groups of proteins analyzed in a different assay such as two-dimensional electrophoresis can be selected from, but are not limited to: putative or known proteases, co-factors of proteases or protease-like proteins; folding modulators, co-factors of folding modulators or proteins that could improve protein folding or solubility; transcription factors; proteins involved in nucleic acid stability or translational initiation; kinases; extracellular or intracellular receptors; metabolic enzymes; metabolic cofactors; envelope proteins; and housekeeping genes.

Metabolome

Proteomic analysis processes allow the abundance and distribution of many proteins to be determined simultaneously. However, the functional consequences of changes to the proteome are reported only indirectly. Another approach is to measure the levels of these small molecules, or metabolites. A genetic profile analyzed in the process of the invention can thus include a metabolomic profile. Processes for analyzing the metabolome of a specific host include gas chromatography, high-pressure liquid chromatography and capillary electrophoresis to separate metabolites according to various chemical and physical properties. The molecules can then be identified using processes such as mass spectrometry.

Detection/Analysis

The process includes analyzing a genetic profile to identify a compensatory gene or gene product that is expressed at a higher level in the recombinant cell. In general, this step includes the monitoring of the expression (e.g. detecting and or quantifying the expression) of a multitude of genes or gene products. The expression is generally monitored by detecting binding of host cell gene products to a transcriptome, proteome or metabolome profile as described above. The analysis of the binding may involve a comparison of binding between a recombinant host cell expressing recombinant protein or peptide and a naïve host cell or a recombinant host cell not expressing the protein or peptide.

Detection

This step includes the monitoring of the expression (e.g. detecting and or quantifying the expression) of a multitude of genes or gene products. The expression is generally monitored by detecting binding of host cell gene products to a transcriptome, proteome or metabolome profile as described above. Typically, at least about 10 genes, or at least about 100, or at least about 1000 and or at least about 10,000 different genes can be assayed at one time. The process can involve providing a pool of identified nucleic acids comprising RNA transcripts of one or more of said genes, or nucleic acids derived from the RNA transcripts; hybridizing the pool of nucleic acids to an array of oligonucleotide probes immobilized on a surface, where the array comprises more than 100 different oligonucleotides and each different oligonucleotide is localized in a predetermined region of said surface, each different oligonucleotide is attached to the surface through at least one covalent bond, and the oligonucleotide probes are complementary to the RNA transcripts or nucleic acids derived from the RNA transcripts; and quantifying the hybridized nucleic acids in the array. A pictoral representation of one technique for monitoring expression of a gene product between two samples is depicted in Figure 12.

The process can also involve providing a pool of cellular proteins. These can be derived from cellular lysates that are made by lysing cells using detergents or surfactants; using osmotic lysis; using thermal changes, such as freeze-thaw cycles; using mechanical means or using pressure changes. Typically chemicals are included in the process of lysing a cell or cell system that inhibit certain proteins, such as proteases, particularly non-specific proteases, to limit degradation of proteins. In addition, cell lysates are typically kept at or below 4°C, and can be kept at or below 0°C or at or below 20°C during processing. Cell

lysates can be separated before further processing, for example by size exclusion chromatography, ion exchange or affinity matrix chromatography such as by using HPLC.

Typically, the identified genetic product, mRNA, cDNA, protein or metabolite is labeled with a detectable marker or probe. The marker or probe can be one or more fluorescent molecules or fluorophores. These can include commercially available molecules such as Cy3 and Cy5 linked to, for example, particular nucleotides that can be incorporated into a reverse transcribed cDNA to provide detectable molecules for screening. In one embodiment, a first fluorophores is incorporated into a sample from the host and a second fluorophore is incorporated into a sample from a host expressing recombinant protein or peptide. In one embodiment, the first fluorophore and second fluorophore emit different wavelengths of light. In this embodiment, the binding of samples from the host and the host expressing recombinant protein can be monitored in the same assay. In another embodiment, the fluorophores are excited at different wavelengths of light. In another embodiment, the first and second fluorophore are excited or emit light at the same wavelength. In this embodiment, the samples from the host and from the host expressing recombinant protein are typically monitored in different assays.

The process can additionally include a step of quantifying the hybridization of the identified nucleic acids or proteins or chemical metabolites. The quantification can include measurement of the levels of transcription of one or more genes. Typically the pool of identified nucleic acids for example, is one in which the concentration of the identified nucleic acids (pre-mRNA transcripts, mRNA transcripts or nucleic acids derived from the RNA transcripts) is proportional to the expression levels of genes encoding those identified nucleic acids.

For transcriptome analysis, the pool of nucleic acids may be labeled before, during, or after hybridization, although typically the nucleic acids are labeled before hybridization. Fluorescence labels are typically used, often with a single fluorophore, and, where fluorescence labeling is used, quantification of the hybridized nucleic acids can be by quantification of fluorescence from the hybridized fluorescently labeled nucleic acid. Such quantification is facilitated by the use of a confocal laser scanner or fluorescence microscope, such as a confocal fluorescence microscope, which can be equipped with an automated stage to permit automatic scanning of the array, and which can be equipped with a data acquisition system for the automated measurement recording and subsequent processing of the fluorescence intensity information. Devices for reading such arrays include the CloneTracker™, ImaGene™, GeneSight™ modules and the GeneDirector™ database,

available from Biodiscovery, Inc., El Segundo, Calif., or the GeneChip™ reader, available from Affymetrix, Inc. of Santa Clara, Calif. In one embodiment, hybridization occurs at low stringency (e.g. about 20°C to about 50°C, or about 30 °C to about 40 °C, or about 37 °C). Hybridization may include subsequent washes at progressively increasing stringency until a desired level of hybridization specificity is reached.

Quantification of the hybridization signal can be by any means known to one of skill in the art. However, in one embodiment, quantification is achieved by use of a confocal fluorescence scanner. Data is typically evaluated by calculating the difference in hybridization signal intensity between each oligonucleotide probe and its corresponding mismatch control probe. Typically, this difference can be calculated and evaluated for each gene. Certain analytical processes are provided herein.

Techniques have been developed to prepare appropriate bacterial hybridization probes (see for eg. Choi et al. (2003) *App. Envir. Microbio.* 69:4737-4742). For example, cells can be stored in an RNA stabilizing agent such as RNA*later* (Ambion, Austin, TX). RNA is generally purified in three steps: (1) isolation of the total RNA, (2) removal of contaminating DNA and (3) clean-up of the total RNA. Total RNA can be isolated and then mixed with random hexamer primers and reverse transcriptase to make cDNA. Typically at least one fluorescent probe is incorporated into the cDNA. In one embodiment, one fluorescent probe is incorporated, in another embodiment more than one probe, for example 2, 3, 4, 5 or more fluorescent probes are incorporated into the same or different samples of cDNA. In a eukaryotic host, the pool of identified nucleic acids can also be the total polyA$^+$ mRNA isolated from a biological sample, or cDNA made by reverse transcription of the RNA or second strand cDNA or RNA transcribed from the double stranded cDNA intermediate.

Fluorescent dyes are typically incorporated into cDNA molecules during the reverse transcription reaction. Due to the different mRNA structure between prokaryotes (bacteria) and eukaryotes (yeast, mammalian cells, etc.), different primers can be used, however random primers can be used in both cases, and oligo-dT primers can be used in eukaryots, which have polyA tails. An alternative process is amino-allyl labeling to increase the signal intensity. This process incorporates nucleotide analogs featuring a chemically reactive group to which a fluorescent dye may be attached after the reverse transcription reaction (Manduchi, E. et al. (2002) Comparison of different labeling processes for two-channel high-density microarray experiments. *Physiol Genomics* 10:169-79).

The pool of identified nucleic acids can be treated to reduce the complexity of the sample and thereby reduce the background signal obtained in hybridization. The terms

"background" or "background signal" refer to hybridization signals resulting from non-specific binding, or other interactions, between the labeled identified nucleic acids and components of the oligonucleotide array (e.g., the oligonucleotide probes, control probes, the array substrate, etc.). In one approach, a pool of mRNAs, derived from a biological sample, is hybridized with a pool of oligonucleotides comprising the oligonucleotide probes present in the array. The pool of hybridized nucleic acids is then treated with RNase A which digests the single stranded regions. The remaining double stranded hybridization complexes are then denatured and the oligonucleotide probes are removed, leaving a pool of mRNAs enhanced for those mRNAs complementary to the oligonucleotide probes in the array.

In another approach to background reduction, a pool of mRNAs derived from a biological sample is hybridized with paired identified specific oligonucleotides where the paired identified specific oligonucleotides are complementary to regions flanking subsequences of the mRNAs complementary to the oligonucleotide probes in the array. The pool of hybridized nucleic acids is treated with RNase H which digests the hybridized (double stranded) nucleic acid sequences. The remaining single stranded nucleic acid sequences which have a length about equivalent to the region flanked by the paired identified specific oligonucleotides are then isolated (e.g. by electrophoresis) and used as the pool of nucleic acids for monitoring gene expression.

A third approach to background reduction involves eliminating or reducing the representation in the pool of particular preselected identified mRNA messages (e.g., messages that are characteristically overexpressed in the sample). This process involves hybridizing an oligonucleotide probe that is complementary to the preselected identified mRNA message to the pool of polyA$^+$ mRNAs derived from a biological sample. The oligonucleotide probe hybridizes with the particular preselected polyA$^+$ mRNA to which it is complementary. The pool of hybridized nucleic acids is treated with RNase H which digests the double stranded (hybridized) region thereby separating the message from its polyA$^+$ tail. Isolating or amplifying (e.g., using an oligo dT column) the polyA$^+$ mRNA in the pool then provides a pool having a reduced or no representation of the preselected identified mRNA message.

Analysis

The identified gene is typically identified by comparing a genetic profile of the host cell expressing the recombinant protein or peptide to a genetic profile of the host cell not

expressing the recombinant protein or peptide. In iterative embodiments, the identified gene to be modified is identified by comparing a genetic profile of the cell that is to be modified (the second cell) to the cell that it was modified from (the first cell). The identified gene is identified by comparing a genetic profile of the second cell to a genetic profile of the first cell and identifying one or more genes the expression of which is increased in the second cell.

cDNA microarrays measure the relative mRNA abundance between two samples. A series of post-induction time point samples can be compared to the pre-induction sample for the same strain (temporal expression profile), or post-induction samples can be compared with different strains at the same time point. The comparison can be through the use of a computer program, such as GeneSight™. For example, when using a microarray using a fluorescent tag, a spot intensity can be measured for each sample attached to the array (for example a DNA sequence). The spot intensity can then be corrected for background and the ratio of the intensity for samples from the host versus the host expressing the recombinant protein or peptide, or for the host expressing the recombinant protein or peptide compared to the modified host expressing the recombinant protein or peptide can be measured. The ratio provides a measure to identify the genes that are up-regulated or the expression of which is increased upon expression of the recombinant protein or peptide, or upon modification of the host cell to allow identification of a identified gene.

To identify whether a gene is up-regulated, a standard or "cut off" ratio is established. The cut off ratio may be designed to overcome the effects of background noise associated with a particular assay. In general, any ratio of greater than 1 between the measurements can designate an up-regulated gene. However, variation between assays can require a ratio higher than 1, for example 1.5, or more than 2, or more than 2.5, or more than 3, or more than 3.5 or more than 4 or more than 4.5, or more than 5 or more than 6, or more than 7, or more than 8, or more than 9 or more than 10. The standard may be established before the process, relying on standards known in the art, or may be established during measurements by comparing ratios of levels of control genes or gene products, such as housekeeper genes.

Step III: Changing expression of the identified compensatory gene or gene product by genetically modifying the cell to provide a modified recombinant cell that achieves an increase in recombinant protein expression, activity or solubility.

Identified Compensatory Genes

The compensatory genes or gene products that are identified in step ii), or homologous analogues, cofactors or subunits thereof, are used to design strategies to genetically modify the cell to either increase, decrease, knock in or knock out expression of one or more identified genes. The gene sequences identified in public databases can be used to design strategies, particularly to design constructs to modulate expression of a gene by techniques described above. Such techniques are well known.

In one embodiment, the identified gene or genes is at least one putative protease, a protease-like protein, a cofactor or subunit of a protease. In other embodiments, the identified gene or genes is at least one folding modulator, putative folding modulator, cofactor or subunit of a folding modulator. In certain embodiments, a identified gene is a subunit of a protease. In one embodiment, the identified gene or genes can be a serine, threonine, cysteine, aspartic or metallo peptidase. In one embodiment, the identified gene or genes can be selected from *hslV, hslU, clpA, clpB* and *clpX*. The identified gene can also be a cofactor of a protease. In another embodiment, the identified gene or genes is a folding modulator. In some embodiments, the identified gene or genes can be selected from a chaperone protein, a foldase, a peptidyl prolyl isomerase and a disulfide bond isomerase. In some embodiments, the identified gene or genes can be selected from *htpG, cbpA, dnaJ, dnaK* and *fkbP*.

Bacterial genes are organized into operons, which are gene clusters that encode the proteins necessary to perform coordinated function, such as biosynthesis of a given amino acid. Therefore, in one embodiment, the identified gene is part of an operon. In a particular embodiment, the identified gene is in an operon that encodes for one or more proteins with protease activity alone or in combination, or is an operon that encodes for one or more proteins with folding modulator activity, including foldases, chaperones, and isomerases.

Proteases

In one embodiment of the invention, the host cell is modified by reducing expression of, inhibiting or removing at least one protease from the genome. The modification can also be to more than one protease in some embodiments. In a related embodiment, the cell is modified by reducing expression of a protease cofactor or protease protein. In another embodiment, the host cell is modified by inhibition of a promoter for a protease or related protein, which can be a native promoter. The gene modification can be to modulate a protein homologous to the identified identified gene.

In the MEROPS database, peptidases are grouped into clans and families. The families are groups of closely related functionally similar peptidases. Families are grouped by their catalytic type: S, serine; T, threonine; C, cysteine; A, aspartic; M, metallo and U, unknown. Over 20 families (denoted S1 - S27) of serine protease have been identified, these being grouped into 6 clans (SA, SB, SC, SE, SF and SG) on the basis of structural similarity and other functional evidence. Structures are known for four of the clans (SA, SB, SC and SE). Threonine peptidases are characterized by a threonine nucleophile at the N terminus of the mature enzyme. The type example for this clan is the archaean proteasome beta component of Thermoplasma acidophilum. Cysteine peptidases have characteristic molecular topologies and are peptidases in which the nucleophile is the sulphydryl group of a cysteine residue. Cysteine proteases are divided into clans (proteins which are evolutionary related), and further sub-divided into families, on the basis of the architecture of their catalytic dyad or triad:

Clan CA contains the families of papain (C1), calpain (C2), streptopain (C10) and the ubiquitin-specific peptidases (C12, C19), as well as many families of viral cysteine endopeptidases.

Clan CD contains the families of clostripain (C11), gingipain R (C25), legumain (C13), caspase-1 (C14) and separin (C50). These enzymes have specificities dominated by the interactions of the S1 subsite.

Clan CE contains the families of adenain (C5) from adenoviruses, the eukaryotic Ulp1 protease (C48) and the bacterial YopJ proteases (C55).

Clan CF contains only pyroglutamyl peptidase I (C15).

Clan PA contains the picornains (C3), which have probably evolved from serine peptidases and which form the majority of enzymes in this clan.

Clans PB and CH contain the autolytic cysteine peptidases.

Aspartic endopeptidases of vertebrate, fungal and retroviral origin have been characterised . Aspartate peptidases are so named because Asp residues are the ligands of the activated water molecule in all examples where the catalytic residues have been identifed, although at least one viral enzyme is believed to have as Asp and an Asn as its catalytic dyad. All or most aspartate peptidases are endopeptidases. These enzymes have been assigned into clans (proteins which are evolutionary related), and further sub-divided into families, largely on the basis of their tertiary structure.

Metalloproteases are the most diverse of the four main types of protease, with more than 30 families identified to date. In these enzymes, a divalent cation, usually zinc, activates

the water molecule. The metal ion is held in place by amino acid ligands, usually three in number. The known metal ligands are His, Glu, Asp or Lys and at least one other residue is required for catalysis, which may play an electrophillic role. Of the known metalloproteases, around half contain an HEXXH motif, which has been shown in crystallographic studies to form part of the metal-binding site. The HEXXH motif is relatively common, but can be more stringently defined for metalloproteases as abXHEbbHbc, where 'a' is most often valine or threonine and forms part of the S1' subsite in thermolysin and neprilysin, 'b' is an uncharged residue, and 'c' a hydrophobic residue. Proline is never found in this site, possibly because it would break the helical structure adopted by this motif in metalloproteases.

The peptidases associated with clan U- have an unknown catalytic mechanism as the protein fold of the active site domain and the active site residues have not been reported.

Certain proteases (e.g. OmpT) can adsorb to the surface of inclusion bodies and may degrade the desired protein while it is being refolded. Therefore, certain identified proteins can be proteases or protease proteins that adhere to inclusion bodies and these can be modified to, for example, reduce attachment.

Proteases or protease proteins can also be classified as Aminopeptidases; Dipeptidases; Dipeptidyl-peptidases and tripeptidyl peptidases; Peptidyl-dipeptidases; Serine-type carboxypeptidases; Metallocarboxypeptidases; Cysteine-type carboxypeptidases; Omegapeptidases; Serine proteinases; Cysteine proteinases; Aspartic proteinases; Metallo proteinases; or Proteinases of unknown mechanism.

Aminopeptidases include cytosol aminopeptidase (leucyl aminopeptidase), membrane alanyl aminopeptidase, cystinyl aminopeptidase, tripeptide aminopeptidase, prolyl aminopeptidase, arginyl aminopeptidase, glutamyl aminopeptidase, x-pro aminopeptidase, bacterial leucyl aminopeptidase, thermophilic aminopeptidase, clostridial aminopeptidase, cytosol alanyl aminopeptidase, lysyl aminopeptidase, x-trp aminopeptidase, tryptophanyl aminopeptidase, methionyl aminopeptidas, d-stereospecific aminopeptidase, aminopeptidase ey. Dipeptidases include x-his dipeptidase, x-arg dipeptidase, x-methyl-his dipeptidase, cys-gly dipeptidase, glu-glu dipeptidase, pro-x dipeptidase, x-pro dipeptidase, met-x dipeptidase, non-stereospecific dipeptidase, cytosol non-specific dipeptidase, membrane dipeptidase, beta-ala-his dipeptidase. Dipeptidyl-peptidases and tripeptidyl peptidases include dipeptidyl-peptidase i, dipeptidyl-peptidase ii, dipeptidyl peptidase iii, dipeptidyl-peptidase iv, dipeptidyl-dipeptidase, tripeptidyl-peptidase I, tripeptidyl-peptidase II. Peptidyl-dipeptidases include peptidyl-dipeptidase a and peptidyl-dipeptidase b. Serine-type carboxypeptidases include lysosomal pro-x carboxypeptidase, serine-type D-ala-D-ala carboxypeptidase,

carboxypeptidase C, carboxypeptidase D. Metallocarboxypeptidases include carboxypeptidase a, carboxypeptidase B, lysine(arginine) carboxypeptidase, gly-X carboxypeptidase, alanine carboxypeptidase, muramoylpentapeptide carboxypeptidase, carboxypeptidase h, glutamate carboxypeptidase, carboxypeptidase M, muramoyltetrapeptide carboxypeptidase, zinc d-ala-d-ala carboxypeptidase, carboxypeptidase A2, membrane pro-x carboxypeptidase, tubulinyl-tyr carboxypeptidase, carboxypeptidase t. Omegapeptidases include acylaminoacyl-peptidase, peptidyl-glycinamidase, pyroglutamyl-peptidase I, beta-aspartyl-peptidase, pyroglutamyl-peptidase II, n-formylmethionyl-peptidase, pteroylpoly-[gamma]-glutamate carboxypeptidase, gamma-glu-X carboxypeptidase, acylmuramoyl-ala peptidase. Serine proteinases include chymotrypsin, chymotrypsin c, metridin, trypsin, thrombin, coagulation factor Xa, plasmin, enteropeptidase, acrosin, alpha-lytic protease, glutamyl, endopeptidase, cathepsin G, coagulation factor viia, coagulation factor ixa, cucumisi, prolyl oligopeptidase, coagulation factor xia, brachyurin, plasma kallikrein, tissue kallikrein, pancreatic elastase, leukocyte elastase, coagulation factor xiia, chymase, complement component c1r55, complement component c1s55, classical-complement pathway c3/c5 convertase, complement factor I, complement factor D, alternative-complement pathway c3/c5 convertase, cerevisin , hypodermin C, lysyl endopeptidase, endopeptidase la, gamma-reni, venombin ab, leucyl endopeptidase, tryptase, scutelarin, kexin, subtilisin, oryzin, endopeptidase k, thermomycolin, thermitase, endopeptidase SO, T-plasminogen activator, protein C, pancreatic endopeptidase E, pancreatic elastase ii, IGA-specific serine endopeptidase, U-plasminogen, activator, venombin A, furin, myeloblastin, semenogelase, granzyme A or cytotoxic T-lymphocyte proteinase 1, granzyme B or cytotoxic T-lymphocyte proteinase 2, streptogrisin A, treptogrisin B, glutamyl endopeptidase II, oligopeptidase B, limulus clotting factor c, limulus clotting factor, limulus clotting enzyme, omptin, repressor lexa, bacterial leader peptidase I, togavirin, flavirin. Cysteine proteinases include cathepsin B, papain, ficin, chymopapain, asclepain, clostripain, streptopain, actinide, cathepsin l, cathepsin H, calpain, cathepsin t, glycyl, endopeptidase, cancer procoagulant, cathepsin S, picornain 3C, picornain 2A, caricain,ananain, stem bromelain, fruit bromelain, legumain, histolysain, interleukin 1-beta converting enzyme. Aspartic proteinases include pepsin A, pepsin B, gastricsin, chymosin, cathepsin D, neopenthesin, renin, retropepsin, pro-opiomelanocortin converting enzyme, aspergillopepsin I, aspergillopepsin II, penicillopepsin, rhizopuspepsin, endothiapepsin,mucoropepsin, candidapepsin, saccharopepsin, rhodotorulapepsin, physaropepsin, acrocylindropepsin, polyporopepsin, pycnoporopepsin, scytalidopepsin a, scytalidopepsin b, xanthomonapepsin, cathepsin e, barrierpepsin, bacterial

leader peptidase I, pseudomonapepsin, plasmepsin. Metallo proteinases include atrolysin a, microbial collagenase, leucolysin, interstitial collagenase, neprilysin, envelysin, iga-specific metalloendopeptidase, procollagen N-endopeptidase, thimet oligopeptidase, neurolysin, stromelysin 1, meprin A, procollagen C-endopeptidase, peptidyl-lys metalloendopeptidase, astacin, stromelysin, 2, matrilysin gelatinase, aeromonolysin, pseudolysin, thermolysin, bacillolysin, aureolysin, coccolysin, mycolysin, beta-lytic metalloendopeptidase, peptidyl-asp metalloendopeptidase, neutrophil collagenase, gelatinase B, leishmanolysin, saccharolysin, autolysin, deuterolysin, serralysin, atrolysin B, atrolysin C, atroxase, atrolysin E, atrolysin F, adamalysin, horrilysin, ruberlysin, bothropasin, bothrolysin, ophiolysin, trimerelysin I, trimerelysin II, mucrolysin, pitrilysin, insulysin, O-syaloglycoprotein endopeptidase, russellysin, mitochondrial, intermediate, peptidase, dactylysin, nardilysin, magnolysin, meprin B, mitochondrial processing peptidase, macrophage elastase, choriolysin, toxilysin. Proteinases of unknown mechanism include thermopsin and multicatalytic endopeptidase complex.

Certain protease of *P. fluorescens* are listed in Table A.

Table A

Class	Family	RXF	Curated Function	Gene	Physiology
MEROPS Homologs					
Aspartic Peptidases					
	A8 (signal peptidase II family)				
		RXF05383	Lipoprotein signal peptidase (ec 3.4.23.36)		Processing of numerous bacterial secreted lipoproteins.
	A24 (type IV prepilin peptidase family)				
		RXF05379	type 4 prepilin peptidase pild (ec 3.4.99.-)		This membrane-bound peptidase cleaves a specialized leader peptide from type 4 prepilin during its secretion from many bacterial species. Once secreted, the processed proteins are required for functions including type 4 pilus formation, toxin and other enzyme secretion, gene transfer, and biofilm formation.

Cysteine Peptidases					
	C15 (pyroglutamyl peptidase I family)				
		RXF02161	Pyrrolidone-carboxylate peptidase (ec 3.4.19.3)		Removal of pyroglutamyl groups from peptides in protein catabolism.
	C40				
		RXF01968	invasion-associated protein, P60		
		RXF04920	invasion-associated protein, P60		
		RXF04923	phosphatase-associated protein papq		
	C56 (PfpI endopeptidase family)				
		RXF01816	protease I (ec 3.4.-.-)		
Metallopeptidases					
	M1				
		RXF08773	Membrane alanine aminopeptidase (ec 3.4.11.2)		
	M3				
		RXF00561	Oligopeptidase A (ec 3.4.24.70)	*prlC*	Degradation of lipoprotein signal peptides, and other Intracellular oligopeptides. Role in maturation of bacteriophage P22 gp7 precursor.
		RXF04631	Zn-dependent oligopeptidases		
	M4 (thermolysin family)				
		RXF05113	Extracellular metalloprotease precursor (ec 3.4.24.-)		
	M41 (FtsH endopeptidase family)				
		RXF05400	Cell division protein ftsH (ec 3.4.24.-)		Proposed role in proteolytic quality control of regulatory molecules and membrane proteins, in yeast.

	M10				
		RXF04304	Serralysin (ec 3.4.24.40)		
		RXF04500	Serralysin (ec 3.4.24.40)		
		RXF01590	Serralysin (ec 3.4.24.40)		
		RXF04495	Serralysin (ec 3.4.24.40)		
		RXF02796	Serralysin (ec 3.4.24.40)		
	M14 (carboxypeptidase A family)				
		RXF09091	Zinc-carboxypeptidase precursor (ec 3.4.17.-)		
	M16 (pitrilysin family)				
		RXF03441	Coenzyme pqq synthesis protein F (ec 3.4.99.-)		
		RXF01918	zinc protease (ec 3.4.99.-)		
		RXF01919	zinc protease (ec 3.4.99.-)		
		RXF03699	processing peptidase (ec 3.4.24.64)		
	M17 (leucyl aminopeptidase family)				
		RXF00285	Cytosol aminopeptidase (ec 3.4.11.1)		Contributes to bacterial nutrition.
	M18				
		RXF07879	Aspartyl aminopeptidase (ec 3.4.11.21)		
	M20				
		RXF00811	Succinyl-diaminopimelate desuccinylase (ec	*dapE*	

			3.5.1.18)		
		RXF04052	Xaa-His dipeptidase (ec 3.4.13.3)		
		RXF01822	Carboxypeptidase G2 precursor (ec 3.4.17.11)		
		RXF04892	N-acyl-L-amino acid amidohydrolase (ec 3.5.1.14)		
	M28 (aminopeptidase Y family)				
		RXF03488	Alkaline phosphatase isozyme conversion protein precursor (ec 3.4.11.-)		
	M42 (glutamyl aminopeptidase family)				
		RXF05615	Deblocking aminopeptidase (ec 3.4.11.-)		
	M22				
		RXF05817	O-sialoglycoprotein endopeptidase (ec 3.4.24.57)		
		RXF03065	Glycoprotease protein family		
	M23				
		RXF01291	Cell wall endopeptidase, family M23/M37		
		RXF03916	Membrane proteins related to metalloendopeptidases		
		RXF09147	Cell wall endopeptidase, family M23/M37		
	M24				
		RXF04693	Methionine		Probable role in cotranslational

			aminopeptidase (ec 3.4.11.18)		removal of N-terminal methionine.
		RXF03364	Methionine aminopeptidase (ec 3.4.11.18)		Probable role in cotranslational removal of N-terminal methionine.
		RXF02980	Xaa-Pro aminopeptidase (ec 3.4.11.9)		Involved in intracellular protein turnover, in bacteria.
		RXF06564	Xaa-Pro aminopeptidase (ec 3.4.11.9)		
	M48 (Ste24 endopeptidase family)				
		RXF05137	Heat shock protein HtpX		
		RXF05081	Zinc metalloprotease (ec 3.4.24.-)		
	M50 (S2P protease family)				
		RXF04692	Membrane metalloprotease		
Serine Peptidases					
	S1 (chymotrypsin family)				
		RXF01250	protease do (ec 3.4.21.-)		
		RXF07210	protease do (ec 3.4.21.-)		
	S8 (subtilisin family)				
		RXF06755	serine protease (ec 3.4.21.-)		
		RXF08517	serine protease (ec 3.4.21.-)		
		RXF08627	extracellular serine protease (ec 3.4.21.-)		
		RXF06281	Extracellular serine protease precursor (ec 3.4.21.-)		
		RXF08978	extracellular serine		

			protease (ec 3.4.21.-)		
		RXF06451	serine protease (ec 3.4.21.-)		
	S9 (prolyl oligopeptidase family)				
		RXF02003	Protease ii (ec 3.4.21.83)		
		RXF00458	Hydrolase		
	S11 (D-Ala-D-Ala carboxypeptidase A family)				
		RXF04657	D-alanyl-D-alanine-endopeptidase (ec 3.4.99.-)		
		RXF00670	D-alanyl-D-alanine carboxypeptidase (ec 3.4.16.4)		
	S13 (D-Ala-D-Ala peptidase C family)				
		RXF00133	D-alanyl-meso-diaminopimelate endopeptidase (ec 3.4.-.-)		Acts in synthesis and remodelling of bacterial cell walls.
		RXF04960	D-alanyl-meso-diaminopimelate endopeptidase (ec 3.4.-.-)		
	S14 (ClpP endopeptidase family)				
		RXF04567	atp-dependent Clp protease proteolytic subunit (ec 3.4.21.92)	*clpP*	Thought to contribute to elimination of damaged proteins in heat shock.
		RXF04663	atp-dependent Clp protease proteolytic subunit (ec 3.4.21.92)	*clpP*	Thought to contribute to elimination of damaged proteins in heat shock.
	S16 (lon protease family)				
		RXF04653	atp-dependent protease La (ec 3.4.21.53)		Thought to contribute to elimination of damaged proteins in heat shock.
		RXF08653	atp-dependent protease		

			La (ec 3.4.21.53)		
		RXF05943	atp-dependent protease La (ec 3.4.21.53)		
	S24 (LexA family)				
		RXF00449	LexA repressor (ec 3.4.21.88)		
		RXF03397	LexA repressor (ec 3.4.21.88)		
	S26 (signal peptidase I family)				
		RXF01181	Signal peptidase I (ec 3.4.21.89)		Cleaves signal peptides from secreted proteins.
	S33				
		RXF05236	Proline iminopeptidase (ec 3.4.11.5)	*pip3*	
		RXF04802	Proline iminopeptidase (ec 3.4.11.5)	*pip1*	
		RXF04808	Proline iminopeptidase (ec 3.4.11.5)	*pip2*	
	S41 (C-terminal processing peptidase family)				
		RXF06586	Tail-specific protease (ec 3.4.21.-)		
		RXF01037	Tail-specific protease (ec 3.4.21.-)		
	S45				
		RXF07170	Penicillin acylase (ec 3.5.1.11)	*pacB2*	
		RXF06399	Penicillin acylase ii (ec 3.5.1.11)	*pacB1*	
	S49 (protease IV family)				
		RXF06993	possible protease sohb (ec 3.4.-.-)		
		RXF01418	protease iv (ec 3.4.-.-)		
	S58 (DmpA aminopeptidase family)				
		RXF06308	D-aminopeptidase (ec 3.4.11.19)		

Threonine Peptidases					
	T1 (proteasome family)				
		RXF01961	atp-dependent protease hslV (ec 3.4.25.-)	*hslV*	Thought to contribute to elimination of damaged proteins in heat shock.
	T3 (gamma-glutamyltransferase family)				
		RXF02342	Gamma-glutamyltranspeptidase (ec 2.3.2.2)	*ggt1*	
		RXF04424	Gamma-glutamyltranspeptidase (ec 2.3.2.2)	*ggt2*	
Unclassified Peptidases					
	U32				
		RXF00428	protease (ec 3.4.-.-)		
		RXF02151	protease (ec 3.4.-.-)		
	U61				
		RXF04715	Muramoyltetrapeptide carboxypeptidase (ec 3.4.17.13)		
	U62				
		RXF04971	PmbA protein	*pmbA*	The product of the PmbA gene ({*Escherichia coli*}) facilitates the secretion of the antibiotic peptide microcin B17, removing an N-terminal, 26-amino acid leader peptide (Madison et al., 1997).
		RXF04968	TldD protein		
Non MEROPS Proteases					
		RXF00325	Repressor protein C2		
		RXF02689	Microsomal dipeptidase (ec 3.4.13.19)		
		RXF02739	membrane dipeptidase		

			(3.4.13.19)		
		RXF03329	Hypothetical Cytosolic Protein		
		RXF02492	Xaa-Pro dipeptidase (ec 3.4.13.9)		
		RXF04047	caax amino terminal protease family		
		RXF08136	protease (transglutaminase-like protein)		
		RXF09487	Zinc metalloprotease (ec 3.4.24.-)		

Certain proteases of *E. coli* origin are listed in Table B.

Table B

Class	**Family**	**Code**	**Peptidase or homologue (subtype)**	**Gene**
Aspartic Peptidases	**A8**	A08.001	signal peptidase II	*lspA*
	A24A	A24.001	type IV prepilin peptidase 1 (EtpN protein (plasmid p0157))	*etpN*
		A24.001	type IV prepilin peptidase 1 (CofP protein)	*cofP*
		A24.001	type IV prepilin peptidase 1 (HofD protein)	*hofD/hopD/hopO*
		A24.003	type IV prepilin peptidase 2 (HopD protein)	*hopD/ECs4188*
		A24 unassigned	family A24A unassigned peptidases (ORF_F310 protein)	*pppA/ORF_F310*
		A24 unassigned	family A24A unassigned peptidases (PilU protein (plasmid R721))	*pilU*
		A24	family A24A unassigned peptidases	*bfpP/bfpG*

		unassigned	(BfpP protein (plasmid pMAR2))	
		A24 unassigned	family A24A unassigned peptidases (PilU protein)	*PILU*
	A26	A26.001	omptin	*ompT/ECS1663/B0565*
		A26.005	proteinase SopA	*sopA*
Cysteine Peptidases	**C26**	C26 unassigned	family C26 unassigned peptidases	*YCJL/Z2490/ECS1875*
	C40	C40.004	spr g.p. (Escherichia-type) (spr protein)	*spr*
		C40 unassigned	family C40 unassigned peptidases (NlpC protein)	*nlpC/C2104//Z2737/ECS2415*
		C40 unassigned	family C40 unassigned peptidases (YafL protein)	*YafL*
		C40 unassigned	family C40 unassigned peptidases (chitinase 3)	
		C40 unassigned	family C40 unassigned peptidases (YdhO protein)	*ydhO*
	C39	C39.005	colicin V processing peptidase (CvaB protein)	*cvaB*
		C39.005	colicin V processing peptidase (MtfB protein)	*mtfB*
		C39 unassigned	family C39 unassigned peptidases (microcin H47 secretion protein MchF)	*mchF/MCLB*
	C56	C56 unassigned	family C56 unassigned peptidases (YhbO protein)	*yhbo*
		C56 unassigned	family C56 unassigned peptidases (c4536 protein)	*c4536*
Metallopeptidases	**M1**	M01.005	alanyl aminopeptidase (proteobacteria)	*pepN*
	M3A	M03.004	oligopeptidase A	*prlC/opdA*
		M03.005	peptidyl-dipeptidase Dcp	*dcp/Z2160/ECS21*

				47
		M03.005	peptidyl-dipeptidase Dcp	*dcp*
	M41	M41.001	FtsH endopeptidase	*hflB/ftsH/ECS405 7*
	M66	M66.001	StcE protease	*stcE*
	M15D	M15 unassigned	subfamily M15D unassigned peptidases (VanX protein)	*ddpX/vanX/B1488/ Z2222/ECS2092*
	M16A	M16.001	pitrilysin	*ptr/ECs3678*
	M16B	M16 unassigned	subfamily M16B unassigned peptidases (PqqL protein)	*pqqL/yddC*
	M17	M17.003	aminopeptidase A (bacteria)	*pepA/xerB*
		M17.004	PepB aminopeptidase	*pepB/Z3790/ECS3 389*
	M24A	M24.001	methionyl aminopeptidase 1	*map*
	M24B	M24.003	X-Pro dipeptidase (bacteria)	*pepQ/ECs4775*
		M24.004	aminopeptidase P (bacteria)	*pepP*
		M24 unassigned	subfamily M24B unassigned peptidases (YqhT protein)	*yqhT/ypdF/B2385/ c2924*
	M20A	M20.010	DapE peptidase (succinyl-diaminopimelate desuccinylase)	*dapE/msgB/C2999*
		M20 unassigned	subfamily M20A unassigned peptidases (YgeY protein)	*ygey*
	M20B	M20.003	peptidase T	*pepT/Z1832/ECS1 572*
	M20C	M20.007	X-His dipeptidase	*pepD/pepH/ECs02 64*
	M20D	M20 unassigned	family M20D unassigned peptidases (YdaJ protein)	*ydaJ/ECs1922*
	M28A	M28 unassigned	subfamily M28A unassigned peptidases (YfbL protein)	*yfbL*
	M28C	M28.005	IAP aminopeptidase	*iap*
	M42	M42 unassigned	family M42 unassigned peptidases (YjhO protein)	*yjhO*

		M42 unassigned	family M42 unassigned peptidases (FrvX protein)	*frvX*
		M42 unassigned	family M42 unassigned peptidases (FrvX protein)	*frvX/b2384/ypdE*
	M38	M38.001	beta-aspartyl dipeptidase	*iadA*
	M22	M22.001	O-sialoglycoprotein endopeptidase	*ygjD*
		M22.002	yeaZ protein	*yeaZ/C2211/Z2850/ECS2516*
	M23B	M23.006	YibP peptidase (YibP protein)	*yibP*
		M23 unassigned	subfamily M23B unassigned peptidases (YebA protein)	*yebA*
	M48B	M48.002	HtpX endopeptidase	*HtpX*
		M48 unassigned	subfamily M48B unassigned peptidases	*YGGG/C3521*
		M48 unassigned	subfamily M48B unassigned peptidases	*YFGC/C3011*
		M48 unassigned	subfamily M48B unassigned peptidases (YggG protein)	*YggG/Z4280/ECS3811*
		M48 unassigned	subfamily M48B unassigned peptidases (YcaL protein)	*ycaL/C1047/Z1255/ECS0992*
	M50A	M50.004	YaeL protease (YAEL protein)	*ecfE/YAEL/B0176/Z0187/ECS0178/C0213*
	M52	M52.001	HybD endopeptidase (HybD protein)	*hybD/ECS3878*
		M52.002	HyaD endopeptidase (HyaD protein)	*hyaD*
		M52.003	HycI endopeptidase (HycI protein)	*hycI/C3277*
Serine Peptidases	**S1B**	S01.260	B1598 endopeptidase	*b1598*
	S1C	S01.273	protease Do	*htrA/degP*
		S01.274	DegQ	*hhoA/degQ/ECS4107/Z4593*
		S01.275	DegS	*hhoB/degS*
	S6	S06.002	EspP g.p. (*Escherichia coli*)	*espP/pssA*

		S06.003	Tsh peptidase (*Escherichia coli*) (Tsh protein)	*tsh/hbp*
		S06.003	Tsh peptidase (*Escherichia coli*)	*c0393*
		S06.004	Pet endopeptidase	*sat*
		S06.004	Pet endopeptidase	
		S06.005	Pic endopeptidase (Shigella flexneri)	*she/pic*
		S6 unassigned	family S6 unassigned peptidases (eatA protein)	*eatA*
		S6 unassigned	family S6 unassigned peptidases (c0350 protein)	*c0350*
		S6 unassigned	family S6 unassigned peptidases (EspC protein)	*espC*
		S6 unassigned	family S6 unassigned peptidases (epeA protein)	*epeA*
		S6 unassigned	family S6 unassigned peptidases	
	S8A	S8 unassigned	subfamily S8A unassigned peptidases	
	S9A	S09.010	oligopeptidase B	*ptrB*
		S09.010	oligopeptidase B	*ptrB/C2255*
	S9X	S9 unassigned	family S9 unassigned peptidases	*YFHR/C3060/b2534/Z3802*
	S11	S11.002	murein-DD-endopeptidase	*pbpG*
		S11.003	penicillin-binding protein 6	*dacC/Z1066/ECS0919*
		S11.003	penicillin-binding protein 6 (penicillin-binding protein pbp-6B)	*dacD/phsE/ECs2812*
		S11.003	penicillin-binding protein 6	*dacA*
	S12	S12 unassigned	family S12 unassigned peptidases (c2452 protein)	*c2452*
		S12 unassigned	family S12 unassigned peptidases (YaiH protein)	*yaiH/C0480*
	S13	S13.001	D-Ala-D-Ala peptidase C	*dacB/ECs4061*

	S14	S14.001	endopeptidase Clp (type 1)	*clpP/lopP/ECS0491*
		S14 unassigned	family S14 unassigned peptidases (ECs0829 protein)	*Z0967/ECS0829*
		S14 unassigned	family S14 unassigned peptidases (ECs2960 protein)	*H0022/Z2112/ECS2960/L34*
	S16	S16.001	lon protease	*lon/deg/ECs0493*
		S16 unassigned	family S16 unassigned peptidases (ECS1039 protein)	*lonB/Z1305/ECS1039*
		S16 unassigned	family S16 unassigned peptidases (c1091 protein)	*c1091*
	S24	S24.001	repressor LexA (LexA protein)	*lexA/exrA*
		S24.003	UmuD protein	
		S24.003	UmuD protein	*umuD/C1631*
	S26	S26A	S26.001	*signal peptidase I*
		S26.014	traF plasmid-transfer protein (TraF protein)	*traF*
	S33	S33 unassigned	family S33 unassigned peptidases (BioH protein)	*bioH/C4189//Z4767/ECS4255*
	S41A	S41.001	C-terminal processing protease-1	*prc/tsp/ECS2540/Z2877//C2239*
	S45	S45.001	penicillin G acylase precursor	*pac*
	S49	S49.001	protease IV	*sppA/ECs2472//C2170*
		S49.002	sohB endopeptidase	*sohB/ECS1844/Z2538//C1737*
	S51	S51.001	dipeptidase E	*pepE*
	S54	S54 unassigned	family S54 unassigned peptidases (c0741 protein)	*c0741*
		S54 unassigned	family S54 unassigned peptidases (glycerophosphate dehydrogenase)	*glpG/C4201//Z4784/ECS4267*
Threonine Peptidases	**T1B**	T01.006	HslV component of HslUV peptidase	*hslV*

	T2	T02.002	asparaginase	*ybiK/Z1051m/C09 13*
	T3	T03.001	gamma-glutamyltransferase 1 (bacterial)	*ggt/C4236*
	S41A	S41.001	C-terminal processing protease-1	*prc/tsp/ECS2540/ Z2877//C2239*
Unclassified Peptidases	**U6**	U06.001	murein endopeptidase	*mepA/ECs3212//C 2874*
		U32	U32 unassigned	*family U32 unassigned peptidases (YdcP protein)*
		U32 unassigned	family U32 unassigned peptidases (YegQ protein)	*yegQ/C2611*
		U32 unassigned	family U32 unassigned peptidases (YhbU protein)	*YHBU/C3911/Z45 19/ECS4039*
		U35	U35 unassigned	*family U35 unassigned peptidases*
		U35 unassigned	family U35 unassigned peptidases (ECs4973 protein)	*ECs4973*
		U49	U49.001	*Lit protease (Escherichia coli)*
		U61	U61.001	*muramoyl-tetrapeptide carboxypeptidase*
		U61 unassigned	family U61 unassigned peptidases (MccF protein)	*mccF*
		U62	U62.001	*microcin-processing peptidase 1*
		U62.002	microcin-processing peptidase 2	*tldD/ECs4117*
		M9G.035	endopeptidase ECP 32 (*Escherichia*	

			coli)	

Certain proteases of S. cerevisiae origin are listed in Table C.

Table C

Class	Family	Code	Peptidase or homologue (subtype)	Gene
Aspartic Peptidases	**A1**	A01.015	barrierpepsin	*bar1*
		A01.018	saccharopepsin	*pep4/pho9*
		A01.030	yapsin 1	*yap3*
		A01.031	yapsin 2	*mkc7*
		A01.035	yapsin 3	*YPS3*
		A01.UPW	family A1 unassigned peptidases	*YPS7/D9476.8/ YDR349C*
		A01.UPW	family A1 unassigned peptidases (YIR039C protein)	*YIR039C*
	A2D	A02.022	Ty3 transposon (Saccharomyces cerevisiae) endopeptidase (retrotransposon Ty3-1)	*POL3/TY3-2 orfB/ TY3B*
	A11B	A11.003	Ty1 transposon (Saccharomyces cerevisiae) endopeptidase (transposon Ty1-17 protein B)	*Ty1B*
		A11.003	Ty1 transposon (Saccharomyces cerevisiae) endopeptidase (transposon Ty1 protein B)	*Ty1B*
		A11.003	Ty1 transposon (Saccharomyces cerevisiae) endopeptidase (transposon Ty1 protein B)	*Ty1B*
	A11X	A11.UPW	family A11 unassigned peptidases (retrotransposon Ty4)	
	A22B	A22.008	YKL100c protein (Saccharomyces cerevisiae)	*YKL100c*
Cysteine Peptidases	**C1B**	C01.085	bleomycin hydrolase (yeast)	*GAL6/YCP1/ LAP3*
	C2	C02.008	calpain-7	*YMR154C/Cpl1/ Rim13*

	C12	C12.002	ubiquitinyl hydrolase YUH1	*yuh1*
	C13	C13.005	glycosylphosphatidylinositol:protein transamidase	*d9798.2*
	C19	C19.002	Ubp1 ubiquitin peptidase	*ubp1*
		C19.003	Ubp2 ubiquitin peptidase	*ubp2*
		C19.004	Ubp3 ubiquitin peptidase	*ubp3*
		C19.005	Doa4 ubiquitin peptidase	*DOA4*
		C19.006	Ubp5 ubiquitin peptidase	*ubp5*
		C19.079	UBP6 (Saccharomyces cerevisiae) (YFR010W protein)	*yfr010w*
		C19.UPW	family C19 unassigned peptidases (YNL186W protein)	*YNL186W*
		C19.UPW	family C19 unassigned peptidases (UBP9)	*ubp9*
		C19.UPW	family C19 unassigned peptidases (YBL067C protein)	*YBL067C*
		C19.UPW	family C19 unassigned peptidases (YBR058C protein)	*UBP12/YBR058C*
		C19.UPW	family C19 unassigned peptidases (ubiquitin carboxy-terminal hydrolase 16)	*UBP16/YPL072W/ LPF12W*
		C19.UPW	family C19 unassigned peptidases (YMR304W protein)	*YMR304W/ ym9952.06*
		C19.UPW	family C19 unassigned peptidases (YMR223W protein)	*YMR223W/ ym9959.05*
		C19.UPW	family C19 unassigned peptidases (UBP7)	*ubp7*
		C19.UPW	family C19 unassigned peptidases (UBP13)	*ubp13*
	C44	C44.971	glucosamine-fructose-6-phosphate aminotransferase	
		C44.971	glucosamine-fructose-6-phosphate aminotransferase (glucosamine-fructose-6-phosphate aminotransferase)	*gfa1*
	C48	C48.001	Ulp1 endopeptidase	*YPL020c*
		C48.005	Ulp2 endopeptidase (Smt4p protein)	*SMT4*

	C50	C50.001	separase	*ESP1/YGR098C*
	C54	C54.001	ATG4 peptidase (Saccharomyces cerevisiae)	*Apg4/Aut2*
	C56	C56.004	YDR533C g.p. (Saccharomyces cerevisiae)	*YDR533C/D9719.36*
		C56.UPW	family C56 unassigned peptidases (YPL280W protein)	*YPL280W*
		C56.UPW	family C56 unassigned peptidases (YOR391C protein)	*YOR391C*
	I34	I34.001	saccharopepsin inhibitor	*PAI3/YMR174C/ YM8010*
Metallopeptidases	**M1**	M01.006	Ape2 aminopeptidase	*lap1/ape2*
		M01.007	Aap1' aminopeptidase	*AAP1*
		M01.007	Aap1' aminopeptidase	
		M01.017	Yin7 g.p. (Saccharomyces cerevisiae)	*yil137C*
		M01.UPW	family M1 unassigned peptidases (ynl045w protein)	*ynl045w*
	M3A	M03.003	saccharolysin	*prd1*
		M03.006	mitochondrial intermediate peptidase	*MIP1*
	M16A	M16.007	Axl1 peptidase	*axl1*
		M16.008	Ste23 peptidase	*ste23*
		M16.UPA	subfamily M16A unassigned peptidases (orf1 protein)	*orf1*
	M16B	M16.003	mitochondrial processing peptidase beta-subunit (beta)	*mas1/mif1*
	M16C	M16.UPC	subfamily M16C unassigned peptidases (YDR430C protein)	*YDR430C*
		M16.UPC	subfamily M16C unassigned peptidases (YOL098C protein)	*YOL098C*
	M16X	M16.971	mitochondrial processing peptidase non-peptidase alpha subunit (alpha)	*mas2/mif2*
		M16.974	UCR2_HUMAN (ubiquinol-cytochrome c reductase core protein 2)	*ucr2/cor2/qcr2*
	M18	M18.001	aminopeptidase I	*ape1/lap4*
		M18.UPW	family M18 unassigned peptidases	*YHR113W*

			(YHR113W protein)	
	M20A	M20.005	cytosolic nonspecific dipeptidase	*YFR044C*
	M20E	M20.002	Gly-X carboxypeptidase	*cps1/cps*
		M20.002	Gly-X carboxypeptidase (pseudogene; deduced from nucleotide sequence by MEROPS)	*AOE110, AOE264, AOE130*
	M22	M22.003	Mername-AA017 peptidase (YKR038C protein)	*YKR038C*
		M22.UPW	family M22 unassigned peptidases (QRI7 protein)	*QRI7*
	M24A	M24.001	methionyl aminopeptidase 1	*map1*
		M24.002	methionyl aminopeptidase 2	*ybl091c*
	M24B	M24.009	aminopeptidase P1	*YLL029w*
		M24.026	aminopeptidase P homologue (YER078C protein)	*YER078C*
		M24.UPB	subfamily M24B unassigned peptidases (YFR006W protein)	*yfr006w*
	M28A	M28.001	aminopeptidase Y	*ape3*
	M28E	M28.006	Mername-AA063 peptidase (YDR415c protein)	*YDR415c*
	M28X	M28.974	glutaminyl cyclase	*YFR018C*
		M28.UPW	family M28 unassigned peptidases (YBR074W protein)	*YBR074W*
	M41	M41.002	Afg3 g.p. (Saccharomyces cerevisiae) (AGF3 protein)	*agf3/yta10*
		M41.003	m-AAA protease (RCA1 protein)	*rca1/yta12*
		M41.004	i-AAA protease	*yme1/yta11/osd1*
	M48A	M48.001	Ste24 endopeptidase	*STE24*
	M48B	M48.018	Oma1 endopeptidase (Saccharomyces cerevisiae) (YKR087C protein)	*YKR087C/YKR407*
	M49	M49.001	dipeptidyl-peptidase III	*YOL057W*
		M49.UPW	family M49 unassigned peptidases	
	M67A	M67.001	Poh1 peptidase	*RPN11/MPR1/ YFR004W*

		M67.002	Jab1/MPN domain metalloenzyme	*YDL216c/D0888*
		M67.973	26S proteasome non-ATPase regulatory subunit 7	*RPN8/YOR261C*
Serine Peptidases	**S1C**	S01.434	Nma111 endopeptidase (Saccharomyces cerevisiae) (YNL123W protein)	*ynl123w*
	S8A	S08.052	cerevisin	*prb1*
		S08.UPA	subfamily S8A unassigned peptidases (YSP3 protein)	*YSP3*
		S08.UPA	subfamily S8A unassigned peptidases (YCR54C protein)	*YCR54C*
	S8B	S08.070	kexin	*kex2*
	S9B	S09.005	dipeptidyl aminopeptidase A	*ste13/yci1*
		S09.006	dipeptidyl aminopeptidase B (fungus)	*dap2*
	S9X	S09.UPW	family S9 unassigned peptidases (Ynl320w protein)	*YNL320W*
	S10	S10.001	carboxypeptidase Y	*prc1*
		S10.007	kex carboxypeptidase	*kex1*
		S10.UPW	family S10 unassigned peptidases (YBR139W protein)	*ybr139W*
	S16	S16.002	PIM1 endopeptidase	*lon/pim1*
	S26A	S26.002	mitochondrial inner membrane protease 1 (1)	*imp1*
		S26.012	mitochondrial inner membrane protease 2 (2)	*imp2*
	S26B	S26.010	signalase (eukaryote) 21 kDa component	*sec11*
		S33.UPW	family S33 unassigned peptidases	*ECM18/YDR125C*
		S33.UPW	family S33 unassigned peptidases	*ECM18/YDR125C*
	S54	S54.007	Pcp1 protein (Saccharomyces cereviseae) (YGR101W protein)	*YGR101W*
	S59	S59.001	nucleoporin 145	*Nup145*
Threonine Peptidases	**T1A**	T01.010	proteasome catalytic subunit 1	*pre3*

		T01.011	proteasome catalytic subunit 2	*pup1*
		T01.012	proteasome catalytic subunit 3	*pre2/prg1*
		T01.983	proteasome subunit beta 3	*pup3*
		T01.984	proteasome subunit beta 2	*pre1*
		T01.986	proteasome subunit beta 1	*pre7/prs3*
		T01.987	proteasome subunit beta 4	*pre4*
	T1X	T01.971	proteasome subunit alpha 6	*prs2/prc2*
		T01.972	proteasome subunit alpha 2	*pre8/prs4*
		T01.973	proteasome subunit alpha 4	*pre9/prs5*
		T01.974	proteasome subunit alpha 7	*pre6*
		T01.975	proteasome subunit alpha 5	*pup2*
		T01.976	proteasome subunit alpha 1	*pre5*
		T01.977	proteasome subunit alpha 3	*pre10/prs1/prc1*
	T3	T03.012	gamma-glutamyltransferase (Saccharomyces) (YLR299w protein)	*L8003.4*
	T5	T05.001	ornithine acetyltransferase precursor	*arg7/emc40/ YMR062C*
Unclassified Peptidases	**U48**	U48.001	prenyl protease 2	*rce1*

Folding Modulators

The identified up-regulated genes or gene products can be one or more folding modulator. Folding modulators can for example be HSP70 proteins, HSP110/SSE proteins, HSP40 (DNAJ-related) proteins, GRPE-like proteins, HSP90 proteins, CPN60 and CPN10 proteins, Cytosolic chaperonins, HSP100 proteins, Small HSPs, Calnexin and calreticulin, PDI and thioredoxin-related proteins, Peptidyl-prolyl isomerases, Cyclophilin PPIases, FK-506 binding proteins, Parvulin PPIases, Individual chaperonins, Protein specific chaperones, or intramolecular chaperones. Folding modulators are generally described in "Guidebook to Molecular Chaperones and Protein-Folding Catalysts" (1997) ed. M. Gething, Melbourne University, Australia.

The best characterized molecular chaperones in the cytoplasm of *E. coli* are the ATP-dependent DnaK-DnaJ-GrpE and GroEL-GroES systems. Based on *in vitro* studies and homology considerations, a number of additional cytoplasmic proteins have been proposed to function as molecular chaperones in *E. coli*. These include ClpB, HtpG and IbpA/B, which,

like DnaK-DnaJ-GrpE and GroEL-GroES, are heat-shock proteins (Hsps) belonging to the stress regulon. The *trans* conformation of X–Pro bonds is energetically favored in nascent protein chains; however, ~5% of all prolyl peptide bonds are found in a *cis* conformation in native proteins. The *trans* to *cis* isomerization of X–Pro bonds is rate limiting in the folding of many polypeptides and is catalyzed *in vivo* by peptidyl prolyl *cis/trans* isomerases (PPIases). Three cytoplasmic PPIases, SlyD, SlpA and trigger factor (TF), have been identified to date in *E. coli*. TF, a 48 kDa protein associated with 50S ribosomal subunits that has been postulated to cooperate with chaperones in *E. coli* to guarantee proper folding of newly synthesized proteins. At least five proteins (thioredoxins 1 and 2, and glutaredoxins 1, 2 and 3, the products of the *trxA*, *trxC*, *grxA*, *grxB* and *grxC* genes, respectively) are involved in the reduction of disulfide bridges that transiently arise in cytoplasmic enzymes. Thus, identified genes can be disulfide bond forming proteins or chaperones that allow proper disulfide bond formation.

Certain folding modulators in *P. fluorescens* are listed in Table D.

Table D

RXF	gene	function	Family
GroES/EL			
rxf02095	*groES*	chaperone	Hsp10
rxf06767::rxf02090	*groEL*	chaperone	Hsp60
RXF01748	*ibpA*	Small heat-shock protein (sHSP) IbpA PA3126;Acts as a holder for GroESL folding	Hsp20
RXF03385	*hscB*	Chaperone protein hscB	Hsp20
Hsp70 (DnaK/J)			
rxf05399	*dnaK*	chaperone	Hsp70
RXF06954	*dnaK*	chaperone	Hsp70
RXF03376	*hscA*	chaperone	Hsp70
RXF03987	*cbpA*	Curved dna-binding protein, dnaJ like activity	Hsp40
RXF05406	*dnaJ*	Chaperone protein dnaJ	Hsp40
RXF03346	*dnaJ*	Molecular chaperones (DnaJ family)	Hsp40
Hsp100 (Clp/Hsl)			
RXF04587	*clpA*	atp-dependent clp protease atp-binding subunit clpA	Hsp100
RXF08347	*clpB*	ClpB protein	Hsp100

RXF04654	*clpX*	atp-dependent clp protease atp-binding subunit clpX	Hsp100
RXF01957	*hslU*	atp-dependent hsl protease atp-binding subunit hslU	Hsp100
RXF01961	*hslV*	atp-dependent hsl protease atp-binding subunit hslV	Hsp100
Hsp33			
RXF04254	*yrfI*	33 kDa chaperonin (Heat shock protein 33 homolog) (HSP33).	Hsp33
Hsp90			
RXF05455	*htpG*	Chaperone protein htpG	Hsp90
SecB			
RXF02231	*secB*	secretion specific chaperone SecB	SecB
Disulfide Bond Isomerases			
RXF07017	*dsbA*	disulfide isomerase	DSBA oxidoreductase
RXF08657	*dsbA/dsbC/dsbG/fernA*	disulfide isomerase	DSBA oxidoreductase
rxf01002	*dsbA/dsbC*	disulfide isomerase	DSBA oxidoreductase/Thioredoxin
rxf03307	*dsbC*	disulfide isomerase	glutaredoxin/Thioredoxin
rxf04890	*dsbG*	disulfide isomerase	glutaredoxin/Thioredoxin
Peptidyl-prolyl cis-trans isomerases			
RXF03768	*ppiA*	Peptidyl-prolyl cis-trans isomerase A (ec 5.2.1.8)	PPIase: cyclophilin type
RXF05345	*ppiB*	Peptidyl-prolyl cis-trans isomerase B.	PPIase: cyclophilin type
RXF06034	*fklB*	Peptidyl-prolyl cis-trans isomerase FklB.	PPIase: FKBP type
RXF06591	*fklB/fkbP*	fk506 binding protein Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	PPIase: FKBP type
RXF05753	*fklB;fkbP*	Peptidyl-prolyl cis-trans isomerase (ec 5.2.1.8)	PPIase: FKBP type
RXF01833	*slyD*	Peptidyl-prolyl cis-trans isomerase SlyD.	PPIase: FKBP

			type
RXF04655	*tig*	Trigger factor, ppiase (ec 5.2.1.8)	PPIase: FKBP type
RXF05385	*yaad*	Probable FKBP-type 16 kDa peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8) (PPiase) (Rotamase).	PPIase: FKBP type
RXF00271		Peptidyl-prolyl cis-trans isomerase (ec 5.2.1.8)	PPIase: FKBP type
pili assembly chaperones (papD like)			
RXF06068	*cup*	Chaperone protein cup	pili assembly papD
RXF05719	*ecpD*	Chaperone protein ecpD	pili assembly papD
RXF03406	*ecpD; csuC*	Chaperone protein ecpD	pili assembly papD
RXF04296	*ecpD; cup*	Chaperone protein ecpD	pili assembly papD
RXF04553	*ecpD; cup*	Chaperone protein ecpD	pili assembly papD
RXF04554	*ecpD; cup*	Chaperone protein ecpD	pili assembly papD
RXF05310	*ecpD; cup*	Chaperone protein ecpD	pili assembly papD
RXF05304	*ecpD; cup*	Chaperone protein ecpD	pili assembly papD
RXF05073	*gltF*	Gram-negative pili assembly chaperone periplasmic function	pili assembly papD

Certain folding modulators in *E. coli* are listed in Table E.

Table E

Uniprot Accession	**Uniprot ID**	**Annotation**	**Family**
GroES/EL			
P05380	CH10_ECOLI	10 kDa chaperonin	Hsp10
P06139	CH60_ECOLI	60 kDa chaperonin	Hsp60
Hsp70 (DnaK/J)			
P04475	DNAK_ECOLI	Chaperone protein dnaK	Hsp70

P77319	HSCC_ECOLI	Chaperone protein hscC	Hsp70
P36659	CBPA_ECOLI	Curved DNA-binding protein cbpA	Hsp40
P31680	DJLA_ECOLI	DnaJ-like protein djlA, rscG	Hsp40
P08622	DNAJ_ECOLI	Chaperone protein dnaJ	Hsp40
P29131	FTSN_ECOLI	Cell division protein ftsN	Hsp40
P09372	GRPE_ECOLI	GrpE protein	GrpE
P31658	HCHA_ECOLI	Chaperone protein hchA	Hsp31
Hsp100 (Clp/Hsl)			
P15716	CLPA_ECOLI	ATP-dependent Clp protease ATP-binding subunit clpA	Hsp100
P03815	CLPB_ECOLI	ClpB protein	Hsp100
P33138	CLPX_ECOLI	ATP-dependent Clp protease ATP-binding subunit clpX	Hsp100
P32168	HSLU_ECOLI	ATP-dependent hsl protease ATP-binding subunit hslU, clpY	Hsp100
Small Heat Shock Proteins			
P29209	IBPA_ECOLI	16 kDa heat shock protein A.	Hsp16
P29210	IBPB_ECOLI	16 kDa heat shock protein B.	Hsp16
Not Part of a Larger Group			
P36662	TORD_ECOLI	Chaperone protein torD	TorD
P15040	SECB_ECOLI	Protein-export protein secB	SecB
P45803	HSLO_ECOLI	33 kDa chaperonin	Hsp33
P10413	HTPG_ECOLI	Chaperone protein htpG	Hsp90
HscAB			
P36541	HSCA_ECOLI	Chaperone protein hscA	Hsp66
P36540	HSCB_ECOLI	Co-chaperone protein hscB	Hsp20
Lipoprotein Carrier Protein			
P61316	LOLA_ECOLI	Outer-membrane lipoprotein carrier protein precursor	LolA
P61320	LOLB_ECOLI	Outer-membrane lipoprotein lolB precursor	LolB
Disulfide Bond Isomerases			
P24991	DSBA_ECOLI	Thiol:disulfide interchange protein dsbA precursor.	
P30018	DSBB_ECOLI	Disulfide bond formation protein B	Disulfide Bond Oxidoreductase

P21892	DSBC_ECOLI	Thiol:disulfide interchange protein dsbC precursor.	
P36655	DSBD_ECOLI	Thiol:disulfide interchange protein dsbD precursor (EC 1.8.1.8) (Protein-disulfide reductase)	
P33926	DSBE_ECOLI	Thiol:disulfide interchange protein dsbE (Cytochrome c biogenesis protein ccmG).	
P77202	DSBG_ECOLI	Thiol:disulfide interchange protein dsbG precursor	Disulfide Bond Oxidoreductase
Peptidyl-prolyl cis-trans isomerases			
P22257	TIG_ECOLI	Trigger factor	PPIase: FKBP type
P45523	FKBA_ECOLI	FKBP-type peptidyl-prolyl cis-trans isomerase fkpA precursor	PPIase: FKBP type
P39311	FKBB_ECOLI	FKBP-type 22 kDa peptidyl-prolyl cis-trans isomerase	PPIase: FKBP type
P22563	FKBX_ECOLI	FKBP-type 16 kDa peptidyl-prolyl cis-trans isomerase	PPIase: FKBP type
P30856	SLYD_ECOLI	FKBP-type peptidyl-prolyl cis-trans isomerase slyD	PPIase: FKBP type
P20752	PPIA_ECOLI	Peptidyl-prolyl cis-trans isomerase A precursor	PPIase: Cyclophilin type
P23869	PPIB_ECOLI	Peptidyl-prolyl cis-trans isomerase B	PPIase: Cyclophilin type
P39159	PPIC_ECOLI	Peptidyl-prolyl cis-trans isomerase C	PPIase: PPIC type
P77241	PPID_ECOLI	Peptidyl-prolyl cis-trans isomerase D	PPIase: PPIC type
P21202	SURA_ECOLI	Survival protein surA precursor	PPIase: Parvulin type
pili assembly chaperones (papD like)			
P53516	AFAB_ECOLI	Chaperone protein afaB precursor	Pili Assembly PapD
P33128	ECPD_ECOLI	Chaperone protein ecpD precursor	Pili Assembly PapD
P31697	FIMC_ECOLI	Chaperone protein fimC precursor	Pili Assembly PapD
P77249	SFMC_ECOLI	Chaperone protein sfmC precursor	Pili Assembly PapD
P75749	YBGP_ECOLI	Hypothetical fimbrial chaperone ybgP precursor	Pili Assembly PapD
P40876	YCBF_ECOLI	Hypothetical fimbrial chaperone ycbF precursor	Pili Assembly PapD
P75856	YCBR_ECOLI	Hypothetical fimbrial chaperone ycbR precursor	Pili Assembly PapD
P33342	YEHC_ECOLI	Hypothetical fimbrial chaperone yehC precursor	Pili Assembly PapD
P77599	YFCS_ECOLI	Hypothetical fimbrial chaperone yfcS precursor	Pili Assembly PapD

P28722	YHCA_ECOLI	Hypothetical fimbrial chaperone yhcA precursor	Pili Assembly PapD
P77616	YQIH_ECOLI	Hypothetical fimbrial chaperone yqiH precursor	Pili Assembly PapD
P42914	YRAI_ECOLI	Hypothetical fimbrial chaperone yraI precursor	Pili Assembly PapD

Certain folding modulators of *S. cervisia* are shown in table F.

Table F

Uniprot Accession	**Uniprot ID**	**GO Source**	**Annotation**	**Family**
GroES/EL				
P19882	HS60_YEAST	GOA:interpro	Heat shock protein 60, mitochondrial precursor	Hsp60
P38228	TC62_YEAST	GOA:interpro	Mitochondrial chaperone TCM62	Hsp60
P38910	CH10_YEAST	GOA:interpro	10 kDa heat shock protein, mitochondrial	Hsp10
Hsp70 (DnaK/J)				
P25491	MAS5_YEAST	GOA:interpro	Mitochondrial protein import protein MAS5, Ydj1	Hsp40
P10591	HS71_YEAST	PMID:9789005	Heat shock protein SSA1	Hsp70
P10592	HS72_YEAST	PMID:9448096	Heat shock protein SSA2	Hsp70
P11484	HS75_YEAST		Heat shock protein SSB1	Hsp70
P40150	HS76_YEAST		Heat shock protein SSB2	Hsp70
P09435	HS73_YEAST	PMID:7867784	Heat shock protein SSA3	Hsp70
P22202	HS74_YEAST		Heat shock protein SSA4	Hsp70
P25294	SIS1_YEAST	GOA:interpro	SIS1 protein	Hsp40
P32527	ZUO1_YEAST	GO:0003754	Zuotin	Hsp40
P35191	MDJ1_YEAST	GOA:interpro	MDJ1 protein, mitochondrial precursor	Hsp40
P12398	HS77_YEAST	PMID:8654364	Heat shock protein SSC1, mitochondrial precursor	Hsp70
P38523	GRPE_YEAST	GOA:interpro	GrpE protein homolog, mitochondrial precursor, MGE1	GrpE
P14906	SC63_YEAST	GOA:spkw	Translocation protein SEC63	Hsp40
P16474	GR78_YEAST		GRP 78, BIP, Kar2	Hsp70
P25303	SCJ1_YEAST	GOA:interpro	DnaJ-related protein SCJ1	Hsp40
P39101	CAJ1_YEAST	GOA:interpro	CAJ1 protein	Hsp40

P48353	HLJ1_YEAST	GOA:interpro	HLJ1 protein	Hsp40
P39102	XDJ1_YEAST	GOA:interpro	XDJ1 protein	Hsp40
P52868	YGM8_YEAST	GOA:interpro	Hypothetical 41.0 kDa protein in CEG1-SOH1 intergenic region	Hsp40
P53940	YNH7_YEAST	GOA:interpro	Hypothetical 58.9 kDa protein in TPM1-MKS1 intergenic region	Hsp40
P38353	SSH1_YEAST		Sec sixty-one protein homolog.	Hsp70
P36016	LHS1_YEAST	GOA:spkw	Heat shock protein 70 homolog LHS1, SSI1	Hsp70
P38788	YHM4_YEAST	PMID:11054575	Heat shock protein 70 homolog YHR064C	Hsp70
Hsp110/Sse				
P32589	HS78_YEAST	PMID:10480867	Heat shock protein homolog SSE1	SSE
P32590	HS79_YEAST		Heat shock protein homolog SSE2	SSE
Hsp100 (Clp/Hsl)				
P31539	H104_YEAST	GOA:interpro	Heat shock protein 104	Hsp100
P33416	HSP7_YEAST	GOA:spkw	Heat shock protein 78, mitochondrial precursor	Hsp100
P38323	MCX1_YEAST	GOA:interpro	Mitochondrial clpX-like chaperone MCX1	Hsp100
Small Heat Shock Proteins				
P15992	HS26_YEAST	PMID:10581247	Heat shock protein 26	Small Hsp
Prefoldin				
P48363	PFD3_YEAST	GOA:interpro	Probable prefoldin subunit 3	Prefoldin
Q04493	PFD5_YEAST	GOA:interpro	Prefoldin subunit 5	Prefoldin
P43573	YFC3_YEAST	GOA:interpro	Hypothetical 91.4 kDa protein in STE2-FRS2 intergenic region	Prefoldin
P46988	PFD1_YEAST	GOA:spkw	Prefoldin subunit 1	KE2
P40005	PFD2_YEAST	GOA:spkw	Prefoldin subunit 2	KE2
P53900	PFD4_YEAST	GOA:spkw	Prefoldin subunit 4	KE2
P52553	PFD6_YEAST	GOA:spkw	Prefoldin subunit 6	KE2
Hsp90				
P02829	HS82_YEAST	GOA:interpro	Heat shock protein HSP82	Hsp90
P15108	HS83_YEAST	GOA:interpro	Heat shock cognate protein HSC82	Hsp90
P06101	CC37_YEAST	GOA:spkw	Hsp90 co-chaperone Cdc37	Cdc37

P33313	CNS1_YEAST	GOA:spkw	Cyclophilin seven suppressor 1	CNS1
P15705	STI1_YEAST	PMID:8972212	Heat shock protein STI1	
Calnexin				
P27825	CALX_YEAST	GOA:spkw	Calnexin homolog precursor	Calnexin
Cytosolic Chaperonins T-complex				
P12612	TCPA_YEAST	GOA:interpro	T-complex protein 1, alpha subunit	TCP-1, Hsp60
P39076	TCPB_YEAST	GOA:interpro	T-complex protein 1, beta subunit	TCP-1, Hsp60
P39078	TCPD_YEAST	GOA:interpro	T-complex protein 1, delta subunit	TCP-1, Hsp60
P40413	TCPE_YEAST	GOA:interpro	T-complex protein 1, epsilon subunit	TCP-1, Hsp60
P39077	TCPG_YEAST	GOA:interpro	T-complex protein 1, gamma subunit	TCP-1, Hsp60
P42943	TCPH_YEAST	GOA:interpro	T-complex protein 1, eta subunit	TCP-1, Hsp60
P47079	TCPQ_YEAST	GOA:interpro	T-complex protein 1, theta subunit	TCP-1, Hsp60
P39079	TCPZ_YEAST	GOA:interpro	T-complex protein 1, zeta subunit	TCP-1, Hsp60
Protein Specific				
P48606	TBCA_YEAST	GOA:spkw	Tubulin-specific chaperone A	protein specific
P53904	TBCB_YEAST	GOA:spkw	Tubulin-specific chaperone B	protein specific
P46670	CIN2_YEAST	GOA:spkw	Tubulin-folding cofactor C Cin2	protein specific
P40987	CIN1_YEAST		Tubulin-folding cofactor D Cin1	protein specific
P39937	PAC2_YEAST	GOA:spkw	Tubulin-folding cofactor E PAC2	protein specific
P21560	CBP3_YEAST	GOA:spkw	CBP3 protein, mitochondrial precursor	protein specific
Q12287	COXS_YEAST	GOA:spkw	Cytochrome c oxidase copper chaperone	protein specific
P40202	LYS7_YEAST	GOA:interpro	Superoxide dismutase 1 copper chaperone	
Q02774	SHR3_YEAST	PMID:10564255	Secretory component protein SHR3	protein specific
P38293	UMP1_YEAST	GOA:spkw	Proteasome maturation factor UMP1	protein specific
P38784	VM22_YEAST	PMID:7673216	Vacuolar ATPase assembly protein VMA22	protein specific

P38072	SCO2_YEAST	GOA:spkw	SCO2 protein, mitochondrial precursor	protein specific
P53266	SHY1_YEAST	PMID:11389896	SHY1 protein	protein specific
P40046	VTC1_YEAST	GOA:spkw	Vacuolar transporter chaperone 1	protein specific
P38958	PT00_YEAST	PMID:11498004	PET100 protein, mitochondrial precursor	protein specific
Disulfide Bond Isomerases				
P17967	PDI_YEAST	PMID:11157982	Protein disulfide isomerase precursor	Disulfide bond oxidoreductase
P32474	EUG1_YEAST	PMID:11157982	Protein disulfide isomerase EUG1 precursor	Disulfide bond oxidoreductase
Q12404	MPD1_YEAST	PMID:11157982	Disulfide isomerase MPD1 precursor	Disulfide bond oxidoreductase
Q99316	MPD2_YEAST	PMID:11157982	Protein disulfide isomerase MPD2 precursor (EC 5.3.4.1)	Disulfide bond oxidoreductase
Q03103	ERO1_YEAST	PMID:9659913	Endoplasmic oxidoreductin 1 precursor (EC 1.8.4.-) (Endoplasmic oxidoreductase protein 1).	Disulfide bond oxidoreductase
P38866	FMO1_YEAST	PMID:10077572	Thiol-specific monooxygenase (EC 1.14.13.-) (Flavin-dependent monooxygenase).	Disulfide bond oxidoreductase
Peptidyl-prolyl cis-trans isomerases				
P14832	CYPH_YEAST	GOA:interpro	Peptidyl-prolyl cis-trans isomerase cyclophilin A/Cpr1/Cyp1 /CPH1/Scc1	PPIase: Cyclophilin Type
P23285	CYPB_YEAST	GOA:interpro	Peptidyl-prolyl cis-trans isomerase cyclophilin B/Cpr2/Cyp2	PPIase: Cyclophilin Type
P25719	CYPC_YEAST	GOA:interpro	Peptidyl-prolyl cis-trans isomerase C/CYP3/CPR3, mitochondrial	PPIase: Cyclophilin Type
P25334	CYPR_YEAST	GOA:interpro	Peptidyl-prolyl cis-trans isomerase CPR4/Scc3	PPIase: Cyclophilin Type
P35176	CYPD_YEAST	GOA:interpro	Peptidyl-prolyl cis-trans isomerase D CypD/Cpr5	PPIase: Cyclophilin Type

P53691	CYP6_YEAST	PMID:10942767	Peptidyl-prolyl cis-trans isomerase CPR6	PPIase: Cyclophilin Type
P47103	CYP7_YEAST	PMID:10942767	Peptidyl-prolyl cis-trans isomerase CYP7	PPIase: Cyclophilin Type
P53728	CYP8_YEAST	GOA:interpro	Peptidyl-prolyl cis-trans isomerase CYP8	PPIase: Cyclophilin Type
Q02770	Q02770	GOA:interpro	Ypl064cp	PPIase: Cyclophilin Type
P20081	FKBP_YEAST	GOA:interpro	FK506-binding protein 1 FKB1/RBP1	PPIase: FKBP Type
P32472	FKB2_YEAST	GOA:interpro	FK506-binding protein 2, FKBP-13/ FKBP-15/ FKB2, FPR2	PPIase: FKBP Type
P38911	FKB3_YEAST	GOA:interpro	FK506-binding nuclear protein FKBP-70/Npi46/Fpr3/	PPIase: FKBP Type
Q06205	FKB4_YEAST	GOA:interpro	FK506-binding protein 4 FPR4	PPIase: FKBP Type
P22696	ESS1_YEAST	GOA:spkw	ESS1 protein	PPIase: Parvulin Type
Miscellaneous poorly characterised				
P27697	ABC1_YEAST	GOA:spkw	ABC1 protein, mitochondrial precursor	ABC1
P53193	YGB8_YEAST	GOA:interpro	Hypothetical 21.8 kDa protein in CKB1-ATE1 intergenic region	Hsp20
P28707	YKL7_YEAST	PMID:9632755	24.1 kDa protein in VMA12-APN1 intergenic region	p23 / wos2
P38932	VP45_YEAST	PMID:11432826	Vacuolar protein sorting-associated protein 45	SEC1 like
Q12019	MDN1_YEAST	GOA:spkw	Midasin	

Genetic manipulation

In step iii), the process includes changing expression of the identified compensatory gene or gene product in the recombinant cell by genetic modification to provide a modified recombinant cell. After identification of one or more up-regulated genes, proteins or metabolic processes, the genome of the host may be modified. Certain genes or gene products, although identified as up-regulated, may not be available for modulation because

they are essential to the cell or are known to affect other processes that may be essential to the cell or organism.

The genome may be modified by including an exogenous gene or promoter element in the genome or in the host with an expression vector, by enhancing the capacity of an identified gene to produce mRNA or protein, or by deleting or disrupting a gene or promoter element, or by reducing the capacity of a gene to produce mRNA or protein. The genetic code can be altered, thereby affecting transcription and/or translation of a gene, for example through substitution, deletion ("knock-out"), co-expression or insertion ("knock-in") techniques. Additional genes for a desired protein or regulatory sequence that modulate transcription of an existing sequence can also be inserted.

Recombination

The genome of the host cell expressing recombinant protein or peptide can be modified via a genetic targeting event, which can be by insertion or recombination, for example homologous recombination. Homologous recombination refers to the process of DNA recombination based on sequence homology. Homologous recombination permits site-specific modifications in endogenous genes and thus novel alterations can be engineered into a genome. One step in homologous recombination is DNA strand exchange, which involves a pairing of a DNA duplex with at least one DNA strand containing a complementary sequence to form an intermediate recombination structure containing heteroduplex DNA (see, for example Radding, C. M. (1982) *Ann. Rev. Genet.* 16: 405; U.S. Pat. No. 4,888,274). The heteroduplex DNA can take several forms, including a three DNA strand containing triplex form wherein a single complementary strand invades the DNA duplex (Hsieh, *et al.*, *Genes and Development* 4: 1951 (1990); Rao, *et al.*, (1991) PNAS 88:2984)) and, when two complementary DNA strands pair with a DNA duplex, a classical Holliday recombination joint or chi structure (Holliday, R., *Genet. Res.* 5: 282 (1964)) can form, or a double-D loop ("Diagnostic Applications of Double-D Loop Formation" U.S. Ser. No. 07/755,462, filed Sep. 4, 1991). Once formed, a heteroduplex structure can be resolved by strand breakage and exchange, so that all or a portion of an invading DNA strand is spliced into a recipient DNA duplex, adding or replacing a segment of the recipient DNA duplex. Alternatively, a heteroduplex structure can result in gene conversion, wherein a sequence of an invading strand is transferred to a recipient DNA duplex by repair of mismatched bases using the invading strand as a template (Genes, 3rd Ed. (1987) Lewin, B., John Wiley, New York, N.Y.; Lopez, *et al.*, *Nucleic Acids Res.* 15: 5643(1987)). Whether by the mechanism of breakage

and rejoining or by the mechanism(s) of gene conversion, formation of heteroduplex DNA at homologously paired joints can serve to transfer genetic sequence information from one DNA molecule to another.

In homologous recombination, the incoming DNA interacts with and integrates into a site in the genome that contains a substantially homologous DNA sequence. In non-homologous ("random" or "illicit") integration, the incoming DNA integrates not at a homologous sequence in the genome but elsewhere, at one of a large number of potential locations. A number of papers describe the use of homologous recombination in mammalian cells.

Various constructs can be prepared for homologous recombination at a identified locus. Usually, the construct can include at least 10 bp, 20 bp, 30 bp, 40 bp, 50 bp, 70 bp, 100 bp, 500 bp, 1kbp, 2 kbp, 4 kbp, 5 kbp, 10 kbp, 15 kbp, 20 kbp, or 50 kbp of sequence homologous with the identified locus. Various considerations can be involved in determining the extent of homology of identified DNA sequences, such as, for example, the size of the identified locus, availability of sequences, relative efficiency of double cross-over events at the identified locus and the similarity of the identified sequence with other sequences.

The targeting DNA can include a sequence in which DNA substantially isogenic flanks the desired sequence modifications with a corresponding identified sequence in the genome to be modified. The substantially isogenic sequence can be at least about 95%, 97-98%, 99.0-99.5%, 99.6-99.9%, or 100% identical to the corresponding identified sequence (except for the desired sequence modifications). The targeting DNA and the identified DNA can share stretches of DNA at least about 10, 20, 30, 50, 75, 150 or 500 base pairs that are 100% identical.

The DNA constructs can be designed to modify the endogenous, identified gene product. The homologous sequence for identifieding the construct can have one or more deletions, insertions, substitutions or combinations thereof designed to disrupt the function of the resultant gene product. In one embodiment, the alteration can be the insertion of a selectable marker gene fused in reading frame with the upstream sequence of the identified gene.

The genome can also be modified using insertional deletion. In this embodiment, the genome is modified by recombining a sequence in the gene that inhibits gene product formation. This insertion can either disrupt the gene by inserting a separate element, or remove an essential portion of the gene. In one embodiment, the insertional deletion includes

insertion of a gene coding for resistance to a particular stressor, such as an antibiotic, or for growth in a particular media, for example for production of an essential amino acid.

The genome can also be modified by use of transposons, which are genetic elements capable of inserting at sites in prokaryote genomes by mechanisms independant of homologous recombination. Transposons can include, for example, Tn*7* in *E. coli*, Tn*554* in *S. aureus*, IS*900* in *M. paratuberculosis*, IS492 from *Pseudomonas atlantica*, IS*116* from *Streptomyces* and IS*900* from *M. paratuberculosis*. Steps believed to be involved in transposition include cleavage of the end of the transposon to yield 3' OH; strand transfer, in which transposase brings together the 3'OH exposed end of transposon and the identified sequence; and a single step transesterification reaction to yield a covalent linkage of the transposon to the identified DNA. The key reaction performed by transposase is generally thought to be nicking or strand exchange, the rest of the process is done by host enzymes.

In one embodiment, a process is provided to increase the level of a identified gene or homologue thereof by incorporating a genetic sequence encoding the gene or homologue into the genome by recombination. In another embodiment, a promoter is inserted into the genome to enhance the expression of the identified gene or homologue. In a separate embodiment, a process is provided for decreasing the expression of a identified gene or homologue thereof by recombination with an inactive gene. In another embodiment, a sequence that encodes a different gene, which can have a separate function in the cell or can be a reporter gene such as a resistance marker or an otherwise detectable marker gene can be inserted into a genome through recombination. In yet another embodiment, a copy of at least a portion of the identified gene that has been mutated at one or more locations is inserted into the genome through recombination. The mutated version of the identified gene can not encode a protein, or the protein encoded by the mutated gene can be rendered inactive, the activity can be modulated (either increased or decreased), or the mutant protein can have a different activity when compared to the native protein.

There are strategies to knock out genes in bacteria, which have been generally exemplified in *E. coli*. One route is to clone a gene-internal DNA fragment into a vector containing an antibiotic resistance gene (*e.g.* ampicillin). Before cells are transformed via conjugative transfer, chemical transformation or electroporation (Puehler, et al. (1984) *Advanced Molecular Genetics* New York, Heidelberg, Berlin, Tokyo, Springer Verlag), an origin of replication, such as the vegetative plasmid replication (the *oriV* locus) is excised and the remaining DNA fragment is re-ligated and purified (Sambrook, et al. (2000) *Molecular cloning: A laboratory manual, third edition* Cold Spring Harbor, New York, Cold Spring

Harbor Laboratory Press). Alternatively, antibiotic-resistant plasmids that have a DNA replication origin can be used. After transformation, the cells are plated onto *e.g.* LB agar plates containing the appropriate antibiotics (*e.g.* 200 μg/mL ampicillin). Colonies that grow on the plates containing the antibiotics presumably have undergone a single recombination event (Snyder, L., W. Champness, et al. (1997) *Molecular Genetics of Bacteria* Washington DC, ASM Press) that leads to the integration of the entire DNA fragment into the genome at the homologous locus. Further analysis of the antibiotic-resistant cells to verify that the desired gene knock-out has occurred at the desired locus is *e.g.* by diagnostic PCR (McPherson, M. J., P. Quirke, et al. (1991) *PCR: A Practical Approach* New York, Oxford University Press). Here, at least two PCR primers are designed: one that hybridizes outside the DNA region that was used for the construction of the gene knock-out; and one that hybridizes within the remaining plasmid backbone. Successful PCR amplification of the DNA fragment with the correct size followed by DNA sequence analysis will verify that the gene knock-out has occurred at the correct location in the bacterial chromosome. The phenotype of the newly constructed mutant strain can then be analyzed by *e.g.* SDS polyacrylamide gel electrophoresis (Simpson, R. J. (2003) *Proteins and Proteomics - A Laboratory Manual.* Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press).

An alternate route to generate a gene knock-out is by use of a temperature-sensitive replicon, such as the pSC101 replicon to facilitate gene replacement (Hamilton, et al. (1989) New process for generating deletions and gene replacements in *Escherichia coli. Journal of Bacteriology* 171(9): 4617-22). The process proceeds by homologous recombination between a gene on a chromosome and homologous sequences carried on a plasmid temperature sensitive for DNA replication. After transformation of the plasmid into the appropriate host, it is possible to select for integration of the plasmid into the chromosome at 44°C. Subsequent growth of these cointegrates at 30°C leads to a second recombination event, resulting in their resolution. Depending on where the second recombination event takes place, the chromosome will either have undergone a gene replacement or retain the original copy of the gene.

Other strategies have been developed to inhibit expression of particular gene products. For example, RNA interference (RNAi), particularly using small interfering RNA (siRNA), has been extensively developed to reduce or even eliminate expression of a particular gene product. siRNAs are short, double-stranded RNA molecules that can target complementary mRNAs for degradation. RNAi is the phenomenon in which introduction of a double-stranded RNA suppresses the expression of the homologous gene. dsRNA molecules are

reduced in vivo to 21-23 nt siRNAs which are the mediators of the RNAi effect. Upon introduction, double stranded RNAs get processed into 20-25 nucleotide siRNAs by an RNase III-like enzyme called Dicer (initiation step). Then, the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process. The siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the cognate RNA (effecter step). Cleavage of cognate RNA takes place near the middle of the region bound by the siRNA strand. RNAi has been successfully used to reduce gene expression in a variety of organisms including zebrafish, nematodes (*C. elegans*), insects (*Drosophila melanogaster*), planaria, cnidaria, trypanosomes, mice and mammalian cells.

Mutation

The genome can also be modified by mutation of one or more nucleotides in a open reading frame encoding an identified gene, particularly an identified protease. Techniques for genetic mutation, for instance site directed mutagenesis are well known in the art. Some approaches focus on the generation of random mutations in chromosomal DNA such as those induced by X-rays and chemicals. Mutagenesis targeted to a defined region of DNA includes many techniques, some more popular than others. *In vitro* approaches to site-directed mutagenesis can be grouped generally into three categories: i) processes that restructure fragments of DNA, such as cassette mutagenesis; ii) localized random mutagenesis; and iii) oligonucleotide-directed mutagenesis.

Oligonucleotide-directed mutagenesis is based on the concept that an oligonucleotide encoding a desired mutation(s) is annealed to one strand of the DNA of interest and serves as a primer for initiation of DNA synthesis. In this manner, the mutagenic oligonucleotide is incorporated into the newly synthesized strand. Mutagenic oligonucleotides incorporate at least one base change but can be designed to generate multiple substitutions, insertions or deletions. Examples include PCR-based processes and practically all of the non-PCR-based processes in use today. These techniques include positive antibiotic selection (Lewis, M.K. and Thompson, D.V. (1990) *Nucl. Acids Res.* 18, 3439; Bohnsack, R.N. (1996) *Meth. Mol. Biol.* 57, 1; Vavra, S. and Brondyk, W.H. (1996) *Promega Notes* 58, 30; *Altered Sites® II in vitro Mutagenesis Systems Technical Manual* #TM001, Promega Corporation), unique restriction site selection (Deng, W.P. and Nickoloff, J.A. (1992) *Anal. Biochem.* 200, 81), uracil incorporation (Kunkel, T.A. (1985) *Proc. Natl. Acad. Sci. USA* 82, 488; Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) *Meth. Enzymol.* 154, 367), and phosphorothioate

incorporation (Taylor, J.W., Ott, J. and Eckstein, F. (1985) *Nucl. Acids Res.* 13, 8764; Nakamaye, K. and Eckstein, F. (1986) *Nucl. Acids Res.* 14, 9679). Oligonucleotides can also encode a library of mutations by randomizing the base composition at sites during chemical synthesis resulting in degenerate or "doped" oligonucleotides. The ability to localize and specify mutations is greatly enhanced by the use of synthetic oligonucleotides hybridized to the DNA insert-containing plasmid vector.

The general format for site-directed mutagenesis is: denaturation of plasmid DNA containing the template of interest (cDNA, promoter, etc.) to produce single-stranded regions; annealing of a synthetic mutant oligonucleotide to the identified strand; synthesis of a new complementary strand using, for example, T4 DNA Polymerase; and sealing the resulting nick between the end of the new strand and the oligonucleotide, for example using T4 DNA Ligase. The resulting heteroduplex is propagated by transformation, for example in *E. coli.* Selection and enrichment processes have been incorporated into mutagenesis processes to greatly improve the efficiency of mutant strand recovery and rates approaching 80-90% are possible. Numerous processes exist to generate different types of mutations and to enhance for the selection of the mutant. Examples of processes to enhance for the selection of the mutant include positive antibiotic selection of the mutant strand, using a uracil-containing DNA strand which can be selectively degraded *in vivo*, and dNTP analog incorporation, which can render one strand of heteroduplex DNA impervious to digestion. Some approaches can be combined, such as cassette mutagenesis and the use of "doped" oligonucleotides to create a library of random mutations in a small, defined region.

An extension of the so-called "standard" processes of site-directed mutagenesis includes those that rely on DNA amplification, specifically the polymerase chain reaction (PCR). The major commonality in site-directed mutagenesis is the use of a mutagenic oligonucleotide. The mutagenic oligonucleotide should hybridize efficiently to the template. For efficient hybridization, there can be, for example, 100% base pairing at either end of the identified sequence without secondary structure formation, but can also be less than 100% identify, such as 98%, 95%, 92%, 90%, 85%, 80%, 70% or only a portion of the sequence can be identical. For small substitutions, 10-15 bases hybridizing on either side of the mismatch are usually sufficient. The composition of the 3′-end of the primer is particularly important as polymerases do not typically extend from a mismatched or poorly hybridized 3′-end.

The basis for site-directed mutagenesis by positive antibiotic selection is that a selection oligonucleotide or oligonucleotides are simultaneously annealed, with the

mutagenic oligonucleotide, to repair an antibiotic resistance gene (10-13). Selection for the mutant strand is enabled by antibiotic resistance of the mutated DNA and sensitivity of the nonmutated strand. This approach offers a very efficient means to generate an indefinite number of the desired mutations with little hands-on time.

Site-directed mutagenesis by the use of a unique restriction site is based on the processes of Deng and Nickoloff (Deng, W.P. and Nickoloff, J.A. (1992) *Anal. Biochem.* 200, 81). In this approach, a selection oligonucleotide containing a mutated sequence for a unique restriction site is annealed simultaneously with the mutagenic oligonucleotide. The selection oligonucleotide renders the nonessential site immune to restriction by the corresponding enzyme. Selection for the mutant strand is enhanced by digesting the resulting pool of plasmids with the unique restriction enzyme. The digestion linearizes the parental plasmid thereby effectively decreasing its ability to transform bacteria.

Site-directed mutagenesis by deoxyuridine incorporation relies on the ability of a host strain to degrade template DNA that contains uracil (U) in place of thymidine (T). A small number of dUTPs are incorporated into the template strand in place of dTTP in a host that lacks dUTPase (*dut*-) and uracil *N*-deglycosidase (*ung*-) activities. (Uracil *per se* is not mutagenic and it base pairs with adenine.) Normally, dUTPase degrades deoxyuridine and uracil *N*-deglycosidase removes any incorporated uracil. Post-mutation replication in a *dut*+ *ung*+ strain is used then to degrade nonidentified strand DNA. This approach requires that single-stranded DNA be used so that only one strand contains the Us which are susceptible to degradation.

The phosphorothioate incorporation approach to site-directed mutagenesis rests on the ability of a dNTP analog containing a thiol group to render heteroduplex DNA resistant to restriction enzyme digestion. The mutant strand is extended from the mutagenic oligonucleotide and synthesized in the presence of dCTPalphaS. Unused template DNA is removed by digestion with an exonuclease. Theoretically, only circular, heteroduplex DNA remains. The heteroduplex is then nicked, but not cut, at the restriction site(s). Exonuclease III is used to digest the nicked strand and the remaining fragment then acts as a primer for repolymerization, creating a mutant homoduplex.

In the polymerase chain reaction (PCR) based approach to generate a mutation in DNA, a template is amplified using a set of gene-specific oligonucleotide primers except that one oligonucleotide, or more in protocols that use multiple amplifications, contains the desired mutation. Variations include altering the hybridization site of the oligonucleotides to produce multiple, overlapping PCR fragments with the mutation in the overlap and the

"megaprimer" approach, which uses three oligonucleotides and two rounds of amplification wherein a product strand from the first amplification serves as a primer in the second amplification.

In the overlap extension approach, complementary oligodeoxyribonucleotide (oligo) primers and the polymerase chain reaction are used to generate two DNA fragments having overlapping ends. These fragments are combined in a subsequent 'fusion' reaction in which the overlapping ends anneal, allowing the 3' overlap of each strand to serve as a primer for the 3' extension of the complementary strand. The resulting fusion product is amplified further by PCR. Specific alterations in the nucleotide (nt) sequence can be introduced by incorporating nucleotide changes into the overlapping oligo primers.

Vector constructs

In a separate embodiment, the host cell is modified by including one or more vectors that encode a identified gene, typically a folding modulator or a cofactor of a folding modulator. In another embodiment, the host cell is modified by enhancing a promoter for a folding modulator or a cofactor for a folding modulator, including by adding an exogenous promoter to the host cell genome.

In another embodiment, the host cell is modified by including one or more vectors that encode an inhibitor of an identified compensatory gene, such as a protease inhibitor. Such an inhibitor can be an antisense molecule that limits the expression of the identified compensatory gene, a cofactor of the identified gene or a homologue of the identified gene. Antisense is generally used to refer to a nucleic acid molecule with a sequence complementary to at least a portion of the identified gene. In addition, the inhibitor can be an interfering RNA or a gene that encodes an interfering RNA. In Eukaryotic organisms, such an interfering RNA can be a small interfering RNA or a ribozyme, as described, for example, in Fire, A. et al.(1998) *Nature* 391:806-11, Elbashir et al. (2001) *Genes & Development* 15(2):188-200, Elbashir et al. (2001) *Nature* 411(6836):494-8, U.S. Patent Nos. 6,506,559 to Carnegie Institute, 6,573,099 to Benitec, U.S. Patent Application Nos. 2003/0108923 to the Whitehead Inst., and 2003/0114409, PCT Publication Nos. WO03/006477, WO03/012052, WO03/023015, WO 03/056022, WO 03/064621 and WO 03/070966. The inhibitor can also be another protein or peptide. The inhibitor can, for example, be a peptide with a consensus sequence for the protease or protease protein. The inhibitor can also be a protein or peptide that can produce a direct or indirect inhibitory molecule for the protease or protease protein in the host. Protease inhibitors can include Amastatin, E-64, Antipain, Elastatinal, APMSF,

Leupeptin, Bestatin, Pepstatin, Benzamidine, 1,10-Phenanthroline, Chymostatin, Phosphoramidon, 3,4-dichloroisocoumarin, TLCK, DFP, TPCK. Over 100 naturally occurring protein protease inhibitors have been identified so far. They have been isolated in a variety of organisms from bacteria to animals and plants. They behave as tight-binding reversible or pseudo-irreversible inhibitors of proteases preventing substrate access to the active site through steric hindrance. Their size are also extremely variable from 50 residues (e.g BPTI: Bovine Pancreatic Trypsin Inhibitor) to up to 400 residues (e.g alpha-1PI: alpha-1 Proteinase Inhibitor). They are strictly class-specific except proteins of the alpha-macroglobulin family (e.g alpha-2 macroglobulin) which bind and inhibit most proteases through a molecular trap mechanism.

An exogenous vector or DNA construct can be transfected or transformed into the host cell. Techniques for transfecting and transforming eukaryotic and prokaryotic cells respectively with exogenous nucleic acids are well known in the art. These can include lipid vesicle mediated uptake, calcium phosphate mediated transfection (calcium phosphate/DNA co-precipitation), viral infection, particularly using modified viruses such as, for example, modified adenoviruses, microinjection and electroporation. For prokaryotic transformation, techniques can include heat shock mediated uptake, bacterial protoplast fusion with intact cells, microinjection and electroporation. Techniques for plant transformation include *Agrobacterium* mediated transfer, such as by *A. tumefaciens*, rapidly propelled tungsten or gold microprojectiles, electroporation, microinjection and polyethelyne glycol mediated uptake. The DNA can be single or double stranded, linear or circular, relaxed or supercoiled DNA. For various techniques for transfecting mammalian cells, see, for example, Keown *et al.* (1990) *Processes in Enzymology* Vol. 185, pp. 527-537.

For recombination events, the constructs can include one or more insertion sequences, which can insert or transpose one or more nucleic acid sequence into a different sequence. However, the construct can be designed for exogenous expression of an identified compensatory gene or homologue thereof without incorporation into the existing cellular DNA/genome.

The constructs can contain one, or more than one, internal ribosome entry site (IRES). The construct can also contain a promoter operably linked to the nucleic acid sequence encoding at least a portion of the identified gene, or a cofactor of the identified gene, a mutant version of at least a portion of the identified compensatory gene, or in the case of proteases, an inhibitor of the identified gene. Alternatively, the construct can be promoterless. In cases in which the construct is not designed to incorporate into the cellular

DNA/genome, the vector typically contains at least one promoter element. In addition to the nucleic acid sequences the expression vector can contain selectable marker sequences. The expression constructs can further contain sites for transcription initiation, termination, and/or ribosome binding sites. The identified constructs can be inserted into and can be expressed in any prokaryotic or eukaryotic cell, including, but not limited to bacterial cells, such as *P. fluorescens* or *E. coli*, yeast cells, mammalian cells, such as CHO cells, or plant cells.

Cloning vectors can include *e.g.* plasmid pBR322 (Bolivar, Rodriguez et al. 1977), the pUC series of plasmids (Vieira and Messing 1982), pBluescript (Short, Fernandez et al. 1988), pACYC177 and pACYC184 (Chang and Cohen 1978). Exogenous promoters for use in such constructs, include, but are not limited to, the phage lambda PL promoter, *E. coli* lac, *E. coli* trp, *E. coli* phoA, *E. coli* tac prornoters, SV40 early, SV40 late, retroviral LTRs, PGKI, GALI, GALIO genes, CYCI, PH05, TRPI, ADHI, ADH2, forglymaldehyde phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, triose phosphate isomerase, phosphoglucose isomerase, glucokinase alpha-mating factor pheromone, PRBI, GUT2, GPDI promoter, metallothionein promoter, and/or mammalian viral promoters, such as those derived from adenovirus and vaccinia virus. Other promoters will be known to one skilled in the art.

Promoters for exogenous vectors, or exogenous promoters designed to be inserted into the genome can be based on specific response elements in a cell. For example, promoters can be responsive to chemical compounds, for example to anthranilate or benzoate, as described in PCT Publication No. WO 2004/005221. The constructs can include one or more promoters. These can be independent, or can be in tandem. For example the promoters can be designed so that a identified compensatory gene is up- or down-regulated in a particular time frame with the recombinant protein or peptide. For example, in a case in which the identified gene is a folding modulator, the folding modulator or cofactor can be induced shortly before induction of the recombinant protein or peptide. Promoters can include, but are not limited to the following:

Promoter	**source**	**regulation**	**induction**
lac	*E. coli*	lacI, $lacI^q$	IPTG
lacUV5	*E. coli*	lacI, $lacI^q$	IPTG
tac (hybrid)	*E. coli*	lacI, $lacI^q$	IPTG
trc (hybrid)	*E. coli*	lacI, $lacI^q$	IPTG

P_{syn} (synthetic)	*E. coli*	lacI, $lacI^q$	IPTG
trp	*E. coli*		tryptophan starvation
araBAD	*E. coli*	araC	l-arabinose
lpp^a	*E. coli*		IPTG, lactose
lpp-lac (hybrid)	*E. coli*	lacI	IPTG
phoA	*E. coli*	phoB (positive) phoR (negative)	phosphate starvation
recA	*E. coli*	lexA	nalidixic acid
proU	*E. coli*		osmolarity
cst-1	*E. coli*		glucose starvation
tetA	*E. coli*		tetracyclin
cadA	*E. coli*	cadR	pH
nar	*E. coli*	fnr	anearobic conditions
p_L	λ	λ cIts857	thermal (shift to 42°C)
cspA	*E. coli*		thermal (shift to below 20°C)
T7	T7	λ cIts857	thermal
T7-lac operator	T7	$lacI^q$	IPTG
T3-lac operator	T3	$lacI^q$	IPTG
T5-lac operator	T5	lacI, $lacI^q$	IPTG
T4 gene 32	T4		T4 infection
nprM-lac operator	*Bacillus*	$lacI^q$	IPTG
VHb	*Vitreoscilla*		oxygen
Protein A	*S. aureus*		

Constructs can include selection markers to identify modified cells. Suitable selectable marker genes include, but are not limited to: genes conferring the ability to grow on certain media substrates, such as the tk gene (thymidine kinase) or the hprt gene

(hypoxanthine phosphoribosyltransferase) which confer the ability to grow on HAT medium (hypoxanthine, aminopterin and thymidine); the bacterial gpt gene (guanine/xanthine phosphoribosyltransferase) which allows growth on MAX medium (mycophenolic acid, adenine, and xanthine). See, for example, Song, K-Y., *et al.* (1987) *Proc. Nat'l Acad. Sci. U.S.A.* 84:6820-6824; Sambrook, J., *et al.* (1989) *Molecular Cloning—A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., Chapter 16. Other examples of selectable markers include: genes conferring resistance to compounds such as antibiotics, genes conferring the ability to grow on selected substrates, genes encoding proteins that produce detectable signals such as luminescence, such as green fluorescent protein, enhanced green fluorescent protein (eGFP). A wide variety of such markers are known and available, including, for example, antibiotic resistance genes such as the neomycin resistance gene (neo) (Southern, P., and P. Berg, (1982) *J. Mol. Appl. Genet.* 1:327-341); and the hygromycin resistance gene (hyg) ((1983) *Nucleic Acids Research* 11:6895-6911, and Te Riele, H., *et al.* (1990) *Nature* 348:649-651). Other selectable marker genes include: acetohydroxy acid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucoronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), red fluorescent protein (RFP), yellow fluorescent protein (YFP), cyan fluorescent protein (CFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracycline. Additional selectable marker genes useful in this invention, for example, are described in U.S. Patent Nos: 6,319,669; 6,316,181; 6,303,373; 6,291,177; 6,284,519; 6,284,496; 6,280,934; 6,274,354; 6,270,958; 6,268,201; 6,265,548; 6,261,760; 6,255,558; 6,255,071; 6,251,677; 6,251,602; 6,251,582; 6,251,384; 6,248,558; 6,248,550; 6,248,543; 6,232,107; 6,228,639; 6,225,082; 6,221,612; 6,218,185; 6,214,567; 6,214,563; 6,210,922; 6,210,910; 6,203,986; 6,197,928; 6,180,343; 6,172,188; 6,153,409; 6,150,176; 6,146,826; 6,140,132; 6,136,539; 6,136,538; 6,133,429; 6,130,313; 6,124,128; 6,110,711; 6,096,865; 6,096,717; 6,093,808; 6,090,919; 6,083,690; 6,077,707; 6,066,476; 6,060,247; 6,054,321; 6,037,133; 6,027,881; 6,025,192; 6,020,192; 6,013,447; 6,001,557; 5,994,077; 5,994,071; 5,993,778; 5,989,808; 5,985,577; 5,968,773; 5,968,738; 5,958,713; 5,952,236; 5,948,889; 5,948,681; 5,942,387; 5,932,435; 5,922,576; 5,919,445; and 5,914,233.

Deletions can be at least about 5 bp, 10 bp, 20 bp, 30 bp, 40 bp or 50 bp, commonly at least about 100 bp, and generally not more than about 20 kbp, where the deletion can

normally include at least a portion of the coding region including a portion of or one or more exons, a portion of or one or more introns, and can or can not include a portion of the flanking non-coding regions, particularly the 5'-non-coding region (transcriptional regulatory region). Thus, the homologous region can extend beyond the coding region into the 5'-non-coding region or alternatively into the 3'-non-coding region. Insertions can generally not exceed 10 kbp, usually not exceed 5 kbp, generally being at least 50 bp, more usually at least 200 bp.

The region(s) of homology can include mutations, where mutations can further inactivate the identified gene, in providing for a frame shift, or changing a key amino acid, or the mutation can correct a dysfunctional allele, etc. Usually, the mutation can be a subtle change, not exceeding about 5% of the homologous flanking sequences.

The construct can be prepared in accordance with processes known in the art, various fragments can be brought together, introduced into appropriate vectors, cloned, analyzed and then manipulated further until the desired construct has been achieved (see, for example Figures 5-11). Various modifications can be made to the sequence, to allow for restriction analysis, excision, identification of probes, etc. Silent mutations can be introduced, as desired. At various stages, restriction analysis, sequencing, amplification with the polymerase chain reaction, primer repair, in vitro mutagenesis, etc. can be employed. Processes for the incorporation of antibiotic resistance genes and negative selection factors will be familiar to those of ordinary skill in the art (see, e.g., WO 99/15650; U.S. Patent No. 6,080,576; U.S. Patent No. 6.136,566; Niwa, *et al., J. Biochem.* 113:343-349 (1993); and Yoshida, et al., *Transgenic Research,* 4:277-287 (1995)).

The construct can be prepared using a bacterial vector, including a prokaryotic replication system, e.g. an origin recognizable by a prokaryotic cell such as *P. fluorescens* or *E. coli.* A marker, the same as or different from the marker to be used for insertion, can be employed, which can be removed prior to introduction into the identified cell. Once the vector containing the construct has been completed, it can be further manipulated, such as by deletion of certain sequences, linearization, or introducing mutations, deletions or other sequences in the homologous sequence. After final manipulation, the construct can be introduced into the cell.

The process can be iterative. In one embodiment, after modification of the host and expression of the recombinant protein in the modified host, a genetic profile of the modified host cell is analyzed to identify one or more further identified genes the expression of which is changed in the modified host cell. In particular, compensatory genes can be those that

show increased expression in the modified host expressing recombinant protein when compared to a modified host cell not expressing the recombinant protein or peptide, or when compared to an unmodified host cell. The process further includes changing the expression of the further identified gene or genes and expressing the protein or peptide in the doubly modified cell. These steps can be iterated to improve protein expression and can be repeated one, two, three, four, five, six, seven, eight, nine, or at least ten times.

Production of protein

The process of the invention optimally leads to increased production of recombinant protein or peptide in a host cell. The increased production can include an increased amount of protein per gram of host protein in a given amount of time, or can include an increase in the length of time the host cell is producing recombinant protein or peptide. The increased production can also include an improvement in the requirements for growth of the recombinant host cell. The increased production can be an increased production of full length protein or peptide. If the improvement is in increased levels of protein, the protein or peptide can be produced in one or more inclusion bodies in a host cell.

The increased production alternatively can be an increased level of active protein or peptide per gram of protein produced, or per gram of host protein. The increased production can also be an increased level of recoverable protein or peptide, such as soluble protein, produced per gram of recombinant or per gram of host cell protein. The increased production can also be any combination of increased total level and increased active or soluble level of protein.

Increased production is typically measured by comparing the level of production after a certain period of induction in a modified cell to the same induction in the unmodified cell.

Soluble/insoluble

The improved expression of recombinant protein can be an increase in the solubility of the protein. The recombinant protein or peptide can be produced and recovered from the cytoplasm, periplasm or extracellular medium of the host cell. The protein or peptide can be insoluble or soluble. The protein or peptide can include one or more targeting sequences or sequences to assist purification.

In certain embodiments, the invention provides a process for improving the solubility of a recombinant protein or peptide in a host cell. The term "soluble" as used herein means

that the protein is not precipitated by centrifugation at between approximately 5,000 and 20,000xgravity when spun for 10-30 minutes in a buffer under physiological conditions. Soluble, active proteins are capable of exhibiting function, and are not part of an inclusion body or other precipitated mass.

The invention can also improve recovery of active recombinant proteins or peptides. For example, the interaction between a identified and a parent polypeptide, polypeptide variant, segment-substituted polypeptide and/or residue-substituted polypeptide can be measured by any convenient in vitro or in vivo assay. Thus, in vitro assays can be used to determine any detectable interaction between a identified and polypeptide, e.g. between enzyme and substrate, between hormone and hormone receptor, between antibody and antigen, etc. Such detection can include the measurement of colorimetric changes, changes in radioactivity, changes in solubility, changes in molecular weight as measured by gel electrophoresis and/or gel exclusion processes, etc. In vivo assays include, but are not limited to, assays to detect physiological effects, e.g. weight gain, change in electrolyte balance, change in blood clotting time, changes in clot dissolution and the induction of antigenic response. Generally, any in vivo assay can be used so long as a variable parameter exists so as to detect a change in the interaction between the identified and the polypeptide of interest. See, for example, US Patent No. 5,834,250.

Cytoplasmic/periplasmic/secreted

In certain embodiments, the protein can also be secreted into the periplasm if fused to an appropriate signal secretion sequence. In one embodiment, the signal sequence can be a phosphate binding protein, a Lys-Arg-Orn binding protein (LAObp or KRObp) secretion signal peptide, an Outer Membrane Porin E (OprE) secretion signal peptide, an azurin secretion signal peptide, an iron (III) binding protein [Fe(III)bp] secretion signal peptide, or a lipoprotein B (LprB) secretion signal peptide.

In one embodiment, no additional disulfide-bond-promoting conditions or agents are required in order to recover disulfide-bond-containing identified polypeptide in active, soluble form from the modified host cell or doubly or multiply modified cell. In one embodiment, the transgenic peptide, polypeptide, protein, or fragment thereof has a folded intramolecular conformation in its active state. It has been found that complex mammalian proteins soluble in the cytoplasm can configure appropriately with the proper positioning of the thiol groups for later disulfide bond formation in the periplasm. In one embodiment, the transgenic peptide, polypeptide, protein, or fragment contains at least one intramolecular

disulfide bond in its active state; and perhaps up to 2, 4, 6, 8, 10, 12, 14, 16, 18, or 20 or more disulfide bonds.

In one embodiment, more than 50% of the expressed, transgenic peptide, polypeptide, protein, or fragment thereof produced will be produced as single, functional peptides, polypeptides, proteins, or fragments thereof in soluble, active form or insoluble easily renatured form in the cytoplasm or periplasm. In another embodiment about 60% , 70%, 75%, 80%, 85%, 90%, 95% of the expressed protein is obtained in or easily renatured into active form.

EXAMPLES

The bacterial strains used in the current study are listed in Table 1. Strains of *P. fluorescens* were grown in shake-flasks at 30°C. OD_{575} was recorded for each strain at various time points.

Table 1. Overview of bacterial strains

Strain	Relevant Strain Genotype	Plasmid	Recombinant Protein
MB214	*P. fluorescens* host strain		
DC206	*pyrF*⁻		
DC240	*pyrF*⁻	pDOW2415	nitrilase
DC271	*pyrF*⁻	pDOW1323	pbp:hGH
DC280	*pyrF*⁻	pDOW1339	vector only plasmid
DC369	*pyrF*⁻	pDOW1426	hGH
DC462	*pyrF*⁻	pDOW3501	GrpE, DnaKJ
DC463	*pyrF*⁻	pDOW3501, pDOW1426	GrpE, DnaKJ, hGH
HJ104	*pyrF*⁻	pDOW1349	hGH-COP
DC370	*pyrF*⁻, *hslU*⁻		
DC372	*pyrF*⁻, *hslU*⁻	pDOW1426	hGH
DC373	*pyrF*⁻, *hslU*⁻	pDOW1323	pbp:hGH
HJ105	*pyrF*⁻, *hslU*⁻	pDOW1349	hGH-COP
DC417	*pyrF*⁻, *hslUV*⁻		
HJ115	*pyrF*⁻, *hslUV*⁻	pDOW1426	hGH
HJ117	*pyrF*⁻, *hslUV*⁻	pDOW1349	hGH-COP

Plasmids used in the following experiments are listed in Table 2.

Table 2: Overview of plasmids

Plasmids	Relevance
pDOW2236	cloning vector
pDOW2240	Ptac *grpE-dnaKJ*, *pyrF*$^+$
pDOW2247	Pmtl no recombinant gene; empty vector
pDOW3501	Pmtl *grpE-dnaKJ*, *pyrF*$^+$
pDOW1349	*pyrF*$^+$, *hGH::COP*
pDOW1426	*pyrF*$^+$, *hGH*
pDOW1261-2	suicide vector, *pyrF*$^+$
pDOW2050	used for construction of the *hslUV* deletion strains

Sample collection and RNA isolation

All samples were collected from a 200ml standard shake flasks experiments. Samples were taken at different time points as indicated in the figures. At each time point, 10 ml of cell culture from the shake flasks was collected and mixed with 10 ml of RNA*later* (Ambion, Austin, TX) reagent to stabilize RNA.

Microarray hybridization and data analysis

For each RNA sample, the fluorescent nucleotides Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia, Piscataway, NJ) were incorporated into cDNA in a reverse transcription (RT) reaction using random hexamer primer (Amersham). The two labeled cDNA pools were combined and applied to a microarray slide. The microarray slides contains 50mer amino-modified oligodeoxyribonucleotides (oligos) representing each ORF of *P. fluorescens*. Each oligo was printed twice for duplicate spots at different location using the SDDC-2 robot (Virtek, Toronto, Canada – now distributed through Bio-Rad Laboratories, Hercules, CA) and SMP3 pins (TeleChem International Inc., Sunnyvale, CA). The microscope slides used were coated with a positively charged epoxy resin for efficient DNA binding (MWG Inc, Alameda, CA). After printing, the slides were post-processed according to MWG's specifications. A software package from BioDiscovery Inc. (El Segundo, CA) was used to facilitate the data analysis. This package consists of CloneTracker™, ImaGene™, GeneSight™ modules and the GeneDirector™ database. Each hybridized slide was scanned using ScanArray 5000 (Packard BioScience, Billerica, MA) to measure fluorescence of the Cy3- and Cy5-labeled cDNA bound to the microarray. The acquired images were quantified in ImaGene™ and raw data was processed in GeneSight™. During the data preparation, the spot intensity for each gene was background-corrected; the signal for the Cy5 channel was normalized to the Cy3 channel using the total signal intensity for the

entire array; the normalized ratio of Cy5 to Cy3 for each gene was log2 transformed, and replicates were combined.

Protein expression analysis by SDS-PAGE

Culture aliquots were harvested at various time points after IPTG induction, normalized to OD_{600} of 10. Cell lysates were separated into soluble and insoluble fractions by centrifugation at 11000 g for 5 min. Aliquots of 2.5 ul were combined with 5 ul 2X NuPAGE LDS sample buffer (Invitrogen, San Diego, CA), 50 uM DTT, and H_2O to 10 μl, then heated at 95°C for 5 min. The proteins were separated and visualized on 12% Nupage gels stained with Coomassie Blue using Simply Blue Safestain (Invitrogen, San Diego, CA).

Fluorescence activity measurement

Protein yield was also measured by fluorescence activity of the fusion of green fluorescence protein (COP) and human growth hormone (hGH). The hgh::COP fusion construct was transformed into wild-type or *hslU* mutant strains and selected on the M9 glucose agar plate without uracil. The IPTG-induced cell culture were normalized to OD_{600} of five. Relative fluorescence (RF) activity was measured using the Spectramax Gemini microplate spectrofluorimeter (Molecular Devices, Sunnyvale, CA) under the appropriate setting (Ex485, Em538530 bandpass filter).

Example 1: Gene expression analysis of strains producing cytoplasmic and periplasmic proteins - *Comparison of different time points*

To study the FMs and protease gene expression during the production of heterologous protein, *P. fluorescens* strains DC206, 280, 240 and 271 were used in the initial microarray experiments. DC206 is the host strain and was used as a control for cell growth; DC280 has a vector-only plasmid and was used as a control for the microarray experiments; DC240 is DC206 with a plasmid encoding cytoplasmic nitrilase enzyme that is soluble; DC271 is DC206 with a plasmid encoding the periplasmic human growth hormone (pbp::hGH) that is partly insoluble. Strains were grown in 200 ml of shake flask medium and cell growth was monitored by measuring OD_{575}. IPTG induction was performed 24 hrs after inoculation. All strains grew similarly and culture samples were taken just before (0 hr) and 4 hrs after induction for RNA isolation and transcriptional profiling (TxP) using DNA microarrays (Figure 1).

The genetic profiles, ie. transcriptional profiles were based on the comparison of the 4 hrs after induction time point sample with that of 0 hr sample, the two samples were labeled with fluorescent dyes, either Cy3-dUTP or Cy5-dUTP, and co-hybridized to the same slide for each strain. Each hybridization was duplicated with dye-swap experiments (*i.e.*, samples were labeled with either Cy3-dUTP or Cy5-dUTP) (Table 3, slides 1 to 6). The hybridized slides were scanned using a confocal laser scanner. Signal intensity for each gene was determined and processed using the microarray software package from Biodiscovery (El Segundo, CA). The expression ratio of the two time points for each gene was calculated and ratios for all the genes across the strains were clustered based on the ratio value and trend among the three strains (DC280, DC240 and DC271) (Figure 2).

Table 3. Summary of microarray experiments performed in Examples 1-3

Experiment	Slide	Cy3	Cy5	Experiment	Slide	Cy3	Cy5
DC280	**1**	4hr sample	0 hr sample	0 hr	**7**	DC240	DC271
	2	0 hr sample	4hr sample		**8**	DC271	DC240
DC240	**3**	4hr sample	0 hr sample	4hr	**9**	DC240	DC271
	4	0 hr sample	4hr sample		**10**	DC271	DC240
DC271	**5**	4hr sample	0 hr sample	DC369	**11**	4hr sample	0 hr sample
	6	0 hr sample	4hr sample		**12**	0 hr sample	4hr sample

To focus on FM and protease gene expression in *P. fluorescens* under the stress imposed by high level recombinant protein production, a list of FM and protease genes was compared to the cluster analysis. After hierarchical clustering analysis of all the genes from DC280, DC240 and DC271, FMs and proteases were identified in two clusters (lines in clusters 6 and 7; Figure 2).

Four genes in cluster 7 show significant higher expression in DC271 expressing mainly insoluble periplasmic human growth hormone as compared to DC240 producing soluble cytoplasmic nitrilase or DC280, which does not overproduce any protein. The four genes are rxf01961 encoding HslV, rxf01957 encoding HslU, rxf03987 encoding CbpA and rxf05455 encoding HtpG. The *E. coli* HslV (ClpQ) and HslU (ClpY) together form a cytoplasmic protease. The small subunit, HslV, is a peptidase related to the proteasomal α-subunits of eukaryotes. The large subunit, HslU, is an ATPase with homology to other Clp family ATPases such as ClpA and ClpX. CbpA of *E. coli* is an analogue of the well-characterized co-chaperone DnaJ as judged from not only its structure but also its function. The phenotype of lesions in DnaJ, such as temperature sensitivity for growth, are restored

upon introduction of the *cbpA* gene on a multicopy plasmid. HtpG of *E. coli* functions as an ATP-independent molecular chaperone *in vitro*. It recognizes and transiently binds non-native folding intermediates, reducing their free concentration in solution and thus preventing unspecific aggregation.

The genes in cluster 6 of Figure 2 were clustered again using hierarchical clustering to identify less pronounced effects. Figure 3 shows that FMs and proteases were identified in two main clusters (lines in cluster 6 and 8). The two FMs in cluster 8 are DnaK and DnaJ, two main chaperones that are well known to work together to fold numerous proteins. Further analysis of expression values of genes from cluster 6 identified an additional FM, ClpX that is higher expressed in DC271 producing pbp::hGH as compared to DC240 producing nitrilase or DC280, which does not overproduce any protein. The *E. coli* ClpX heat shock protein is homologous to members of prokaryotic and eukaryotic HSP100/Clp ATPases family. ClpX of *E. coli* was isolated as a specific component of the ATP-dependent Clp proteases, which maintain certain polypeptides in a form competent for proteolysis by the ClpP protease subunit. ClpX can act as a molecular chaperone, in the absence of ClpP, by activating the initiation proteins involved in DNA replication. Identified FMs and proteases important for periplasmic hGH production are listed in Table 4.

Table 4. List of FM and protease genes whose steady-state mRNA ratio levels are higher in DC271 as compared to DC240 and DC280. The values listed are the ratio of 4 hr after IPTG induction to 0 hr.

Gene ID	**DC280 (4hr vs. 0hr)**	**DC240 (4hr vs. 0hr)**	**DC271 (4hr vs.0hr)**	**Gene**	**Function**
RXF05455_1	0.8	0.6	5.3	*htpG*	Chaperone protein HtpG
RXF03987_1	1.0	0.5	5.2	*cbpA*	Curved DNA-binding protein
RXF01961_1	0.9	0.4	5.0	*hslV*	ATP-dependent protease HslV (ec 3.4.25.-)
RXF01957_1	1.0		4.8	*hslU*	ATP-dependent Hsl protease, ATP-binding subunit HslU
RXF05399	1.0	0.6	3.3	*dnaK**	Chaperone protein DnaK
RXF05399_1	1.3	0.6	3.0	*dnaK**	Chaperone protein DnaK
RXF05406_1	1.2	0.7	3.0	*dnaJ*	Chaperone protein DnaJ

RXF04654_1	1.1	0.9	2.0	*clpX*	ATP-dependent Clp protease, ATP-binding subunit ClpX

*For *dnaK*, two probes are present on the microarray chip and thus two gene expression values are provided.

Example 2 - Gene expression analysis of strains producing cytoplasmic and periplasmic proteins - *Direct comparison of different strains*

In order to confirm the results obtained above, additional microarray experiments were performed by direct comparison of the two strains DC271 and DC240 (slides 7 to 10 in Table 3). The comparison of the two strains at the 4 hrs after induction time point confirmed that an almost

identical set of FM and protease genes were up-regulated in cells expressing partially soluble pbp::hGH (Table 5). All genes listed in Table 5 are significantly (*i.e.* ≥ 2-fold) higher expressed in strains producing the partly insoluble hGH as compared to cells producing fully soluble nitrilase. In the direct comparison of DC271 to DC240, a few additional proteins were identified as compared to the time point comparison (see Table 4) that showed significantly higher gene expression values during partially insoluble hGH production. Those genes included rxf08347 encoding ClpB, rxf04587 encoding ClpA, and rxf05753 encoding FkbP. The *E. coli* ClpB homologue is involved in reactivation of inclusion bodies together with DnaKJ-GrpE. ClpA from *E. coli* has a chaperone function or, when together with ClpP, degrades proteins. In *E. coli*, FkbP

functions as a peptidyl-prolyl isomerase.

Table 5. List of FM and protease genes whose steady-state mRNA levels are higher in DC271 as compared to DC240. The values listed are the ratio of DC271 to DC240 at 4 hr after IPTG induction.

Gene ID	**DC271 vs. DC240 at 0hr**	**DC271 vs. DC240 at 4hr**	**Gene**	**Function**
RXF03987_1	0.8	10.8	*cbpA*	Curved DNA-binding protein
RXF01957_1	0.9	10.0	*hslU*	ATP-dependent Hsl protease, ATP-binding subunit HslU
RXF01961_1	0.7	10.0	*hslV*	ATP-dependent protease HslV (ec 3.4.25.-)

RXF05455_1	0.7	7.8	*htpG*	Chaperone protein HtpG
RXF05406_1	1.0	4.7	*dnaJ*	Chaperone protein DnaJ
RXF08347_1	0.6	3.8	*clpB*	ClpB protein
RXF05399_1	1.0	3.7	*dnaK**	Chaperone protein DnaK
RXF05399	0.9	2.9	*dnaK**	Chaperone protein DnaK
RXF04587_1	0.9	2.8	*clpA*	ATP-dependent Clp protease, ATP-binding subunit ClpA
RXF05753_1	1.1	2.1	*fkbP*	Peptidyl-prolyl cis-trans isomerase (ec 5.2.1.8)
RXF04654_1	1.2	2.0	*clpX*	ATP-dependent Clp protease, ATP-binding subunit ClpX

*For *dnaK*, two probes are present on the microarray chip and thus two gene expression values are provided.

Example 3: Gene expression analysis of a strain producing an insoluble cytoplasmic protein

Since DC271 expresses partially periplasmic human growth hormone (pbp::hGH), it was investigated if similar or different FMs and protease genes were up-regulated in a strain expressing mainly insoluble cytoplasmic hGH. DC369 was used in this experiment. The 4 hrs after induction sample was compared with that of the 0 hr time point sample, and microarray experiments were performed as shown in Table 3 (slides 11 and 12). Again, similar FM and protease genes were found to be up-regulated indicating that the identified genes are involved in

cytoplasmic rather than periplasmic folding and protein degradation (Table 6). A summary of which genes were identified in which experiment along with the fold up-regulation is shown in the Venn diagram of Figure 4.

Table 6. List of FM and protease genes whose steady-state mRNA levels are higher in DC369 at 4 hrs after induction as compared to time zero. The values listed are the ratio of 4 hr after IPTG induction to 0 hr (just before induction).

Gene ID	**DC369 (4hr vs. 0hr)**	**Gene**	**Function**
RXF01961_1	4.8	*hslV*	ATP-dependent protease HslV (ec 3.4.25.-)

RXF01957_1	4.3	*hslU*	ATP-dependent Hsl protease, ATP-binding subunit HslU
RXF03987_1	4.1	*cbpA*	Curved DNA-binding protein
RXF05455_1	3.3	*htpG*	Chaperone protein HtpG
RXF05406_1	2.3	*dnaJ*	Chaperone protein DnaJ
RXF08347_1	2.2	*clpB*	ClpB protein
RXF05399_1	2.1	*dnaK**	Chaperone protein DnaK
RXF02095_1	2.0	*groES*	10 kDa Chaperonin GroES
RXF06767_1	2.0	*groEL*	10 kDa Chaperonin GroEL
RXF05399	1.8	*dnaK**	Chaperone protein DnaK
RXF04587_1	1.7	*clpA*	ATP-dependent Clp protease, ATP-binding subunit ClpA

*For *dnaK*, two probes are present on the microarray chip and thus two gene expression values are provided.

Example 4: Generation of an *hslU* mutant strain in *P. fluorescens* DC206

The two genes *hslVU* were found to be among the most highly up-regulated identified genes. HslU is a cytoplasmic ATPase. The homologous protein in *E. coli* can act in combination with a second protein to promote energy dependent protein degradation in *E. coli*. HslU interacts with HslV, a protein with homology to the α subunits of proteasome. The *E.coli* HslVU homologues were reported to be involved in overall proteolysis of misfolded proteins in Missiakas, D., et al. (1996) Identification and characterization of HsIV HsIU (ClpQ ClpY) proteins involved in overall proteolysis of misfolded proteins in *Escherichia coli. Embo J* 15:6899-909. DNA sequence analysis suggested that the *P. fluorescens hslVU* genes are likely to be part of a bicistronic operon (Figure 5).

In order to verify that HslVU are indeed involved in the degradation of hGH, an *hslU* knockout strain was constructed. Such a strain was generated by insertional inactivation of *hslU* (Figure 6). An approximately 550 bp DNA fragment internal to *hslU* was cloned into the kanamycin-resistant pCR2.1-TOPO vector. Since this vector has an origin of replication (ColE1) that is functional in *E. coli* but not in *P. fluorescens*, the constructed plasmids will integrate into the chromosome of DC206 through homologous recombination in order to confer kanamycin resistance. The correct insertion site for the kanamycin resistant colonies was confirmed by diagnostic colony PCR using primers that hybridize to the outside of the

originally amplified region and within the plasmid backbone (Table 3). The constructed *hslU* mutant strain was designated DC370.

Primers were designed that would amplify a ~550 bp internal region of the *hslU* gene (Table 7). The internal fragment was amplified using Taq Polymerase (Promega), purified, and cloned into pCR2.1-TOPO vector (Invitrogen, San Diego, CA). The plasmids were transformed into competent *P. fluorescens* DC206 and selected on the M9 glucose agar plates supplemented with 250 μg/ml uracil and 50 μg/ml kanamycin.

Table 7. Primers

Primers	Sequence	Purpose
hslU_sens	accgaagtcggctatgtggg (SEQ ID NO:1)	used in PCR amplification of the internal *hslU* fragment
hslU_antis	aatcgcgctgcacgccttcg (SEQ ID NO:2)	
hsl_F2	ttcatcaaggtcgaagcg (SEQ ID NO:3)	used in diagnostic PCR
hsl_R2	tcagtcttgaccatgcc (SEQ ID NO:4)	
M13_R	caggaaacagctatgac (SEQ ID NO:5)	
M13_F	taaaacgacggccag (SEQ ID NO:6)	
hsl-Up	gtggcagccaccaaggctgc (SEQ ID NO:7)	used in SOE PCR the up- and down- DNA fragment of *hslUV*
hsl_middleUp	cccacattgagtgaggcttacaaggggagagtctccacg (SEQ ID NO:8)	
hsl_middleDown	cgtggagactctccccttgtaagcctcactcaatgtggg (SEQ ID NO:9)	
hsl_down	ggccaatggttggccacgcg (SEQ ID NO:10)	
hsl_UpUp	tgccgacgccacaggtgc (SEQ ID NO:11)	used in diagnostic PCR
hsl_DownDown	gcctggtactgcgactcg (SEQ ID NO:12)	
RC199	atatactagtaggaggtaacttatggctgacgaacagacgca (SEQ ID NO:13)	used in cloning the *grpE-DnaKJ*
RC200	atattctagattacaggtcgccgaagaagc (SEQ ID NO:14)	

Protein expression comparison by SDS-PAGE analysis

To study the effect of the *hslU* gene knockout, two exogenous protein expression were compared between the parent strain DC206 and the newly constructed mutant strain DC370. The plasmids harboring the gene encoding pbp::hGH (pDOW1323), and hGH (pDOW1426) were each transformed into competent DC370 cells and resulted in strains

DC373 and DC372, respectively. Standard shake-flask growth experiments were performed with the four strains. Figure 7 shows that the wild-type and mutant strains have similar growth rates. Samples were run on SDS-PAGE gels (Figure 8 and 9). The results suggest that the mutant produced higher amounts of proteins due to the deletion of the protease subunit HslU.

Protein expression comparison by fluorescence activity

Since the observed effect of the lack of HslU on the yield of hGH is difficult to quantitate using SDS-PAGE analyses, the temporal profile of protein production was monitored by the fluorescence of a fusion protein between COP green fluorescent protein and hGH. A plasmid containing an hGH::COP fusion was constructed and transformed into the parent strain DC206 and the *hslU* gene deletion strain DC370 resulting in strains HJ104 and HJ105 (Table 1). Standard shake flask experiments were performed and samples were taken at various time points for fluorescence measurements (Figure 10). The readings from the fluorimeter clearly showed that the *hslU* protease mutant strain had significantly higher protein expression levels compared to that of the parental strain (Figure 11). This finding corroborates the results obtained by SDS-PAGE analysis. Comparing to the wild type strain, the *hslU* mutant increased 33.05% of the relative fluorescence at 24 hrs after induction (see insert in Figure 11).

Example 5: Construction of an *hslUV* clean knockout strain

The Hsl protease consists of two subunits: : an ATP-binding subunit encoded by *hslU*, and a protease subunit encoded by *hslV*. The previously constructed Hsl protease knock-out strain is an insertional inactivation of the *hslU* gene. To remove the concern that HslV might still function as a protease by being able to couple with an ATP-binding subunit of another protease, a deletion strain was constructed that had both the *hslU* and *hslV* genes removed from the chromosome.

As shown in Figure 13, plasmid pDOW2050 was constructed by PCR amplification of two DNA fragments flanking the *hslUV* region, the two fragments were subsequently fused using the Splicing by Overlap Extension (SOE) PCR method (see Ho, S.N. (1991) Method for gene splicing by overlap extension using the polymerase chain reaction. Application: US patent 89-3920955023171). The fused DNA fragments were then ligated into the *Srf*I site of vector pDOW1261-2. The deletion plasmid was named pDOW2050 after the insert was confirmed by DNA sequencing.

Plasmid pDOW2050 was electroporated in DC206 and plated onto M9 agar plates supplemented with 1% glucose and 15 μg/ml tetracycline. Tetracycline-resistance is due to an integration event that recombines the entire plasmid into the chromosome at one of the two homologous regions within the genome (Figure 13). To select for cells that have a deletion of the *hslUV* genes resulting from a second homologous recombination between the integrated plasmid and the homologous DNA region in the chromosome, the tetracycline resistant colonies were grown to stationary phase in LB medium supplemented with 250 μg/ml uracil. Cells are then plated onto LB agar plates supplemented with 500 μg/ml 5-fluoroorotic acid (5-FOA). Cells that lost the integrated plasmid by a second recombination event also have lost the *pyrF* gene and thus are resistant to 5-FOA, resulting in the desired chromosomal *hslUV* deletion strain, called DC417.

Phenotypic analysis of *hslUV* deletion strain

SDS-PAGE analysis of the *hslUV* deletion strain expressing hGH protein (strain HJ115) showed much higher protein yield than the wild-type strain DC369, similar to what was observed earlier using the *hslU* insertional mutant strain DC372 (data not shown).

Protein yield was also measured by fluorescence activity of the hGH::COP fusion using the same method described earlier. Plasmid pDOW1349 containing the hGH::COP fusion was transformed into wild-type and mutant strains resulting in strains HJ104 and HJ117, respectively. Standard shake flask experiments were performed and samples were taken at various time points for relative fluorescence measurements (Figure 14). The readings from the fluorimeter indicated that the *hslUV* protease deletion strain had significantly higher proteins expression levels (about 50% yield increase) as compared to that of the wild-type strain. This result is similar to what was observed previously with the *hslU* knock-out strain.

Example 6: Iterative target identification using DNA microarray technology

To investigate if a new set of proteases are up-regulated in the *hslUV* protease deletion strain, DNA microarray experiments were conducted. Standard shake flask experiments were performed using the wild type (DC369) and mutant strain (HJ115) expressing hGH. For each strain, the 4 hrs after induction samples were compared to that of the 0 hr time point sample (just before heterologous protein induction) and DNA microarray experiments were performed. Comparing the ratio of the two time points between the wild-type and mutant strains, a new list of protease genes that are up-regulated in the *hslUV* protease deletion strain was identified (Table 8). These newly identified genes encoding

proteases can now be the targets for a second round of gene deletion events to further improve the yield of heterologous protein production.

Table 8: Protease genes whose steady-state mRNA levels are higher in the *hslUV* protease deletion strain (HJ115) as compared to the wild type strain (DC369), based on the ratio of 4hr after IPTG induction to 0 hr (just before induction).

ORFID	Curated Function	Sequence
rxf00133	D-alanyl-meso-diaminopimelate endopeptidase (ec 3.4.-.-)	atgcactttggaaaatggtttcacaccagcaccctgctggtcggcttgagttttgtgctgggcgg ctgcgccagcgtctcccaaacctccaccccggcaaccctggataagctgttgagcgacccgg cgctgcaaggcgccaccgtctcgctgatggtgcgtgatgcccgcacaggcaccacgctgtat cagcacaacccacgcacccggctggtgcccgcgtccaacctcaagctgttgaccacggcgg cagccatggatgtattggggccgcagtaccgcttcgccacgcaactgctgagcaatggcctac gccagggcgaccggctgactggcaacctgtacctgcgtggcttgggcgacccgagtattcag tttgccgactatcaggcgctcgccgcgcaattggccagccagggcgtgcgccaggtgcagg gtgacctggtgttcgacgacacttggttcgatgccgagcggctgggcgtggactggtcccatg atgatgaaaccacctactacggcgcgcagatttcagcgctgaccgtggcgcccaataccgact ttgatgctggcagcgtgctggtcaccgccaaggcgccgttgcacgtcggctcgccggtcggc gtggagatctacccgcccaccgactacctgcaactgaataaccgcgccgtcagcgggccgg gtaacagctatgggatcaaccgtcgccatggcaccaacctgctgcagctcagcggcgcggtg gcgcctggccggcagagccagcaattgatcagcgtgtgggagccgacgcaactggtggcca acctgtttgagcaagccttggcgcagcagggcatcaaggtgctggggcgtcgggtgatgggc ggggcaagtcctgctggggtgacggtgctggccgagcaccaatcggcgccgttgcaggagc tgatcgtgccgctgctcaagctctcgaacaacgccatgtccgaagccgtgctcaaggccatgg gccgccagacggccagcagcggcacggcggcggcgggcgccgtggcggtggccgacttt ctcaagcgccaggggctggacaccagcgctgtgagccaagtggacggctccggcctgtcgc ggcgtaacctggtgtcgtcgcaaaccctcaccgacctgctgctggcggccagcaaacaaccc tggttcgacgcctggtacaacgcgctgccggttgccggcaatgccgaccgtatgaccggcgg cagcctgggttaccgcctgcgcggcacggctgcggaaaataacctgcatgccaagaccggct ccatggccggcgtgtcgtcattgagcggttacatcaccgatgctcacgggcgcaagctggtgtt cgcgatgttgaccaacaactatgtggtcgctggcgcgcaggtaaaagccgtggaaaaccgcg tcgccgtggccctgtcccacagcgaagactga
rxf01918	zinc protease (ec 3.4.99.-)	atgagtgatcgcaaaaacagccgcctgatcctgcccggcctgatcgccgtcaccctgatggcg gccagcgccgtttacttcttgcgccccagcgagtcggtcgccagccaggccctggacaaggc tcaaacggccagcaccctgcaatccctggcggaactggatggcaaggcaccgaccaaccgc aagctcgacgtacaaacctggaccaccgccgaaggcgccaaggtgctgttcgtcgaagccca tgagttgccgatgttcgacatgcgcctgctgttcgccgccggcagcagccaggatggcgacgt gccaggcctggcgctgatgaccaacgccatgctcaacgaaggcgtgccgggcaaggacgtc agccagatcgccagtggcttcgaaggcctgggggccgactttggcaacggcgcctaccgcg acatggcgctggtgaccctgcgcagcctgagcgacagcgccaagcgcgacgccgccctgtc actgttcaaccaggtgatcggccagccgactttcccggcagactcactggcacgcatcaagaa ccagatcctggccggtttcgagtaccagaagcagaaccccggcaaactggcgagcatcgaac tgttcaagcgcctgtacggcgaccacccttacgcacacccgagcgaaggcacccccgagag cgtgccgaagattaccctggcgcagttgcaggcgttccacgccaaggcctatgcagcgggta acgcggtgattgcagtggtgggcgacctgacccgcgccgaagctgaagccatgacggccaa ggtgtccgcgtcgctgcccaaaggcccggctatggccaagatcgcccagccgaccgagcca aaagccggcctgagccgtatcgagttcccgtccaagcaaacccacctgctgtttgcgcagttg

		ggcatcgaccgtgccgacccggattacgcagccttgtccctgggtaaccagatcctcggcgg cggtggcttcggcacccgcttgatgagcgaagtgcgtgaaaagcgcggcctgacctacggc gtgtattccggtttctcaccaatgcaggcgcgcggcccgttcatgatcaacctgcagacccgcg ccgaaatgagcggtggcaccttgcgcctggtggaggacgtactggctgactacctcaagacc ggcccgacgcaaaaggaactggatgacgccaagcgcgagctggccggcagcttcccgctgt ccaccgccagcaacgccgatatcgtcgggcagttgggcgccatgggtttctacaacctgccg ctgagctatctggaagatttcatgaaacaatcccaggccctgaccgtcgatcaggtcaaggctg caatgaataaacacttgagcgccgacaagatggtcatcgtgaccgccggcccgacgattgcg caaaagccactaccgccccccactgataaacctgccgagcagccgctcggggttccggagc attaa
rxf0268 9	Microsomal dipeptidase (ec 3.4.13.19)	ttgtcgtggattgacgctttcggcaattcccctgtcgtttttgcacccggctccgtcggtgcctgg gcatatgctggccccaaagcgccggcagacgattcggcgcatgaatcgccaataaggggac gcctgatgagcccagccgagttgcacgccgacagcatcgttatcgacggtctgattattgccaa gtggaaccgcgacctgttcgaagacatgcgcaaaggtggcctcaccgccgccaattgcacgg tgtcggtgtgggaaggcttccaggccacgatcaataacatcgttgccagccagaccctgatcc gcgaaaacagcgacctggtgatcccggtgaaaaccaccgccgacatccgccgcgccaagg agctgggcaagactggcatcatcttcggcttccagaatgcccatgcctttgaggaccagctcgg ctatgtcgagatcttcaagcagctcggcgtgggcgtggtgcagatgtgctacaacacccagaa cctggtgggcaccggttgctacgagcgcgatggcggcctgtcgggtttcgggcgtgagatcg tcggcgagatgaaccgcgtcggcatcatgtgcgacctgtcccacgtgggctccaagaccagc gaagaggtcatcctcgaatcgaaaaagccggtgtgctactcccactgtctgccgtccgggctta aagagcacccgcgcaacaagtccgatgaagagctgaagttcatcgccgaccatggcggattt gtcggtgtgaccatgttcgcgccgtttttggccaagggcatcgactcgactatcgacgactatgc cgaagccatcgaatacaccatgaacatcgtcggcgaagacgccatcggcatcggcaccgact tcacccagggccatggccaggatttcttcgaaatgctcacccatgacaagggctacgcccgcc gcctgaccagcttcggcaagatcatcaacccgctgggcatccgcaccgtgggtgagttcccca acctcaccgagaccctgctcaagcgcggccacagcgagcgcgtggtgcgcaagatcatggg cgagaactgggtcaacgtgctcaaggacgtctggggcgaataa
rxf0511 3	Extracellular metalloprotea se precursor (ec 3.4.24.-)	atgacaatttggcccaggggcgaacacagggctatcctgaaaaccgttacccggacgttcac cacacgccaaaaaggagccagctcatgtgtgttcgccaaccgcgcaacccgatttttgcctga tcccgccgtacatgctcgaccagatcgcacgccacggcgacaaagcccaacgggaagtcgc attacgcacgcgtgccaaggacagcacgtttcgttcgttgcgcatggtcgcggtacccgccaa ggggccggcccgcatggcactggccgtgggcgccgagaagcaacgctcgatctacagtgc cgaaaacaccgacagcctgcccggcaagctgatccgcggcgaagggcagcccgccagtgg cgatgccgcggtggacgaagcctatgacggcctgggcgcgaccttcgatttttttgaccaggtc tttgatcgcaattccatcgacgatgcgggcatggcgctggacgccacggtgcacttcggccag gactacaacaatgcgttctggaattcgacccagatggtgttcggcgatggcgaccagcagttgt tcaaccgctttaccgtggcactcgacgtcattgggcatgagttggcccatggcgtgactgagga tgaggccaagctgatgtacttcaaccagtccggtgcgctgaacgagtcgttgtcggacgtgttc ggttcgctgatcaagcagtacgcgttaaagcaaacggccgaggatgccgactggttgatcgg caaggggttgtttaccaaaaagatcaagggcacggcgctgcgctcgatgaaggcgccaggc actgcgtttgatgacaagctgctgggcaaagacccgcagcctgggcacatggatgattttgtgc aaacttacgaggacaatgggggcgtgcatatcaattccggcattcccaaccatgcgttctacca ggtggcgatcaatataggcgggttcgcctgggagcgtgccgggcgtatctggtatgacgcact gcgcgattcgcggttgcggcccaattccgggttcttgcgttttgcgcgcattacccacgatattg ccggccagctttatggcgtgaacaaagctgagcagaaggcagtcaaggaaggctggaaagc ggtgggcataaacgtttga
rxf0540 0	Cell division protein FtsH (ec 3.4.24.-)	ttgaacgatatggcaaagaatctgatcctgtggttgatcatcgcggctgtcctggtgacggtgat gaacaacttctccagccctaacgagccgcagaccctcaactattccgacttcatccagcaagtt aaggatggcaaggtcgagcgcgtagcggttgatggctacgtgattaccggtaagcgcaacga

		tggcgacagcttcaagaccattcgtcctgccattcaggacaacggtctcatcggtgacctggtg gataacaaggtcgttgtggaaggcaagcagcctgaacagcaaagcatctggacccagctcct ggtggccagcttcccgatcctggtgattatcgccgtgttcatgttcttcatgcgccagatgcaag gcggtgcgggaggcaagggcgggccgatgagcttcggcaaaagcaaggcgcgcctgctct ccgaagaccaggtgaagaccaccctggctgacgtcgcaggttgcgacgaagccaaggaag aagtcggtgagttggtcgagttcctgcgtgatccgggcaagttccagcgcctgggtggccgtat tcctcgcggtgtgctgatggtggggcctccgggtaccggtaaaaccttgctggccaaggcgat tgccggcgaagccaaggtgcctttcttcacgatttccggttctgacttcgtcgagatgtttgtcgg cgtcggcgccagccgtgttcgcgatatgttcgagcaggccaagaagcacgcgccatgcatca tcttcatcgacgaaatcgatgccgttggtcgccatcgtggcgcgggcatgggggtggtcacg atgagcgtgagcagaccctcaaccagttgctggtagagatggatggtttcgagatgaatgacg gcattatcgtcatcgccgcaaccaaccgtcccgacgttctcgaccctgcgttgctgcgtccggg ccgtttcgaccgtcaggttgtggtcggcctgccggacattcgtggtcgtgagcagatcctgaaa gtacacatgcgcaaggtgccaatgggtgacgacgtggctccggctgtgatcgcccgtggtact cccggtttctccggtgctgatctggcgaacctggtcaacgaggcttcgctgttcgctgcccgtac tggcaagcgcatcgttgagatgaaagagttcgaattggcgaaagacaagatcatgatgggcg ccgagcgcaaatccatggtcatgtccgagaaagagaagcagaacaccgcttatcacgaggcc ggtcacgccattgtaggtcgcgttgtgcctgagcatgaccccgtctacaaagtgtcgatcattcc tcgtggtcgggcactgggtgtgaccatgttcctgccggaagaagatcgctacagcctctccaa gcgtgcgctgatcagccagatctgctcgctgtatggcggtcgtattgctgaggaaatgaccctt ggcttcgacggtgtgaccactggtgcctccaatgacatcatgcgtgccagccagatcgcacga aacatggtgaccaagtggggcttgtcggaaaaactcggcccattgatgtacgccgaagagga aggcgaagtgttcctggggcgtggcggcggtgggcaaagcgccagcttctcgggtgagaca gccaagctgatcgactccgaagttcgcagcatcattgaccagtgctatggcacggccaagcag attttgactgacaaccgtgacaagctggacgccatggctgatgcgttgatgaagtacgaaacca tcgatgccgaccagatcgacgacatcatggcgggccgtacgccgcgtgagccgcgcgactg ggaaggtggttcgggtacttcgggcactccgcctgtggtgcagaatgagcgccctgaaacgc ctatcggcggcccggcagctgatcactaa

Example 7: Co-overexpression of folding modulators increases solubility of target protein hGH

Based on the transcriptional profiling data shown in Figure 4, expression of folding modulators (FMs) DnaK and DnaJ was increased in strains producing recombinant protein compared to control strains (see Tables 4 and 5). A strain that co-overproduced GrpE, DnaK and DnaJ along with hGH was produced and tested to identify if this resulted in the accumulation of increased levels of soluble hGH.

Construction of grpE-dnaKJ-containing plasmid for co-overexpression with hGH

The *P. fluorescens grpE-dnaKJ* genes were amplified using chromosomal DNA isolated from MB214 (DNeasy; Qiagen, Valencia, CA) as a template, RC199 (5'-ATATACTAGTAGGAGGTAACTTATGGCTGACGAACAGACGCA-3') and RC200 (5'-ATATTCTAGATTACAGGTCGCCGAAGAAGC-3') as primers, *PfuTurbo* (Stratagene, La

Jolla, CA) was used following the manufacturer's recommendations. The resulting PCR product (4 kb) was digested with *Spe*I and *Xba*I (restriction sites underlined in the primers above) and ligated to pDOW2236 to create pDOW2240 containing the *grpE-dnaKJ* genes under the control of the *tac* promoter. Plasmid pDOW2240 was digested with *Spe*I and *Hind*III and the resulting *grpE dnaKJ*-containing 4.0 kb fragment was gel-purified using Qiaquick (Qiagen) and ligated to pDOW2247 also digested with *Spe*I and *Hind*III. The resulting plasmid, pDOW3501, containing *grpE-dnaKJ* under the control of the mannitol promoter, was transformed into DC388 by selecting on M9 glucose plates supplemented with 250 μg/ml uracil. Finally, pDOW1426 was electroporated into the above strain (DC462) and selected on M9 glucose plates, resulting in strain DC463 with two inducible plasmids: 1) pDOW1426 carrying P_{tac} *hGH* and 2) pDOW3501 carrying P_{mtl} *grpE-dnaKJ*.

Shake flask fermentation, sample collection and analysis

Duplicate cultures of DC463 were grown in shake flasks. Protein induction was accomplished by addition of 0.1 mM IPTG for hGH and 0.5% mannitol for GrpE-DnaKJ at 24hrs after inoculation. Samples were collected at 0, 4, 8, 24 and 48 hours after induction. At each time point, 20 OD_{600} cells normalized in 1 mL were harvested, lysed using EasyLyse™ (Epicentre, Madison, WI) and separated into soluble and insoluble fractions by centrifugation at 14000 rpm for 30 minutes. Equal volumes of samples were combined with BioRad (Hercules, CA) 2x Laemmli buffer, heated at 95°C for 5 minutes with 30 μL loaded onto a BioRad 15% Tris HCl Criterion gel using 1x Tris Glycine SDS running buffer (BioRad). The proteins were visualized with Simply Blue Safestain (Invitrogen, Carlsbad, CA) as shown in Figure 15. The resulting Coomassie-stained gels were scanned using a Molecular Devices Personal Densitometer (Molecular Devices, Sunnyvale, CA) with analyses performed using ImageQuant and Excel. As shown in Figure 15, co-overexpression of GrpE, DnaKJ significantly increased the solubility of hGH, converting almost 100% of the target protein into the soluble fraction, albeit at a lower total protein yield. Additional experiments repeating growth and induction of DC463 using the simultaneous addition of IPTG and mannitol closely mimicked the results shown here, although with a varying degree of hGH solubility (between 50-100%; data not shown), when GrpE DnaKJ were co-overproduced. These findings further demonstrate that targeted strain engineering based on transcriptional profiling can lead to a rational strain design to increase solubility and/or yield of a recombinant protein.

The invention has been described with reference to certain embodiments and non-limiting examples. It will be clear to one of skill in the art that other embodiments of the invention are also possible.

Claims

1. A process for improving the expression of a recombinant protein or peptide in a host cell or organism comprising:
    i) expressing the recombinant protein or peptide in the recombinant host cell or organism;
    ii) analyzing a genetic profile of the recombinant cell to identify one or more compensatory gene(s) or gene product(s) that are expressed at a higher level in the recombinant cell than in either a host cell that has not been modified to express the recombinant protein or a recombinant cell that is not expressing the recombinant protein; and
    iii) changing expression of the identified compensatory gene or gene product in the recombinant cell by genetic modification to provide a modified recombinant cell that achieves an increase in recombinant protein expression, activity or solubility.
2. The process of claim 1 further comprising expressing the protein or peptide in the modified recombinant cell.
3. The process of claim 1 further comprising:
    (a) expressing the recombinant protein or peptide in the modified recombinant cell;
    (b) analyzing a genetic profile of the modified recombinant cell to identify at least one second gene(s) or gene product(s) that are differentially expressed in the modified recombinant cell;
    (c) changing expression of the second identified gene product in the modified recombinant cell to provide a doubly modified cell; and
    (d) expressing the protein or peptide in the doubly modified recombinant cell.
4. The process of claim 3 further comprising repeating steps a) to d).
5. The process of claim 4 comprising repeating steps a) to d) until cell viability is affected by changing the expression of the identified gene(s) or gene product(s).
6. The process of claim 4 comprising repeating steps a) to d) until expression of the recombinant protein or peptide reaches a targeted endpoint.
7. The process of claim 1 wherein a genetic profile is analyzed by comparing a genetic profile of the recombinant cell to a second genetic profile of the host cell.
8. The process of claim 1 wherein the genetic profile is a transcriptome profile.
9. The process of claim 8 wherein the transcriptome profile is determined through a microarray.

10. The process of claim 1 wherein the genetic profile is a proteome profile.
11. The process of claim 10 wherein the proteome profile is determined through two dimensional gel electrophoresis, ICAT or LC/MS.
12. The process of claim 10 wherein the proteome profile is determined through a peptide array.
13. The process of claim 12 wherein the peptide array is an antibody array.
14. The process of claim 1 wherein the identified gene product is a protease, a subunit of a protease, a cofactor of a protease, a cellular or a genetic modulator affecting expression of a protease.
15. The process of claim 14 wherein the identified gene product is a protease.
16. The process of claim 14 wherein the identified gene product is a subunit of a protease.
17. The process of claim 14 wherein the identified gene product is a cofactor of a protease.
18. The process of claim 14 wherein the identified gene product is a cellular or genetic modulator affecting expression of a protease.
19. The processof claim 14 wherein the identified gene product is selected from the group consisting of D-alanyl-meso-diaminopimelate endopeptidase, zinc protease, microsomal dipeptidase, extracellular metalloprotease precursor, cell division protein ftsH and gene products derived from genes *hslV, hslU, clpX, clpA* and *clpB*.
20. The process of claim 14 wherein identified gene product mRNA level is up-regulated when the recombinant protein or peptide is expressed in the host cell.
21. The process of claim 14 wherein the identified gene product is removed from a host cell genome.
22. The process of claim 21 wherein the identified gene product is removed by homologous recombination.
23. The process of claim 1 wherein the identified gene product is a folding modulator, a subunit of a folding modulator, a cofactor of a folding modulator, or a cellular or genetic modulator affecting the expression of a folding modulator.
24. The process of claim 23 wherein the identified gene product is a folding modulator.
25. The process of claim 23 wherein the identified gene product is a subunit of a folding modulator.
26. The process of claim 23 wherein the identified gene product is a cofactor of a folding modulator.
27. The process of claim 23 wherein the identified gene product is a cellular or genetic modulator affecting the expression of a folding modulator.

28. The process of claim 23 wherein the folding modulator is a chaperone protein.
29. The process of claim 23 wherein the folding modulator is selected from the group consisting of gene products of the genes *cbpA, htpG, dnaK, dnaJ, fkbP2,groES* and *groEL*.
30. The process of claim 23 wherein the expression of the identified gene product is changed by increasing expression of the identified gene, a cofactor of a identified gene, or a cellular or genetic modulator of the identified gene.
31. The process of claim 30 wherein the increased expression is by inclusion of a DNA encoding the identified gene product.
32. The process of claim 30 wherein the increased expression is by insertion of a promoter into a host cell genome.
33. The process of claim 30 wherein the increased expression is by inclusion of an exogenous vector into the host cell.
34. The process of claim 1 wherein the host cell is a microbial cell.
35. The process of claim 1 wherein the host cell is a Pseudomonad.
36. The process of claim 1 wherein the host cell is a *P. fluorescens* cell.
37. The process of claim 1 wherein the host cell is an *E. coli* cell.
38. The process of claim 1 wherein the host cell is selected from the group consisting of an insect cell, a mammalian cell, a yeast cell, a fungal cell and a plant cell.
39. The process of claim 9 wherein the microarray comprises samples of binding partners to at least 50% of a genome of the host cell.
40. The process of claim 9 wherein the microarray technique comprises samples of binding partners to at least 80% of a genome of the host cell.
41. The process of claim 9 wherein the microarray comprises samples of binding partners to at least 90% of a genome of the host cell.
42. The process of claim 9 wherein the microarray comprises samples of binding partners to at least 95% of a genome of the host cell.
43. The process of claim 1 wherein the improved expression is an increase in the amount of recombinant protein or peptide.
44. The process of claim 1 wherein the improved expression is an increased solubility of the recombinant protein or peptide.
45. The process of claim 1 wherein the improved expression is an increased activity of the recombinant protein or peptide.
46. The process of claim 1 wherein the genetic profile is a profile of genes in a gene family.

47. The process of claim 1 wherein the profile comprises proteases and folding modulators.
48. The process of claim 46 wherein the profile consists essentially of proteases.
49. A host cell or organism that expresses a recombinant protein that has been genetically modified to reduce the expression of at least two proteases.
50. A host cell or organism that expresses a recombinant protein that has been genetically modified to reduce the expression of at least one protease selected from the group consisting of D-alanyl-meso-diaminopimelate endopeptidase, zinc protease, microsomal dipeptidase, extracellular metalloprotease precursor, cell division protein ftsH and gene products derived from genes *hslV, hslU, clpX, clpA* and *clpB*.
51. A host cell or organism that expresses a recombinant mammalian derived protein that has been genetically modified to reduce the expression of at least one protease.
52. The host cell or organism of claims 52 wherein the recombinant protein is human growth hormone.
53. A host cell or organism that expresses a recombinant protein that has been genetically modified to increase the expression of at least two folding modulators that are not folding modulator subunits.