Advances in *Escherichia coli* production of therapeutic proteins

James R Swartz

Escherichia coli offers a means for the rapid and economical production of recombinant proteins. These advantages, coupled with a wealth of biochemical and genetic knowledge, have enabled the production of such economically sensitive products as insulin and bovine growth hormone. Although significant progress has been made in transcription, translation and secretion, one of the major challenges is obtaining the product in a soluble and bioactive form. Recent progress in oxidative cytoplasmic folding and cell-free protein synthesis offers attractive alternatives to standard expression methods.

Addresses
Department of Chemical Engineering, Stanford University, Stanford, CA 94305-5025, USA; e-mail: swartz@chemeng.stanford.edu

Current Opinion in Biotechnology 2001, 12:195–201

0958-1669/01/$ – see front matter

Abbreviations

bGH	bovine growth hormone
PEP	phosphoenol pyruvate
PTS	phosphotransferase system
t-PA	tissue plasminogen activator

Introduction

Escherichia coli was the first host used to produce a recombinant DNA (rDNA) pharmaceutical, enabling the approval of Eli Lilly's rDNA human insulin in 1982. This is especially noteworthy, because insulin was already a 'mature' pharmaceutical. Its selling price of less than $400/g, its large volume of production and its chronic administration regimen combine to require a cost-efficient, highly effective and scaleable process. Later, the marketing of Monsanto's bovine growth hormone (bGH) product in 1994 set a new standard for pharmaceutical protein production from *E. coli*. Monsanto's bGH product sells for only $11.60/g (http://www.monsanto.com/dairy/3_econom.html). This suggests bulk production costs of less than $5/g even though the Food and Drug Administration (FDA) required high-quality standards for product approval. Even more impressive is the fact that both insulin and bGH require oxidative protein folding and that insulin is a heterodimer [1,2]. Both examples attest to the versatility and economic potential of *E. coli*-based production.

Until the mid-90s, *E. coli* was the dominant host (in terms of economic value) for the production of protein pharmaceuticals; however, in recent years, *E. coli* has been overtaken by mammalian cell production. This has been driven by the desire to produce more complex proteins and, in particular, antibodies. In many cases, *E. coli* is unable to fold these products into their proper conformation. Although the insulin and bGH examples suggest potential economic feasibility, most producers are unwilling to implement an *in vitro* protein folding process. In addition, *E. coli* cannot prepare or attach mammalian glycosylation chains. However, for many products and applications glycosylation is not required. Also, significant progress has been made in the expression and *in vivo* folding of mammalian proteins in *E. coli* and cell-free protein synthesis is now emerging as an option. This review focuses on recent advances in protein expression and, especially, on protein folding using *E. coli*. It is intended to complement the excellent summary offered by Baneyx [3].

Although *in vivo* production is an established technology, the large *E. coli* knowledge base and *E. coli*'s genetic flexibility empower continued development. Cell-free methods are still expensive and are generally conducted on a very small scale, but they allow the production of toxic proteins and offer considerable flexibility as well as scale-up. As we enter the 'proteomic era', cell-free synthesis also offers the potential for the efficient, multiplexed expression of protein libraries.

In vivo production: expression vectors

Expression vectors were well covered by Baneyx [3], but it is still useful to review and expand this fundamental topic. A variety of expression vectors are now available commercially. Most use moderate-to-high copy number plasmids. These can drive rapid protein expression, but usually require active selection pressure, for example, by antibiotics. However, a case can be made for low copy number plasmids or for expression cassettes incorporated into the chromosome.

Low gene dosage

Typically, the more rapid the intracellular product accumulation, the greater the probability of product aggregation. This is desirable for inclusion body production and, as the insulin and bGH examples teach, may provide an economically attractive production process. However, our usual objective is soluble, active protein. For this and for protein secretion, slower and more sustained production is preferred [4,5••], and high copy number may not be necessary or even desirable. The important parameter is probably the cell-specific rate of protein synthesis (translation). The final rate of protein synthesis will normally depend on several factors: gene dosage, promoter strength, mRNA stability and the efficiency of translation initiation. If the latter three factors are favorable, low gene dosage may be adequate for many applications. High product yields will then depend upon sustaining the production period.

Jones and Keasling [6] describe the use of F-plasmid-based expression vectors that are stably maintained at 1–2 copies per cell. Clearly, the total accumulation of expressed protein (β-galactosidase) was lower than with

higher copy number vectors, even when the mRNA was stabilized with a 5′ hairpin secondary structure [7]. But, if used with a stronger promoter, for example T7, and if developed for longer expression periods, this lower rate of expression may still provide significant product accumulation while avoiding aggregation and/or saturation of the secretion pathway. This same concept can be explored with chromosomal insertion of the expression cassette. Olson *et al.* [8•] followed this path to avoid infringing a variety of expression patents, but also showed that chromosomally based expression provided genetically stable organisms and acceptable expression levels.

Chromosomal integration and F-type plasmids also avoid the need for antibiotic presence during protein production. Although antibiotics are normally removed readily during product purification, validation of removal is necessary and may be expensive. If high copy number is still desired, for example, for the production of inclusion bodies, Williams *et al.* [9] of Cobra Therapeutics (Keele, UK) suggest an alternative approach that doesn't require antibiotics. The *lac* operator is placed upstream of an essential gene on the chromosome and is also present on the plasmid. With the *lac* repressor expressed from the chromosome, the plasmid-borne copy of the operator must titrate the repressor away from the chromosome in order for the cell to grow. The only required plasmid sequence is the short (26 base pair) *lac* operator. Although Williams and colleagues described the use of the kanamycin resistance gene on the chromosome, more recent experiments (JAJ Hanak of Cobra, personal communication) used a construction where the *lac* operator controlled transcription from the *dapD* gene, essential for peptidoglycan synthesis. No antibiotics were required for the stable maintenance of a high copy number plasmid.

Promoter choice

A variety of promoters are now used for protein expression [3]. Yet, there is still a need for a promoter with little or no expression before induction and with reliable, adjustable expression. Possibly, the arabinose promoter system comes closest to fulfilling these objectives [10]; however, Siegele and Hu [11] have observed induction heterogeneity among an induced population. This apparently arises from variable initial concentrations or induction rates for the arabinose transport proteins. It would appear that more reliable promotion would arise from a system with either the *araE* or the *araFG* genes (or both) constitutively expressed from the plasmid or chromosome and with the *araBAD* operon deleted from the chromosome. In this case, low concentrations of arabinose would be expected to provide equivalent and controlled induction of all cells.

Inexpensive and automatic (i.e. without operator intervention) induction is often desirable for large-scale production. The *phoA* (alkaline phosphatase) promoter can be used in such cases as it is tightly controlled and induced when phosphate becomes depleted from the medium. Induction requires phosphate-starvation, however, and could limit the duration of protein synthesis. Expression yields were improved from the *phoA* promoter without loss of control when the PhoS (PstS) phosphate-sensing protein was mutated to allow promoter induction at higher phosphate concentrations [P1]. Phosphate could then be fed continually (to avoid phosphate starvation) without repressing the promoter.

mRNA stability

The stability of mRNA can affect expression rates. Carrier and Keasling [12] investigated a series of 5′ mRNA secondary structures to identify those that improved messenger half-life. However, these structures had relatively little effect on β-galactosidase expression (even though this protein requires a large, unstable mRNA) as long as transcription rates were high. With lower transcription rates caused either by weaker promoter induction or by lower plasmid copy number, the 5′ hairpins did improve expression. These results can be compared with those of Lopez *et al.* [13•]. They truncated the C-terminal portion of RNaseE to inactivate its RNase activity, thereby significantly decreasing total mRNA degradation. In the mutated host, specific β-galactosidase accumulation increased by more than 20-fold from the T7 promoter (to 46,000 units/mg total protein). Interestingly, β-galactosidase accumulation was slightly lower in the RNaseE mutant relative to the wild-type cell when the *lac* promoter was used (3950 versus 4600 units/mg total protein). As the RNaseE mutants described by Lopez *et al.* are reported to grow well, they frequently may prove to be beneficial, especially when the highly processive T7 RNA polymerase is used.

Translation initiation

Although this is a factor that is often overlooked, it can have a huge impact on expression efficiency. Simmons and Yansura [4] used this to advantage to slow translation so that the secretion apparatus was not overwhelmed. Counterintuitively, higher product secretion and accumulation resulted from less effective ribosomal binding. As reviewed by Sprengart and Porter [14], translation initiation is affected by several factors. A consensus Shine–Dalgarno (SD) sequence and the proper spacing and sequence before the initiation codon are certainly beneficial. A downstream box (DB) may also be helpful [15]; however, an even more important factor may be possible mRNA secondary structures that block ribosome binding [16]. A single base change that affected secondary structure stability near the SD region caused a 500-fold change in the expression of the coat protein of RNA bacteriophage MS2. These secondary structures can possibly be disrupted by RNA helicases such as the DEAD protein of *E. coli*. Iost and Dreyfus [17] showed that overexpression of the DEAD protein stimulated β-galactosidase expression 30-fold from the T7 promoter, but not from the *lac* promoter. Without the DEAD protein overexpression, β-galactosidase production from the T7 promoter was tenfold lower than from the *lac* promoter even though transcription was tenfold faster.

Further experiments suggested that the DEAD protein stabilized the transcript, apparently independent of ribosome coverage. Yet, the *E. coli* DEAD-box proteins share important homologies with proteins having demonstrated RNA helicase activities [18,19]. It is still tempting to recommend testing the overexpression of these proteins for genes with suspected problematic mRNA secondary structure. Of course, an alternative approach is to modify the 5′ coding sequence (without changing the amino acid sequence) to discourage predicted secondary structure.

In vivo production: host design considerations

The complete sequence of the *E. coli* chromosome and a variety of genetic tools now allow almost any modification of our host organism. Bass, Gu and Christen [20] describe a modification of the method of Metcalf *et al.* [21] to allow the precise insertion of DNA into the chromosome without leaving a drug resistance or other marker. One obvious application is the insertion of expression cassettes as mentioned earlier. Another is modifying the chromosome to augment promoter control, as with the PhoS mutation, or to select for plasmid retention as with the Cobra method. Still more exciting, is the possibility of modifying the production cell for more efficient metabolism, for stabilization of the protein product, and for more efficient protein folding.

Improving host metabolism

Perhaps the most frequent target has been the reduction of acetate formation. For example, Chou, Bennett and San [22] decreased specific glucose uptake by inactivating the *ptsG* gene encoding the glucose-specific enzyme II of the phosphotransferase system (PTS). Other enzyme IIs could still mediate glucose transport, although at a lower rate. In this way, glycolytic flux was decreased to reduce acetate spillage from acetyl-CoA accumulation. A similar, but more dramatic approach was taken by Flores *et al.* [23]. They completely inactivated the PTS system to avoid phosphoenol pyruvate (PEP) hydrolysis by the PTS and then selected for increased glucose flux through the galactose permease transporter. Both approaches as well as the more traditional use of limited glucose feeding can slow glucose transport into the cell thereby avoiding acetyl-CoA accumulation. In a potentially complementary approach, Farmer and Liao [24] reduced acetate formation without limiting glucose consumption. The overexpression of PEP carboxylase (PPC) converted some of the PEP directly to oxaloacetate, bypassing acetyl-CoA. In addition, inactivating the *fadR* regulatory gene allowed the expression of the glyoxylate shunt enzymes to further increase anaplerotic pathway fluxes and avoid acetyl-CoA accumulation. Acetate formation was reduced significantly.

Several years ago, Dong, Nilsson and Kurland [25] reported that the expression of recombinant proteins in *E. coli* leads to a dramatic loss of ribosomes and cell viability. Also, it has been known for several years that *Vitreoscilla* hemoglobin promotes growth and protein production for microaerobic *E. coli* [26]. Recently, Nilsson *et al.* [27] reported that the *Vitreoscilla* hemoglobin increased intracellular ribosome and tRNA concentrations late in an oxygen-limited 30 h fermentation. The concentration of expressed β-lactamase was also increased. Even better performance was achieved by coexpressing a fusion protein comprised of *Vitreoscilla* hemoglobin linked to the FAD- and NAD-binding domains of the *Alcaligenes eutrophus* flavohemoprotein (FHP) [28]. This appears to be one means for preserving ribosome concentrations, but there is still a need for more fundamental solutions.

Another interesting host-cell manipulation was described by Rowe and Summers [29•]. They found that the overexpression of a small RNA called Rcd in *hns* mutant strains (to avoid production of the H-NS histone-like nucleoid structuring protein) produced a quiescent cell state. After Rcd induction, these cells significantly slowed their production of host proteins, but continued to express the recombinant product. Chloramphenicol acetyltransferase accumulated to more than 40% of the total cell protein. Although this approach needs to be demonstrated for high cell density production, it offers the attractive features of focussing cellular resources more fully on product synthesis and of possibly avoiding the secondary induction of proteases.

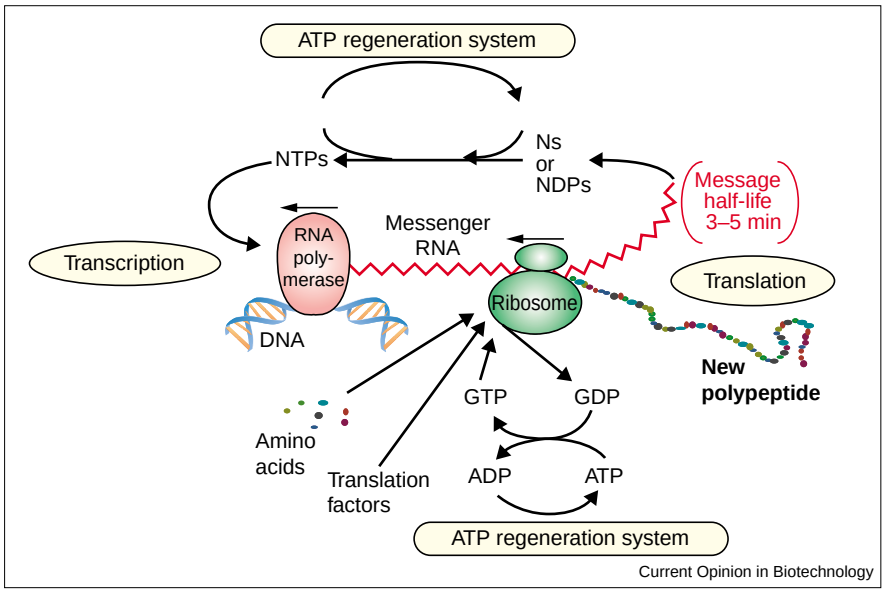
In vivo production: protein folding

Folding from inclusion bodies

Heterologous protein accumulation, either in the cytoplasm or periplasm of *E. coli*, often occurs in inclusion bodies. Evidently, hydrophobic sequences are not adequately protected by chaperonins and intermolecular interactions produce stable aggregates. These aggregates can usually be isolated by differential centrifugation and provide a useful concentration and purification step. In at least one case, however, periplasmic inclusion bodies could not initially be efficiently recovered from the lysed cells. Evidently, the inclusion bodies were entangled with the peptidoglycan. When the bacteriophage T4 lysozyme was expressed near the end of the fermentation, inclusion body recovery increased from less than 50% to nearly 100% following differential centrifugation [P2].

Obtaining properly folded protein from inclusion bodies requires control of aggregation and is often successful. Methods have been recently reviewed [30,31•]. In addition to a number of commercial pharmaceuticals (including a derivative of tissue plasminogen activator [t-PA] containing multiple disulfide bonds), success has been reported with multimeric proteins [32], truncated proteins [33] and *de novo* designed chemically synthesized proteins [34]. Finding the optimal refolding conditions is still relatively empirical, with the best results obtained from evaluating a matrix of conditions affecting solubility and disulfide-bond formation and isomerization. The use of either soluble or immobilized folding aids can improve results. One of the best examples is given by Altamirano *et al.* [35•]. They immobilized a minichaperone, a prolyl isomerase and the *E. coli* periplasmic oxidoreductase DsbA and improved the

Figure 1



Schematic to illustrate cell-free protein synthesis, showing the coupled processes of transcription and translation obtained with *E. coli* cell extracts. First, cells are grown and lysed and the cell extract prepared. Substrates and salts are then added to the extract and protein synthesis is initiated by adding the template.

folding yield of the scorpion toxin Cn5 from less than 5% to more than 85%.

Folding in the cytoplasm

It is still desirable, however, to obtain soluble, bioactive proteins without the requirement for refolding. Cytoplasmic folding is often enhanced at lower temperatures and the use of cold-inducible promoters may facilitate this approach [36]. In addition, the overexpression of intracellular chaperones has produced mixed but often encouraging results [37]. Probably the most exciting recent development is a system that allows efficient disulfide-bond formation and isomerization in the *E. coli* cytoplasm [38••]. Bessette *et al.* [38••] found mutants that grew well in spite of their inability to make active thioredoxin reductase and glutathione reductase. When the *E. coli* periplasmic protein disulfide isomerase DsbC was expressed in the cytoplasm by removing its secretion leader sequence, even complicated proteins such as a truncated t-PA with nine disulfide bonds could be expressed in an active form in this new mutant. G Georgiou announced at the 2000 American Institute of Chemical Engineers (AIChE) meeting in Los Angeles that this system is also showing promise for the production of Fab antibody fragments, especially when GroEL/GroES is overexpressed. The cytoplasmic space naturally provides a number of chaperonins as well as a supply of ATP.

Folding in the periplasm

It would seem that the periplasm is an ideal place to fold mammalian proteins because it already has the ability to form and isomerize disulfide bonds. Indeed, a number of secreted, disulfide-bond-containing proteins fold readily in the periplasm including human growth hormone and a very large number of single-chain Fv antibody fragments. In addition, even though the periplasm has not evolved to support the folding of large, complex proteins with many disulfide bonds, the folding environment can be modified; for example, the overexpression of DsbC facilitated the folding of even t-PA with 17 disulfide bonds [39]. However, in the case of insulin-like growth factor I (IGF-I) with three disulfide bonds, periplasmic overexpression of either DsbA or DsbC actually decreased the yield of folded product, although it significantly increased IGF-I accumulation in inclusion bodies [40]. Jeong and Lee [5••] have offered an impressive counter-example, accumulating soluble leptin in the periplasm up to 26% of the cell protein. They used a novel *Bacillus* endoxylanase signal peptide to improve translocation and overexpressed DsbA to increase folding.

The overexpression of other helper proteins may assist with some products. Skp overexpression as well as the periplasmic prolyl isomerase, FkpA, can help in the folding of secreted single-chain Fvs [41]. In many ways, the periplasmic space can be considered as a reaction vessel in which the proper environment, 'catalyst' concentrations, and substrate feed rates must be maintained for optimal protein folding.

Extracellular production

Many attempts have been made to selectively release recombinant proteins from *E. coli* into the surrounding medium. It has proven very difficult and, apparently, such systems have not been commercialized. In one recent example, the third topological domain of TolA was secreted into the periplasm [42]. When induced, however, most of the periplasmic proteins were released and the culture suffered a three order of magnitude loss in viability.

Another recent publication may suggest at least partial feasibility. Miksch *et al.* [43] have reported successful use of the *kil* gene of the *E. coli* ColE1 plasmid when controlled by the *fic* stationary phase promoter. The *kil* gene product mediates controlled export of periplasmic proteins. Apparently the system worked even better in *Klebsiella planticola*, producing large quantities of extracellular β-glucanase [44•]. The *kil* gene has been evaluated in the past with mixed results, and it will be interesting to know the range of proteins compatible with this new system.

Cell-free protein synthesis

Efficient protein synthesis

Cell-free methods appear to introduce a new degree of complexity because the cells must first be grown and the cell extracts prepared. However, the cell-free approach offers many potential advantages. Certainly, it can allow the synthesis of proteins toxic to cell division. It also allows most of the metabolic resources to be focussed only on product synthesis. More importantly, it provides incredible flexibility in manipulating protein synthesis and folding. The general approach is illustrated in Figure 1, and these methods are reviewed by Nakano and Yamane [45].

One of the most dramatic recent successes was reported by Kigawa *et al.* [46••]. Using the semicontinuous system described by Kim and Choi [47] (also called the continuous exchange cell-free [CECF] system by Spirin and associates [P3]), Kigawa *et al.* synthesized up to 6 mg/ml of chloramphenicol acetyl transferase (CAT). In this system, a larger volume of support reagents communicates with the reaction volume via a dialysis membrane. Consumable small molecular weight reagents are replenished and reaction products are removed. It should be noted that the Kigawa results required concentration of the cell extract, an incubation period of 20 h, and the use of a surrounding solution 20 times larger than the reaction volume. These factors increase production cost, but the large protein yield is still very impressive.

The results from Kigawa build upon the pioneering work of the Spirin laboratory [48] that demonstrated synthesis for 40 h in a continuous translation system. The continuous system demonstrated the potential robustness of the cell-free approach, but has proven to be rather cumbersome and expensive. The semicontinuous system offers high yields with a simpler format, but the excess volume of reagents adds significant expense, particularly for the nucleotides and energy source.

More recently, progress has been made with batch and fed-batch modes by carefully analyzing reaction stoichiometries and reaction sensitivities. Because the high-energy bond ATP regeneration reagents are expensive and because the released phosphate inhibits protein synthesis, a new system was developed using pyruvate and pyruvate oxidase for ATP regeneration [49]. Pyruvate is much less expensive than the other energy sources currently used, and the system recycles the phosphate to avoid accumulation; however, pyruvate oxidase requires oxygen and this poses a scale-up challenge. As an alternative approach, periodic additions of PEP, arginine, cysteine, tryptophan and magnesium allowed the standard PEP system to produce over 400 μg/ml of CAT [50•]. Addition of oxalic acid to inhibit PEP synthetase decreased the loss of ATP and further increased protein yields [51]. These results have since been extended by adding NAD and coenzyme A so that additional ATP can be generated from pyruvate, the product of PEP hydrolysis. The NAD and coenzyme A additions even allow high protein yields using glucose 6-phosphate as the energy source [52]. This approach has provided yields exceeding 1 mg/ml in a fed-batch mode. More importantly, the results indicate that ancillary metabolic pathways can be reactivated in the *E. coli* extracts in order to support protein synthesis.

As we are entering the era of 'proteomics', the ability to produce large numbers of proteins rapidly in a parallel manner is increasingly important, and many of the preceding cell-free synthesis techniques are amenable to high-throughput screening technologies. The ability to synthesize proteins directly from PCR fragments would help considerably. Several investigators have demonstrated feasibility; for example, Nakano *et al.* [53] have produced respectable yields from PCR products using a hollow-fiber membrane reactor. Further stabilization of the PCR-produced DNA templates will enable efficient batch and fed-batch production in multiplexed systems.

Protein folding with cell-free systems

A 1998 review has suggested considerable promise for obtaining bioactive proteins from cell-free systems [54]; for example, Ryabova *et al.* [55] reported that functional single-chain Fv antibody fragments were synthesized in a cell-free translation system. Using the flexibility offered by the cell-free format, they also showed that the addition of chaperonins and a glutathione redox buffer improved folding yields. More recently, Kolb *et al.* [56••] have described the cotranslational folding of firefly luciferase (62 kDa) in an *E. coli* translation system. They show very convincingly that the enzyme folds as it emerges from the ribosome, just as it presumably does in the eukaryotic cell. Such results underscore the potential for obtaining high yields of bioactive, complex proteins from this versatile system.

Conclusions

Although many alternative organisms and expression systems are now being used for recombinant protein production, exciting progress continues to be made with *E. coli*. Rapid growth and protein production rates combine with voluminous physiological knowledge and advanced genetic tools to make it one of the most powerful and versatile expression systems. The newly demonstrated ability to conduct oxidative protein folding in the cytoplasm and exciting new results from cell-free experiments now open entirely new opportunities to exploit this simple, but highly productive organism.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Kroeff EP, Owens RA, Campbell EL, Johnson RD, Marks HI: Production scale purification of biosynthetic human insulin by reversed-phase high-performance liquid chromatography. *J Chromatogr* 1989, 461:45-61.

2. Storrs SB, Przybycien TM: Commercial-scale refolding of recombinant methionyl bovine somatotropin. In *Protein Refolding*. Edited by Georgiou G, DeBernardez-Clark E. Washington, DC: American Chemical Society; 1991:197-205.

3. Baneyx F: Recombinant protein expression in ***Escherichia coli.*** *Curr Opin Biotechnol* 1999, 10:411-421.

4. Simmons LC, Yansura DG: Translational level is a critical factor for the secretion of heterologous proteins in ***Escherichia coli.*** *Nat Biotechnol* 1996, 14:629-634.

5. •• Jeong KJ, Lee SY: Secretory production of human leptin in ***Escherichia coli***. *Biotechnol Bioeng* 2000, 67:398-407.
An excellent example of combining a new secretion leader sequence, controlled expression rates and overexpression of a periplasmic helper protein in order to produce impressive quantities of soluble, bioactive leptin.

6. Jones KL, Keasling JD: Construction and characterization of F plasmid-based expression vectors. *Biotechnol Bioeng* 1998, 59:659-665.

7. Carrier T, Jones KL, Keasling JD: mRNA stability and plasmid copy number effects on gene expression from an inducible promoter system. *Biotechnol Bioeng* 1998, 59:666-672.

8. • Olson P, Zhang Y, Olsen D, Owens A, Cohen P, Nguyen K, Ye JJ, Bass S, Mascarenhas D: High level expression of eukaryotic polypeptides from bacterial chromosomes. *Protein Express Purif* 1998, 14:160-166.
This paper describes the construction and amplification of chromosomal expression cassettes. These appear to provide high and stable product expression without the need to maintain plasmids.

9. Williams SG, Cranenburgh RM, Weiss AME, Wrighton CJ, Sherratt DJ, Hanak JAJ: Repressor titration: a novel system for selection and stable maintenance of recombinant plasmids. *Nucleic Acids Res* 1998, 26:2120-2124.

10. Guzman L-M, Belin D, Carson MJ, Beckwith J: Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J Bacteriol* 1995, 177:4121-4130.

11. Siegele DA, Hu JC: Gene expression from plasmids containing the ***araBAD*** promoter at subsaturating inducer concentrations represents mixed populations. *Proc Natl Acad Sci USA* 1997, 94:8168-8172.

12. Carrier TA, Keasling JD: Library of synthetic 5′ secondary structures to manipulate mRNA stability in ***Escherichia coli***. *Biotechnol Prog* 1999, 15:58-64.

13. • Lopez PJ, Marchand I, Joyce SA, Dreyfus M: The C-terminal half of RNase E, which organizes the ***Escherichia coli*** degradosome, participates in mRNA degradation but not rRNA processing ***in vivo***. *Mol Microbiol* 1999, 33:188-199.
The authors suggest that protein expression can be improved by slowing the general rate of RNA degradation (apparently without slowing growth).

14. Sprengart ML, Porter AG: Functional importance of RNA interactions in selection of translation initiation codons. *Mol Microbiol* 1997, 24:19-28.

15. Etchegaray J-P, Inouye M: Translational enhancement by an element downstream of the initiation codon in ***Escherichia coli***. *J Biol Chem* 1999, 274:10079-10085.

16. De Smit MH, van Duin J: Secondary structure of the ribosome binding site determines translational efficiency: a quantitative analysis. *Proc Natl Acad Sci USA* 1990, 87:7668-7672.

17. Iost I, Dreyfus M: mRNAs can be stabilized by DEAD-box proteins. *Nature* 1994, 372:193-196.

18. Iost I, Dreyfus M, Linder P: Ded1p, a DEAD-box protein required for translation initiation in ***Saccharomyces cerevisiae,*** is an RNA helicase. *J Biol Chem* 1999, 274:17677-17683.

19. Linder P, Daugeron M-C: Are DEAD-box proteins becoming respectable helicases? *Nat Struct Biol* 2000, 7:97-99.

20. Bass S, Gu Q, Christen A: Multicopy suppressors of prc mutant ***Escherichia coli*** include two HtrA (DegP) protease homologs (HhoAB), DskA, and a truncated R1pA. *J Bacteriol* 1996, 178:1154-1161.

21. Metcalf WW, Jiang W, Wanner BL: Use of the ***rep*** technique for allele replacement to construct new ***Escherichia coli*** hosts for maintenance of R6Kγ origin plasmids at different copy numbers. *Gene* 1994, 138:1-7.

22. Chou C-H, Bennett GN, San K-Y: Effect of modified glucose uptake using genetic engineering techniques on high-level recombinant protein production in ***Escherichia coli*** dense cultures. *Biotechnol Bioeng* 1994, 44:952-960.

23. Flores N, Xiao J, Berry A, Bolivar F, Valle F: Pathway engineering for the production of aromatic compounds in ***Escherichia coli***. *Nat Biotechnol* 1996, 14:620-623.

24. Farmer WR, Liao JC: Reduction of aerobic acetate production by ***Escherichia coli***. *Appl Environ Microbiol* 1997, 63:3205-3210.

25. Dong H, Nilsson L, Kurland CG: Gratuitous overexpression of genes in ***Escherichia coli*** leads to growth inhibition and ribosome destruction. *J Bacteriol* 1995, 177:1497-1504.

26. Khosla C, Curtis JE, DeModena J, Rinas U, Bailey JE: Expression of intracellular hemoglobin improves protein synthesis in oxygen-limited ***Escherichia coli***. *Biotechnology* 1990, 8:849-853.

27. Nilsson M, Kallio PT, Bailey JE, Bulow L, Wahlund K-G: Expression of ***Vitreoscilla*** hemoglobin in ***Escherichia coli*** enhances ribosome and tRNA levels: a flow field-flow fractionation study. *Biotechnol Prog* 1999, 15:158-163.

28. Frey AD, Bailey JE, Kallio PT: Expression of ***Alcaligenes eutrophus*** flavohemoprotein and engineered ***Vitreoscilla*** hemoglobin-reductase fusion protein for improved hypoxic growth of ***Escherichia coli***. *Appl Env Microbiol* 2000, 66:98-104.

29. • Rowe DCD, Summers DK: The quiescent-cell expression system for protein synthesis in ***Escherichia coli***. *Appl Env Microbiol* 1999, 65:2710-2715.
This paper describes an interesting and potentially important approach to slow general protein expression so that more metabolic resources can be focused on product formation; the approach still needs to be demonstrated for high cell density production.

30. Lilie H, Schwarz E, Rudolph: Advances in refolding of proteins produced in ***E. coli***. *Curr Opin Biotechnol* 1998, 9:497-501.

31. • De Bernardez Clark E, Schwarz E, Rudolph R: Inhibition of aggregation side reactions during ***in vitro*** protein folding. *Methods Enzymol* 1999, 309:217-236.
An excellent description of fundamental methods for protein folding.

32. Tran-Moseman A, Schauer N, De Bernardez Clark E: Renaturation of ***Escherichia coli***-derived recombinant human macrophage colony-stimulating factor. *Protein Express Purif* 1999, 16:181-189.

33. Richter W, Hermsdorf T, Lilie H, Eferland U, Rudolph R, Kronbach T, Dettmer D: Refolding, purification, and characterization of human recombinant PDE4A constructs expressed in ***Escherichia coli***. *Protein Express Purif* 2000, 19:375-383.

34. Lilie H, Haehnel W, Rudolph R, Baumann U: Folding of a synthetic parallel beta-roll protein. *FEBS Lett* 2000, 470:173-177.

35. • Altamirano MM, Garcia C, Possani LD, Fersht AR: Oxidative refolding chromatography: folding of the scorpion toxin Cn5. *Nat Biotechnol* 1999, 17:187-191.
A strong example of immobilized effectors improving *in vitro* protein folding.

36. Mujacic M, Cooper KW, Baneyx F: Cold-inducible cloning vectors for low-temperature protein expression in ***Escherichia coli***: application to the production of a toxic and proteolytically sensitive fusion protein. *Gene* 1999, 238:325-332.

37. Thomas JG, Baneyx F: Divergent effects of chaperone overexpression and ethanol supplementation on inclusion body formation in recombinant ***Escherichia coli***. *Protein Express Purif* 1997, 11:289-296.

38. •• Bessette PH, Aslund F, Beckwith J, Georgiou G: Efficient folding of proteins with multiple disulfide bonds in the ***Escherichia coli*** cytoplasm. *Proc Natl Acad Sci USA* 1999, 96:13703-13708.
The authors describe an exciting new approach for the *in vivo* oxidative folding of complex proteins; altering the cytoplasmic redox potential and providing

disulfide isomerization catalysts allows the convincing production of proteins with multiple disulfides.

39. Qiu J, Swartz JR, Georgiou G: Expression of active human tissue type plasminogen activator in ***Escherichia coli***. *Appl Env Microbiol* 1998, 64:4891-4896.

40. Joly JC, Leung WS, Swartz JS: Overexpression of ***Escherichia coli*** oxidoreductases increases recombinant insulin-like growth factor I accumulation. *Proc Natl Acad Sci USA* 1998, 95:2773-2777.

41. Bothmann H, Pluckthun A: The periplasmic ***Escherichia coli*** peptidylprolyl ***cis***,***trans***-isomerase Fkp I: increased functional expression of antibody fragments with and without ***cis***-prolines. *J Biol Chem* 2000, 275: 17100-17105.

42. Wan EW, Baneyx F: TolAIII co-overexpression facilitates the recovery of periplasmic recombinant proteins into the growth medium of ***Escherichia coli***. *Protein Express Purif* 1998, 14:13-22.

43. Miksch G, Neitzel R, Fiedler E, Friehs K, Flaschel E: Extracellular production of a hybrid β-glucanase from ***Bacillus*** by ***Escherichia coli*** under different cultivation conditions in shaking cultures and bioreactors. *Appl Microbiol Biotechnol* 1997, 47:120-126.

44. • Miksch G, Neitzel R, Friehs K, Flaschel E: High-level expression of a recombinant protein in ***Klebsiella planticola*** owing to induced secretion into the culture medium. *Appl Microbiol Biotechnol* 1999, 51:627-632.
Expression of the *kil* gene with a stationary phase promoter allowed product release to the surrounding medium in good yields.

45. Nakano H, Yamane T: Cell-free protein synthesis systems. *Biotechnol Adv* 1998, 16:367-384.

46. •• Kigawa T, Yabuki T, Yoshida Y, Tsutsue M, Ito Y, Shibata T, Yokoyama S: Cell-free production and stable-isotope labeling of milligram quantities of proteins. *FEBS Lett* 1999, 442:15-19.
The authors demonstrate the potential of cell-free protein synthesis. Although the yield is impressive, there is still much room for improvement.

47. Kim D-M, Choi C-Y: A semicontinuous prokaryotic coupled transcription/translation system using a dialysis membrane. *Biotechnol Prog* 1996, 12:645-649.

48. Spirin AS, Baronov VI, Ryabova LA, Ovodov SY, Alakhov YB: A continuous cell-free translation system capable of producing polypeptides in high yield. *Science* 1988, 242:1162-1164.

49. Kim D-M, Swartz JR: Prolonging cell-free protein synthesis with a novel ATP regeneration system. *Biotechnol Bioeng* 1999, 66:180-188.

50. • Kim D-M, Swartz JR: Prolonging cell-free protein synthesis by selective reagent additions. *Biotechnol Prog* 2000, 16:385-390.
The authors use straightforward stoichiometric analysis and fed-batch protocols for significant yield improvement from simple cell-free protein synthesis systems.

51. Kim D-M, Swartz JR: Oxalate improves protein synthesis by enhancing ATP supply in a cell-free system derived from ***Escherichia coli***. *Biotechnol Lett* 2000, 22:1537-1542.

52. Kim D-M, Swartz JR: Regeneration of ATP from glycolytic intermediates for cell-free protein synthesis. *Biotechnol Bioeng*, in press.

53. Nakano H, Shinbata T, Okumura R, Sekiguchi S, Fujishiro m, Yamane T: Efficient coupled transcription/translation from PCR template by a hollow-fiber membrane bioreactor. *Biotechnol Bioeng* 1999, 64:194-199.

54. Jermutus L, Ryabova LA, Plukthun A: Recent advances in producing and selecting functional proteins by using cell-free translation. *Curr Opin Biotechnol* 1998, 9:534-548.

55. Ryabova LA, Desplancq D, Spirin AS, Plukthun A: Functional antibody production using cell-free translation: effects of protein disulfide isomerase and chaperones. *Nat Biotechnol* 1997, 15:79-84.

56. •• Kolb VA, Makeyev EV, Spirin AS: Co-translational folding of a eukaryotic multidomain protein in a prokaryotic translation system. *J Biol Chem* 2000, 275:16597-16601.
A convincing demonstration that cotranslational folding can occur efficiently during cell-free protein synthesis and is much more effective than post-translational *in vitro* protein folding.

Patents

P1. Bass S, Swartz JR: Method of controlling polypeptide production in bacterial cells. US Patent 5,304,472, 1994.

P2. Leung W-LS, Swartz JR: Process for bacterial production of proteins. US Patent 6, 180,367, 2001.

P3. Alakhov JB, Baronov VI, Ovodov SJ, Ryabova LA, Spirin AS, Morozov IJ: Method of preparing polypeptides in cell-free translation system. US Patent 5,478,730, 1995.