



# Bacterial Systems Breed New Expectation

Dr Søren M Madsen and Sean A MacDonald, Bioneer A/S consider how to meet the production requirements of the next generation of protein therapeutics



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Advances in molecular biology have resulted in the sequencing of several hundred complete genomes including, perhaps most notably, that of humans. Ever-increasing information on the genes and proteins involved in disease have led to a large number of novel potential drug targets and an even larger number of new molecules being tested against those targets. Proteins are becoming more desirable as potential therapeutics to help overcome some of the shortcomings of NCEs, as well as to help the biotechnology and pharmaceutical industries to restock their pipelines with promising new candidates.

The increasing demand and variety of pharmaceutical proteins requires a production process that is safe, economically viable, scalable to industrial dimensions, and that ultimately results in a pure product. Proteins are too complex to be produced by chemical synthesis, and isolation from natural sources often results in low yields and has inherent safety risks. Past experience has shown that protein production in recombinant hosts is often the only cost-effective way to generate sufficient yields to make products commercially viable. As the industry continues to face additional price pressure, finding the ideal match of protein and recombinant host organism becomes all the more important in order to maximise the production yields.

Since the approval of human insulin produced in *Escherichia coli*, a Gram negative bacteria, almost 25 years ago, more than 100 new proteins produced using recombinant DNA technology have been approved for human administration (1). The majority of these biopharmaceuticals have been produced in either *E coli*, or *Saccharomyces cerevisiae*, more commonly known as baker's yeast. These microorganisms have been model organisms for scientists since the beginnings of

molecular biology, and as a consequence, well established genetic tools and fermentation technologies have been developed. Also, due to their extensive use in the manufacturing of recombinant proteins, regulatory authorities around the world are familiar with the safety risks of these expression platforms, thus removing one more unknown when trying to get new products approved.

Driven by the shortcomings of *E coli* and *S cerevisiae*, scientists began making use of other production hosts, such as mammalian cell lines like Chinese hamster ovary cells (CHO). Mammalian cell lines, despite being more difficult to handle and maintain, are still preferred for production of complex glycosylated proteins, where the generation of an authentic product is essential and the complexity cannot be handled by less 'sophisticated' microbial systems.

The increase in the number and diversity of new recombinant proteins, coupled with the commercial pressures that the industry is facing to keep prices down, has led groups to diversify production systems. There are now a variety of technologies that aim to harness the protein expression capacity of insect cells, transgenic plants and animals, as well as new improved variations on the 'basic three' bacteria, yeast and mammalian cells.

## REVIEWING THE EXPRESSION PLATFORM

The choice of an expression system is a key decision in the development of a new protein biopharmaceutical. Every system has its advantages and drawbacks; each has an impact on production for a specific protein. As such, general

guidelines are available to assist with selection, but predictions of success are impossible. This makes it important to match the right expression system to the protein of interest through experimentation. Although it requires more short-term effort and resources, it is recommended that researchers try to find this match early in development and examine a variety of different expression platforms in parallel. This is important in order to identify the organism that has optimal performance with respect to the quantity and quality of the recombinant protein. The added short-term cost is offset by the minimised risk of time and cost problems later on in the development process, which can potentially have a much more significant impact.

Despite the fact that approved biopharmaceuticals have been produced in a limited number of organisms, the current literature is full of new and unexploited expression platforms that will probably have potential in the production of future biopharmaceutical proteins. Even with the introduction of these new systems, bacteria remain important in the protein expression 'arsenal' due to their scalability, ease of use and regulatory acceptability. A large number of expression platforms based on Gram negative, as well as Gram positive, bacteria have been described in the literature (*Pseudomonas*, *Caulobacter*, *Lactococcus*, *Lactobacillus*, *Staphylococcus*, and so on) and provide various benefits and drawbacks depending on the specific features of the protein of interest.

In this review, we examine three bacterial gene expression systems: namely *E coli*, still the 'gold standard'; and two new promising bacterial alternatives – the Gram negative *Pseudomonas fluorescens* and the Gram positive *Lactococcus lactis*. These two systems still harness the advantages of bacterial expression, but offer some alternative features that can be attractive in specific cases where *E coli* may still work, but is not the ideal choice.

#### ***E COLI*: THE 'GOLD STANDARD'**

*E coli* was the first organism to be used to produce proteins recombinantly. Since then, a large number of biopharmaceutical proteins produced in *E coli* have successfully reached the market (1,2). This organism is well known throughout both academia and industry, and the vast majority of scientists working in the biotechnology industry have been exposed to it at some point in their careers. Its popularity has led companies like Novagen and Invitrogen to develop and commercialise plasmids and host strains with specialised features to meet the needs of different situations.

#### Molecular Biology

Several plasmids with inducible promoters, like the T7 phage promoter induced by isopropyl-thiogalactopyranoside (IPTG) or the pBAD promoter controlled by L-arabinose, are commercially available. Despite the very strong and elegant

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T7 promoter system, problems regarding promoter leakiness have been observed, which is detrimental in the case of a toxic gene product. Another drawback of this system is the induction of the T7 promoter. The T7 polymerase that recognises the T7 promoter is induced by addition of IPTG to the culture. This compound is not ideal due to its high cost and toxicity. In contrast, the *pBAD* promoter controlling the *araBAD* operon results in high level expression, tight regulation and cost-effective induction. A variety of fusion partners used for detection and purification (his-tag, GST-tag, and so on) have been engineered into the plethora of expression vectors that are now available. A number of optimised host strains are available and include: strains deficient in proteases and strains that facilitate disulfide formation, as well as strains that overexpress certain tRNA molecules that could otherwise be in short supply and thereby hamper protein production.

### Production

In *E coli*, recombinant proteins usually accumulate in the cytoplasm, and examples where recombinant protein constitutes up to 30 per cent of total cellular protein can be found in the literature (3). However, excessive production is not without drawbacks, as the recombinant protein will sometimes misfold and aggregate into so-called inclusion bodies. While inclusion body formation might be advantageous in some cases due to resistance to proteolytic degradation, the subsequent solubilisation and refolding of the inclusion bodies is expensive and results in reduced yield. Furthermore, Gram negative bacteria like *E coli* contain lipopolysaccharides (LPS) or endotoxins in their cell wall. Recovery of the recombinant protein from the cytoplasm results in the release of the pyrogenic endotoxins, which must

be removed from the final product. This adds to the cost of the downstream process.

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Due to its aerobic lifestyle, *E coli* can reach cell densities far above 100g/L of dry weight (DW) during controlled fed-batch fermentations. However, its aerobic lifestyle also creates the need for a carefully designed feeding strategy to avoid problems with solubility or precipitation of media components, as well as growth inhibition due to formation of metabolic byproducts. The yield of recombinant protein in *E coli* can reach the 1-10g/L scale (2).

### A GRAM NEGATIVE ALTERNATIVE: *PSEUDOMONAS FLUORESCENS*

Recently a new, interesting expression platform based on the Gram negative bacterium *P fluorescens* was described (4). Strains of *P fluorescens* are usually found outside in the soil, water or on the surfaces of plants. It is a non-pathogenic bacterium and American Tissue Culture Collection has designated strains of *P fluorescens* as Biosafety Level 1 – making it an attractive production organism when trying to navigate the regulatory system. However, this expression platform still suffers from the presence of endotoxins, which creates an extra burden for the purification of the protein.

### Molecular Biology

The host strains are derived from *P fluorescens* biovar I (MB101) and can be grown to high cell densities in



bioreactors (DW>100g/l) using a simple defined media containing mineral salts, ammonia and glucose without oxygen enrichment. A number of expression vectors that are stable, non-conjugative and of varying copy number have been developed. Heterologous gene expression is controlled by promoters such as *tac* or *lac*, which permits induction by IPTG or lactose. Furthermore, native *P fluorescens* promoters that are better suited to industrial scale fermentations have been identified. Two promoters driving expression of enzymes that degrade aromatic hydrocarbons were isolated and shown to respond to sodium benzoate and anthranilate. The potential of these two promoters for heterologous protein production were demonstrated in 20L scale (5).

Plasmids and strains have been developed that eliminate the necessity for using antibiotic resistance genes for maintenance of the expression vector. Essential genes involved in the intermediary metabolism were placed on the expression vector and the corresponding genes on the chromosome were deleted, resulting in the same yield in 20L scale fermentation when compared to a fermentation using antibiotic selection (4).

#### Production

*Pseudomonas* has the capability to secrete proteins both to the periplasm as well as to the extracellular environment using different types of secretion pathways. Once again, this possibility could save handling time and costs in downstream processing. However, so far most data concerning the system has been restricted to cytoplasmic and periplasmic production of recombinant proteins.

A number of proteins were expressed in *P fluorescens* and compared side-by-side with the T7 expression platform of *E coli*. Intracellular expression of human gamma interferon (IFN) results in 2-4g/l of insoluble protein in *E coli*, whereas the *P fluorescens* system produced 4g/l and approximately 95 per cent was found in a soluble and fully active form (4). Similarly, intracellular expression of a single chain antibody, gal13, was increased from 0.5g/l in *E coli* to 4g/l in *P fluorescens*. While only 48 per cent was found in a soluble form in *E coli*, the corresponding number in *P fluorescens* was 96 per cent. Expression of an industrial enzyme, nitrilase was produced at 25g/l and corresponded to more than 50 per cent of total cell protein, clearly demonstrating the power of the *Pseudomonas* system (6).

#### A GRAM POSITIVE ALTERNATIVE: *LACTOCOCCUS LACTIS*

Another promising expression platform is based on the Gram positive bacteria *L lactis*. It has a long history in the food industry and has attained generally recognized as safe (GRAS) designation by regulatory authorities worldwide. This organism is easy to handle and, with improved understanding on the genetic level, the last couple of decades has resulted in the development of a number of gene expression systems based on *L lactis* (7,8). In contrast to Gram negative bacteria, *L lactis* does not produce endotoxins and inclusion body

formation has not been described, making downstream processing less complex.

#### Molecular Biology

As a Gram positive organism, there is only one cell membrane allowing heterologous proteins to be secreted directly to the extracellular milieu using the *sec*-pathway. The fact that *L lactis* only secretes a limited number of endogenous proteins, as well as few native proteases, makes the fermentation broth relatively 'clean', and the recombinant protein is accessible for simple purification. A number of inducible promoters from *L lactis* have been isolated and used in the design of new expression platforms (7,8).

The NICE system is based on the auto-inducible *nisA* promoter and two cognate genes involved in the sensing of the inducer nisin. This system acts very like IPTG inducible promoters in *E coli* or *P fluorescens*. Increasing amounts of nisin result in a linear dose-response curve with respect to the yield of recombinant protein. The NICE system has been used for production of a variety of proteins, and in a recent optimisation study the yield of an antimicrobial protein, lysostaphin, was increased from 100mg/l to 300mg/l by systematically optimisation of the fermentation and induction conditions (9).

Another *L lactis* expression platform is based on an auto-inducible promoter: P170 (7,10). The P170 promoter was originally described to be induced by low pH in the transition to stationary phase. Induction of promoter activity at this time point is advantageous, as it separates production of biomass from the phase where production of recombinant proteins takes place. *L lactis* is a fermentative organism where sugar consumption gradually increases the concentration of lactate in the culture. Physiological characterisation of the promoter showed that the real inducer of P170 was the lactate concentration. The P170 Expression System is induced when a certain threshold of lactate is reached in the culture. This type of auto-induction eliminates the need for the addition of exogenous components (such as IPTG, sodium benzoate, anthranilate, nisin, and so on) to induce recombinant protein production. Optimised P170 promoter variants have been combined with optimised signal peptides, resulting in secretion of the recombinant proteins. A number of expression vectors of varying copy number is available, which includes a non-antibiotic based plasmid selection are available.

#### Production

A fermentation medium composed of non-animal derived components, which sustains growth up to 200D<sub>600</sub> units (DW ~6g/l) in batch fermentations, is available. However, the production of lactic acid – the primary end product of glucose metabolism – will have a limiting effect on biomass production in *L lactis*. Lactic acid inhibits growth even when the acid is neutralised by the addition of base to keep pH constant. As a consequence, the yield in biomass production is below that of other expression systems with cell densities of approximately

200D<sub>600</sub> units. With this limited cell density, the expression system has reached 300mg/L of secreted protein, which is similar to the levels obtained using the NICE system previously described. Although this level is acceptable for some high value proteins, in most cases higher production levels are desirable.

New technologies have recently helped to overcome this cell density problem, the most successful of which is the Reverse Electro Enhanced Dialysis (REED) process developed by JURAG Separations A/S located in Denmark. Using a membrane filtration technology, low-molecular weight negative ions like lactate can be removed on a continuous basis from the fermentation broth. In this way, both pH and lactate content in the culture are kept constant. By applying this technology, the exponential growth phase is prolonged, resulting in a substantial increase in the final yield of biomass and recombinant protein. The effect of the REED unit resulted in a nine-fold increase in biomass and an increase for 300mg/L to 2,000mg/L for a secreted test protein.

## CONCLUSION

Although useful in many cases, *E coli* is not a generic solution to the industry's protein production problem, and that the diversity of new proteins being discovered demands the application of alternative systems. There are other practical alternatives that offer many of the same benefits of *E coli* as a bacterial system – namely scalability, regulatory 'acceptability', and overall low costs of production – but also offer some advantages in cases where the current 'gold standard' may not be the optimal system. With price pressures across all markets impacting not just generic drugs but patented ones as well, it's important for the industry to shift from using *E coli* voluntarily, rather than being forced to change once they realise that it is not ideal for large scale production. The cost and complication of switching production systems at a later stage of development can result in major delays, which can have consequences ranging from increased costs for larger companies, to losing partners or backers for smaller biotech companies.

It is therefore important for biotechnology companies seeking to develop protein therapeutics to reconsider the protein production issue – it is time to focus on finding the right system from the start in order to prevent problems developing. Rather than letting *E coli* be one of the determining factors in which product to bring forward, find the system to match the most promising therapeutic candidates in the pipeline. With advances being made in alternative bacterial expression systems that are being actively marketed and distributed by a variety of different groups, there is really no reason to settle for a 'one size fits all' paradigm in an industry that requires tailormade solutions. Bacterial systems are not the ultimate solution either, and testing of more complex alternatives, despite their added difficulties of use, scalability, cost and regulatory issues, should be explored. ♦

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