Biodrugs 2004; 18 (1): 51-62
1173-8804/04/0001-0051/$31.00/0

Expression Systems for the Production of Recombinant Pharmaceuticals

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Abstract

The new generation of biological products are largely the result of genetic engineering. The qualitative and quantitative demand for recombinant proteins is steadily increasing. Molecular biologists are constantly challenged by the need to improve and optimise the existing expression systems, and also develop novel

approaches to face the demands of producing the complex proteins of tomorrow. This continuous evolution is paralleled by growing concerns about the safety of these novel pharmaceuticals, with health authorities setting high standards for certification. One of the strategies used by researchers in this field involves sourcing new genetic elements for incorporation into expression systems by systematically analysing the rich natural diversity of microorganisms and plant-based expression systems. There are, in addition, numerous tools for modifying microorganisms and for re-engineering existing biological pathways or processes to meet the needs of the pharmaceutical industry. The aim of this review is to present the conventional and alternative expression systems, focusing on prokaryotic expression systems and briefly exploring other complementary recombinant protein production systems and their unique features.

Technologies based on genetic recombination are paving the way towards the therapeutic usage of complex molecules. An increasingly large number of biological products in current use are recombinant proteins. These proteins are proving useful in numerous areas; from the treatment of hemophilia to complex cancer immunotherapies targeting effector molecules.

The number of vaccine formulations consisting of or containing a recombinant protein is presently very limited. Nevertheless, the design of potential vaccines and screening strategies could open the way for extensive use of recombinant antigens in the near future. Meanwhile, evolving standards of health authorities, such as limiting the use of antibiotics, has further encouraged the design of new systems.

The most commonly described expression systems are still prokaryotic or mammalian cell based. Significantly, alternative host/vector combinations or new hosts such as Gram-positive bacteria, novel yeast strains, filamentous fungi and plant-based systems are progressively invading a field traditionally occupied by *Escherichia coli* and mammalian cells. Recently, *in vitro* systems such as continuously-fed cell-free translation (CFCF) are being used for some specific applications. The present review focuses on prokaryotic expression systems and briefly explores other complementary recombinant protein production systems. (see table I).

1. Prokaryotic Systems: General Considerations

1.1 Current Status

A large number of prokaryotic expression vectors have been described and are available either commercially or through collaborations with research laboratories. Among these only a limited number are suitable for industrial use. For example, its hard to imagine the well-described PR or PL promoter from λ phage under the control of a temperature-sensitive repressor being suitable for a large-scale fermentation process. Additionally, regulatory concerns would prohibit the use of toxic inducers or components of animal origin in a culture medium.

Intellectual property ownership is another consideration; some expression systems or genetic constitutive elements have been described for more than 20 years, while others have never been patented. Moreover, different components of a given system can be described in different patent applications not necessarily originating from the same company or laboratory.

1.2 Strains

A large number of bacterial hosts have been selected and/or modified to improve recombinant protein expression. Generally these strains are defective in proteases such as Lon or the outer membrane protease, OmpT. The preferred choice has been BL21, and the sister strain BL21 λDE3 is suitable for expression vectors containing the T7-RNA polymerase promoter.

1.3 T7, Arabinose and Others

The most popular expression systems in current use are T7 or its derivatives.[1] The T7 promoter is very efficient and is easily regulated in appropriate environments. For example, it is down-regulated in the presence of another plasmid expressing the T7 lysozyme, a natural inhibitor of T7-RNA polymerase. The original system using the strain BL21 λDE3 is now complemented by a wide range of strains or vectors[2] allowing for the addition of Tags and leader sequences, for the expression of fusions or for intracytoplasmic disulfide-bond assembly.

The intrinsic feature of the T7 promoter of immediate, high expression upon induction[3] makes it less attractive for toxic antigens and proteins with a propensity to form inclusion bodies.

The arabinose promoter is tightly regulated and is used when an expression level requires fine tuning; it can be considered as complementary to T7.[4] Stringent control of the expression is

Table I. Summary of the most commonly available expression systems (only major advantages and drawbacks are presented)

System	Advantages	Drawbacks	Scale-up	Cost effectiveness	Stage of development
Prokaryotic					
Escherichia coli	High yield	No post-translational modifications	Very good potential	Low to moderate	Production
	Large choice of genetic elements	Secretion difficult			
Bacillus	Secretion	No post-translational modifications	Very good potential	Low to moderate	Production/development
	Low protease				
Caulobacter crescentus	Surface display	No post-translational modifications	Very good potential	Low to moderate	Research/development
	Easy purification				
Lactobacillus zeae	Secretion	No post-translational modifications	Very good potential	Low to moderate	Development
Exotic hosts (cold bacteria)	Adapted to temperature-sensitive products	Restricted to specific applications	Unknown	Unknown	Research
Eukaryotic					
Mammalian cells	Secretion	Additives	Good potential	High to very high	Production
	Suitable for complex molecules	Low yield			
Insect cells	High yield	Glycosylation profile	Good potential	High	Production/development
	Simple media				
	Viral safety				
Vegetal	Biomass	Glycosylation profile	Unlimited	Low	Production/development
	Secretion				
	Viral safety				
Yeast	Biomass	Glycosylation profile	Very good potential	Low to moderate	Production
	Secretion				
Nonconventional yeast	Growing capacity in extreme conditions/waste material	Genetic still needs to be explored	Good potential (to be confirmed)	Low to moderate (to be confirmed)	Research
Trypanosome	Mammalian-like glycosylation	Genetic still needs to be explored	Medium potential (to be confirmed)	High to very high	Development
Transgenic animals	Suitable for complex molecules	Time consuming	Related to animal size	Very expensive	Research/development
		Restricted to very high added-value products			
Cell-free translation					
CECF/CFCF systems	Suitable for toxic molecules	Glycosylation	100mg range	High	Research
	Incorporation of unnatural amino acids	Disulfide bond			

CECF = continuous-exchange cell-free; **CFCF** = continuous-flow cell-free.

however only possible for re-engineered strains with a genetic knockout in the arabinose locus.

The tryptophan (Trp) promoter has also been extensively used for the large-scale production of proteins. The patent-free status of the Trp promoter makes it attractive,[5] the only potential problem being a marked leakage of expression that is difficult to control even in the presence of large amounts of Trp in the culture medium. A tight regulation of the Trp promoter is obtained with the ICONE 200 (Improved Cell for Over and Nonleaky Expression) strain, a genetically engineered *E. coli* mutant.[6] The capacity to express a wide range of proteins including toxic proteins such as HIV-1 protease and poliovirus 2B protein has been demonstrated.

1.4 Auto-Inducible Systems

Auto-induction of expression occurring near the end of the log growth phase is definitely an advantage, since the process does not require the operator's intervention or the addition of inducers. Several models have been described making use of quorum sensing,[7] or taking advantage of so-called auto-inducible promoters, which are upregulated upon changes in the culture medium (see *Lactococcus lactis* in section 4.1), or which control a stationary-phase inducible gene.[8]

The exploitation of cell-cycle regulator RNA to generate quiescent-cell expression in *E. coli* has been used for protein overexpression.[9] A small RNA called regulator of cell division *(Rcd)* is encoded within the *Cer* locus. *Rcd* causes a reversible arrest of the cell cycle, leaving time for multimer resolution. *Rcd*, when overexpressed, can improve production efficiency by generating a quiescent stage. Quiescent cells no longer produce biomass, but have their metabolic resources directed towards the expression of plasmid-borne genes.

1.5 Absolute Downregulation

An elegant and original way to overcome the problem of expression leakage was proposed by a group from the University of Gdansk, Poland.[10] The latest version of their expression vector consists of a construct in which the gene of interest, flanked by convergent attP and attB sites derived from the phage λ, is cloned in reverse orientation defining an 'off' position. In the 'off' position, the cloned gene can not be expressed, and only its antisense mRNA can be produced. A change in orientation, allowing for expression, occurs upon induction of the phage λ recombinase expressed by the host cell. This places the gene under direct control of the P(tac)/P(lac) promoters, resulting in very high expression.

1.6 Codon Bias

Codon bias is often considered a limiting factor potentially affecting the expression level of a given recombinant protein. Some transfer RNAs (tRNAs) corresponding to arginine, proline, isoleucine, and leucine are found in very limited amounts in *E. coli*. Different approaches can be envisaged to overcome this problem: one of them makes use of strains engineered or cotransformed with an accessory plasmid[11,12] to contain extra copies of tRNAs rarely found in *E. coli*. The application of this method resulted in a marked increase of expression in the case of plasmodium or other AT-rich parasite genes.[13] Other alternatives involve constructing a 'synthetic gene' in which the majority of rare codons have been replaced by the ones more frequently used in *E. coli*. Synthetic gene constructs can be obtained either by systematic site-directed mutagenesis or by the stepwise assembly of overlapping oligonucleotides. In some cases surprising and undesired effects have been obtained. For example, in a work described by Lammertyn et al.,[14] codon adjustment led to decreased mRNA stability and protein yield. Finally, it seems that the relationship between heterologous expression and codon bias is not as simple as it is sometimes suggested,[15] and may be sequence context dependent.[16]

A given microorganism can have different codon usages associated with classes of genes,[17] representative of different groups of proteins[18] or growth phases.[19] A recent study presented a mathematical model capable of predicting gene expression levels from codon biases in α-proteobacterial genomes.[20]

It is important to note that codon bias should not be perceived as the only factor affecting expression, but be considered within a wider context that includes mRNA secondary structure.[21]

1.7 Folding

A large number of recombinant proteins have the propensity to misfold when overexpressed in *E. coli*, resulting in an accumulation of cytoplasmic inclusion bodies. Chaperones constitute a heterogeneous group of molecules that mediate the folding and assembly of proteins.[22] Among characterized chaperones are heat-shock proteins such as Hsp70, or others like GroEL/GroES, DnaK/DnaJ/GrpE exhibiting distinct reaction mechanisms.[23] These can be used both *in vivo* and *in vitro*, although rational use of chaperones individually or in combination requires a dedicated protocol. Details of the pathway involved in chaperone function

that would permit further fine-tuning of the process are not fully elucidated.

This is illustrated in a recent study,[24] which describes the chaperone-assisted expression of recombinant nitrile hydratase in *E. coli*. In this system, the subunits of GroES and GroEL are expressed simultaneously using a cotransforming plasmid that confers additional antibiotic resistance. A potential drawback is the subsequent over-solicitation of the expression machinery, which may lead to an undesired increase in chaperone production at the expense of the recombinant protein.

1.8 Fusions

Fusion can provide a shortcut to boost expression levels, increase solubility or facilitate the downstream purification process. A number of fusion partners, such as the maltose-binding protein domain product of Mal E, glutathione S-transferase (GST), or thioredoxin have been extensively used and are easily accessible as components of commercially available vectors. Other less conventional fusion partners, such as nuclease A from *Staphylococcus aureus*, are dedicated to eukaryotic membrane proteins.[25]

Short-sequence tags expressed either as N-terminal or C-terminal fusions enable easy and efficient purification procedures.[26,27] The usefulness of such fusions has been assessed in large-scale screening procedures as well as in the context of laboratory production. Obviously, these extra sequences have to be excised from the final product if is to be used as an injectable therapeutic or vaccine. Numerous methods for precise excision of tag sequences have been described, making use of proteases (thrombin, enterokinase, etc.) or chemicals (iodobenzoic acid, cyanogen bromide or Cu++ ions). In some cases original target sites for cleavage are enhanced through site-directed mutagenesis.[28] Current techniques based on the action of site-specific proteases, such as factor Xa, are quite inefficient, expensive, and may be inapplicable if internal protease target sites are present. There remains a need for an efficient tag excision technique. Nevertheless, several attractive systems have been described, such as the biotin-ubiquitin tagging[29] or the IMPACT™[1] (Intein Mediated Purification with an Affinity Chitin-binding Tag) system making use of a self-cleavable fusion partner and thus not requiring any additional protease treatment.[30]

The expression of molecules devoid of additional amino acids or tags requires a dedicated purification process. Production of unmodified molecules can be accomplished via another category of vectors termed 'bicistronic'. A good illustration of this is seen in a study describing the production of an 'authentic' human interleukin (IL)-18.[31]

Bicistronic vectors are also designed to directly enhance the expression of poorly expressed proteins without the help of a fusion partner. In these constructs, a sequence encoding a short peptide efficiently expressed in *E. coli* is linked to the 5'end of the gene of interest, strictly as a transcriptional fusion. As an example, the production of bovine pancreatic ribonuclease has been rescued in such a way.[32] More recently, AT-rich genes from *Pyrococcus furiosus*, have been overexpressed thanks to the introduction of an overlapping leader open-reading frame.[33]

2. Prokaryotic Systems: Antibiotic-Free Systems

The increasing regulatory requirements to which biological agents are subjected will have a great impact in the field of recombinant protein expression and production. There is an expectation that in the near future, there may be 'zero tolerance' towards antibiotic-based selection and production systems. Besides the antibiotic itself, the antibiotic resistance gene is an important consideration. Kanamycin and tetracycline, to a lesser extent, are still acceptable to the health authorities. In contrast, the use of β-lactams, which are often responsible for allergic responses, is strictly prohibited. In addition, the complete absence of antibiotic-resistance genes is the only way to ensure that there is no propagation in the environment or transfer of resistance to pathogenic strains.

Different approaches can be envisaged to overcome the need for antibiotics in the process of recombinant protein production, including increasing plasmid stability and maintenance during the fermentation process, and the use of alternative, 'antibiotic-free' selection markers.

2.1 Plasmid Stabilization

Plasmid stabilization can be achieved by insertion of a genetic element such as the *Cer* locus, which allows stable inheritance of ColE1 and related plasmids by preventing the runaway accumulation of multimers known as 'dimer catastrophe'. Multimer resolution is achieved through action of the XerCD site-specific recombinase at the *Cer* site.[34]

Cloning of the *Cer* locus into various expression vectors has been extensively documented, and the proof of concept largely established in high-cell density cultures.[35]

1 The use of tradenames is for product identification purposes only and does not imply endorsement.

Other systems, such as *Hok/Sok*, described for the first time in 1986,[36] are based on post-segregational killing of plasmid-free cells. The translation of the *Hok* (host killing) messenger, encoding a toxin lethal to the bacteria, is completely blocked by the antimessenger *Sok* (suppression of killing). In the absence of plasmid, *Sok*, which is not as stable as *Hok*, is lost first, allowing the translation of the *Hok* mRNA and expression of the toxin. Other stabilization elements derived from the *par* locus have been reported.[37]

2.2 Nonantibiotic Selection

The most common way to achieve selection in the absence of antibiotics is via complementation of an essential gene utilising an expression vector in a strain with a defect in the same essential gene. The *dapD* gene, which has a role in the lysine biosynthetic pathway as well as cell wall assembly, has been selected as a candidate by several authors. Mutations in the dap pathway are lethal.[38] A very promising system was recently described in the literature: the so-called 'operator repressor titration for antibiotic-free plasmid maintenance' proposed by Cobra Therapeutics,[39] is a model in which the plasmid loss induces the downregulation of the essential *dapD* gene, and thus bacterial death.

Other systems such as pCOR,[40] based on the complementation of an amber mutation, have also been established. Nevertheless, the requirement for a minimal medium for culture means these systems are less likely to be used for overexpression.

The various complementation-based expression systems have the common drawback of being strain dependent, since genetic knockout or modification of an essential gene is not easily transferable from one strain to another and has to be done independently for each strain. Strain modification by chromosomal mutagenesis is still a tedious approach, notwithstanding the existence of technologies recently described and the use of counter-selectable vectors[41] or PCR-amplified fragments.[42]

3. Prokaryotic Systems: Secretion

3.1 Secretion and Export

According to the literature, the word 'secretion' is ambiguously used to characterize true secretion in the culture medium as well as export to the periplasm of Gram-negative bacteria. Efficient secretion has proven to be highly protein dependent and very difficult to achieve in *E. coli*. Among the few examples reported, one requires the help of a signal sequence from *Streptomyces lividans*.[43] An example of the dependence of secretion on protein structure is the greatly-enhanced secretion of a Fab superantigen fusion after the alteration of some key residues located in the framework regions.[44] In a different context, two nonadjacent regions of *Klebsiella oxytoca* pullulanase, fused to β-lactamase, have proven to be necessary and sufficient to direct the translocation of the enzyme across the outer membrane.[45]

In contrast, periplasmic expression has been widely described as a means to overcome solubility and folding problems.[46] Nevertheless, periplasmic production requires adherence to a number of requirements such as the appropriate choice of a leader sequence and optimisation of the translational level. Some signal peptides such as OmpA[47] or PelB[48] have been shown to efficiently target various molecules, including eukaryotic proteins, to the periplasm of *E. coli*. An increasing number of leader sequences with this ability are being identified, including some of eukaryotic origin.[18] The dependence of secretion on a narrow range of translational levels has been demonstrated.[49] Optimizing rather than maximizing translation is required to ensure high-level secretion to the periplasm.

Attaining correctly-folded antibody fragment expression is probably one of the most difficult tasks, as assembly of both intra- and intermolecular disulfide bonds is necessary. This may explain why new antibody 'formats', such as single chain Fv fragments (scFv), have been designed. Nevertheless, there are numerous examples of Fab fragments expressed in *E. coli*, even though the amounts obtained seem to be sequence dependent. Finally, an interesting study by Simmons et al. has described the production of full-length 'aglycosylated' antibodies within the periplasm of *E. coli*.[50]

3.2 Regulated Release Into the Growth Medium

Studies have shown that bacteriocin release protein (BRP) stimulates the 'escape' of protein through the cell membrane rather than being actively secreted.[51] BRP is responsible for transient pore formation in the bacterial cell wall, through which periplasmic proteins are released into the growth medium. This elegant system makes use of a BRP-encoding gene delivered by an extra plasmid. The system is difficult to modulate however, as release efficiency is highly protein dependent and high levels of BRP expression may prove lethal to the expressing cells.[51] This feature makes such a system less likely to be the basis for a large-scale industrial process. In spite of inherent difficulties in achieving an adequate balance between appropriate pore formation and cell death, investigators have reported success with this approach,

for example, with the secretory expression of the thermostable alkaline phosphatase from *Bacillus stearothermophilus*.[52]

A very similar approach making use of the kil protein has allowed the production of recombinant murine IL-2 at levels of up to 16 mg/L.[53]

3.3 Glycosylation in *Escherichia Coli*?

N-linked glycosylation is the most common post-translational modification of secretory proteins in eukaryotes. Glycosylation is not restricted to eukaryotic cells since an N-linked glycosylation system was found in the bacterium *Campylobacter jejuni*. Wacker and collaborators[54] have recently established that a functional N-linked glycosylation pathway could be transferred into *Escherichia coli*. Although bacterial N-glycans differ structurally from their eukaryotic counterparts, the cloning of a glycosylation pathway cassette in *E. coli* opens up the possibility of expressing recombinant glycoprotein structures in this bacterial host. This interesting example should not let us underestimate the difficulties inherent in the 'humanisation' of nonmammalian hosts after genetic transfer of complete glycosylation pathways.

4. Alternative Bacterial Hosts

4.1 *Lactococcus lactis*

Like other lactobacilli, *L. lactis* is designated as a 'GRAS' ('generally regarded as safe') organism. It can be used for the expression of recombinant proteins and is capable of secretion. In a system proposed by the Biotechnological Institute (Horsholm, Denmark), expression is controlled by the *L. lactis* P170 promoter that is upregulated at a low pH during transition to the stationary phase. In a model system, up to 500 mg/L of *S. aureus* nuclease can be secreted into the culture medium when its sequence is coupled to the SP310 signal peptide from *L. lactis*.[55] Many vaccine antigens for use in humans have been expressed in such a system, among them a 105 kDa protein from *Mycobacterium tuberculosis* and different malaria antigens.[56] In addition, lactic bacterial hosts such as *L. lactis* or *Lactococcus plantarum* have potential for live vaccine design. Nevertheless important issues need to be addressed.[57] These include:

- viability of re-engineered commensal bacteria in mucosal compartments;
- their ability to present the antigen to the right effector of the immune response;
- possible transmission to pathogenic counterparts of selection markers used to engineer the strain.

A promising passive immunotherapy protocol has been recently described for the treatment of dental caries, in which the development of *Streptococcus mutans* can be substantially reduced by *Lactobacillus zeae* expressing scFv fragments.[58] Another therapeutic approach involves *L. lactis* secreting human IL-10 for the treatment of inflammatory bowel disease.[59]

As well as *Lactobacilli*, several other human commensal bacteria are currently under investigation as vehicles for *in situ* delivery of effector molecules like antibody fragments.[60]

4.2 *Bacillus*

Bacillus is very often considered as a 'prototype Gram-positive bacteria', as well as an attractive alternative to *E. coli* for the following reasons.

- It is suitable for high-density cultures in fermenters.
- Most of the *Bacillus* strains are devoid of membrane proteases.
- *Bacillus* strains are more prone to secretion than *E. coli*.
- A wide variety of expression vectors and strong promoters have been documented.

A large number of *Bacillus* vectors have been described; they can be based on strong constitutive promoters or derived from bacteriophages. In the vast majority of cases an intermediate 'shuttle' vector is necessary, since the low transfection efficiency of *Bacillus* makes it somewhat difficult to use directly for cloning. The various secretory pathways of *Bacillus subtilis* have been extensively investigated and in some cases the corresponding genes have been overexpressed in order to improve the production of a given recombinant protein.[61]

An additional means of protein exportation into the culture medium has been investigated in *Bacillus*. The TAT (Twin Arginine Translocation) pathway for secretion[62] was successfully used for trafficking recombinant proteins through the cell wall.

4.3 *Caulobacter crescentus*

Caulobacter crescentus is a commercially available expression system (Invitrogen Corporation) allowing the production and easy purification of recombinant proteins. Heterologous proteins/peptides are expressed as fusions to RsaA, an abundant protein involved in forming the bacterium's paracrystalline surface layer.[63] Moreover, this component of the S-layer protein is directly exported to the host's cell surface. The major limitation seems to be the size of the foreign protein, as expression of fusion partners exceeding 150 amino acids is often problematic. A subunit vaccine against the Infectious Hematopoietic Necrosis Virus (INHV), consisting of a glycoprotein fused to the S-layer protein from *C.*

crescentus, has demonstrated some degree of efficiency in rainbow trout fry.[64] Similarly, recombinant proteins based on the pilus tip epitope ('adhesintope') of *P. aeruginosa*, in fusion with the C-terminus of RsaA or inserted into full-length RsaA, have been tested as vaccine candidates.[65]

4.4 *Streptomyces lividans*

Under appropriate fermentation conditions, significant amounts of recombinant proteins can be recovered from culture supernatants of *Streptomyces lividans*. Numerous studies describing the secretion of biopharmaceuticals, in amounts ranging from 40–200 mg/L, make *Streptomyces* a rational choice.[66] In some cases, the protein was recovered in its native state, allowing rapid purification and facilitating downstream processing.[67]

4.5 Cold-Adapted Bacteria

Tutino et al.[68] have described the use of naturally occuring 'cold-adapted' bacteria as hosts for protein production at low temperatures (even around 0°C). Two systems have been designed based on plasmids derived from 'cold-adapted' Antarctic bacteria, capable of replicating in several psychrophilic hosts. An *in vivo* promoter-trap assay has allowed the isolation of 'cold' promoters, such as heat/cold inducible, heavy metal and oxidative/reductive stress-responsive elements. Cold-adapted bacteria may play a role in solving peculiar problems like the expression of enzymes with low stability at room temperature.

5. Eukaryotic Cells

5.1 Yeast

Yeasts are eukaryotic cells capable of growing at very high density, secreting large amounts of recombinant protein and performing glycosylation. The glycosylation pattern is somewhat different from the original, and overglycosylation (in terms of polysaccharide molecular weight) is often observed. *Saccharomyces cerevisiae* has been considered a 'prototype' for many years, but other yeast strains have recently become accessible.[69] Genetic tools have been developed for use in *Kluyveromyces lactis*,[70] *Pichia pastoris*[71] and *Hansenula polymorpha*,[72] and in several cases these strains have proven to have significant advantages over *Saccharomyces*, both in terms of production yields and secretion capacity.

The non-conventional host *Zygosaccharomyces bailii* offers many potential advantages over *S. cerevisiae*. This yeast strain exhibits efficient growth on low-cost or waste substrates, as well as the ability to grow under severe culture conditions such as acidic environments (as low as pH 2.2) and high partial CO_2 pressures. It can also survive in high concentrations of preservatives (600 mg/L of benzoic acid or sorbic acid). The data presented by P. Branduardi[73] demonstrates the potential of protein expression in *Z. bailii*, using plasmids and genetic elements derived from *S. cerevisiae* or other more conventional yeast strains.

Yarrowia lipolytica is also a nonconventional yeast strain, capable of growing on industrial fats, in which a large number of recombinant proteins have been successfully expressed, particularly industrial enzymes.[74] Different strains have been constructed, harboring deletions of protease-encoding genes and capable of secretion under specific conditions.[75]

5.2 Mammalian Cell Lines

Mammalian expression systems are frequently employed for producing recombinant proteins for which the glycosylation pattern is of some relevance to the molecule's intrinsic activity. They are also considered as the ultimate resource for very complex and high molecular weight proteins such as blood clotting factors or multimeric antibodies.

Regulatory and safety concerns from health authorities regarding mammalian cells in culture are nothing new,[76] and in recent years these issues have gained prominence, leading to regulatory requirements becoming more stringent.[77] In parallel to improvements in culture processes and quality of documentation, alternatives to mammalian cells are being developed.

5.3 Insect Cell Lines

Insect cell lines such as Sf-9 and High-Five have the ability to support cell growth and recombinant protein production with some theoretical advantages over mammalian cells. They grow on well defined culture media, devoid of animal-origin compounds, and have a stronger viral safety profile than their mammalian counterparts.

Nevertheless, the adaptability of insect cell lines to large-scale procedures, for example, using microcarrier systems, appears to be troublesome.[78] In addition, it appears that even if insect cells have the genetic potential to perform sialylation of glycoproteins, this is a highly specialized function that probably rarely occurs. Thus, the production of sialylated recombinant glycoproteins in the baculovirus insect-cell system will require metabolic engineering efforts to extend the native protein glycosylation pathways of insect cells.[79]

5.4 Plant-Based Systems

The high potential of transgenic plants used as bioreactors is of increasing interest in the pharmaceutical and biotechnology industry for the following reasons.

- They constitute an alternative and cost-effective eukaryotic system.
- Expected yields of recombinant proteins are >1kg.
- They are compatible with cheap, simple, well defined and industrially compliant culture media.
- They have a strong viral safety component.

Particularly, in the field of vaccine production, transgenic plants can accommodate the 'edible vaccine' concept. The concept of 'edible vaccines' has sparked interest and controversy for varying reasons, including demonstration of the proof of principle and standardization of the antigen dose. These issues lie beyond the scope of this review.

The most promising results to date involve 'plantibodies', topically administered to inhibit the recolonization of cavities by *Streptococcus mutans*[80] or elicit mucosal protection against genital herpes.[81] Even though some issues such as differences in glycosylation patterns and subsequent influence on the half-life of injected plant-derived products still remain unresolved, the future of vegetal bioreactors is promising.

Several characteristics of vegetables may be applicable in recombinant protein expression. The continuous release of recombinant proteins from their roots into the culture medium, also termed 'rhizosecretion' is an attractive feature,[82] which has been further improved by exploiting the ability of *Agrobacterium rhizogenes* to induce the formation of large amounts of root tissue.[83] Another elegant example of a vegetal protein expression system was illustrated in a paper by Mayfield and coworkers,[84] demonstrating the potential of chloroplasts from the unicellular green algae *Chlamydomonas reinhardtii* to express fully active antibodies.

5.5 Transgenic Animals

The advent of microinjection, embryonic stem cell manipulation, as well as other techniques that have led to the development of transgenic models such as mice, have also paved the path towards genetic manipulation of larger animals and their potential application as bioreactors. An example is the production of recombinant molecules in the mammary glands of transgenic cattle.[85] Such an approach has to be carefully considered in terms of cost, technical difficulty and production time, among other factors, and therefore would be restricted to certain pharmaceuticals of very high value. A good illustration is the laborious expression of blood clotting factor VIII in the mammary glands of transgenic sheep,[86] and the somewhat better recovery of the same molecule in transgenic rabbits.[87] Other complex molecules like CTLA4Ig, a fusion protein with immunosuppressive properties, are under investigation using transgenic mice as a preliminary step.[88] Finally, some species seem to be more adaptable to genetic manipulation and more prone to produce large amounts of recombinant proteins; as, for example, the BELE (Breed Early Lactate Early) goat.[89]

6. Alternative Systems

6.1 Cell-Free Translation

Since the early 1950's, it has been known that disrupted cells are still capable of synthesizing proteins. Based on this observation, 'cell-free translation' systems have been developed and used to obtain small amounts of labeled proteins for identification or characterization. The next step was to move from qualitative to semiquantitative or quantitative use of this technique, and to further define the so-called 'continuous-flow cell-free translation systems' (CFCF)[90] or 'continuous-exchange cell-free translation systems'(CECF). These new-generation systems are able to produce milligram amounts of a desired product and are suitable for automation.[91] Recent improvements include the possibility of expressing proteins with disulfide bonds, and cotranslation of disulfide isomerase and/or chaperones to assist with protein folding.[92] Moreover, cell-free expression opens the way to the synthesis of unexpected new molecules such as polypeptides containing non-natural amino acids. For instance, modified amino acids artificially linked to supressor tRNAs can be incorporated, through recognition of amber codons, at specific sites within a sequence. Selective labeling of amino acids has become an indispensable technique associated with multidimensional nuclear magnetic resonance (NMR) spectroscopy. In this context, cell-free translation has a marked advantage over traditional *in vivo* labeling protocols, which suffer from reduced efficiency due to metabolic scrambling.[93]

A generally encountered problem of proteolysis during synthesis has been recently overcome by the 'PURE' (Protein-synthesising Using Recombinant Elements) system. Briefly, it consists of a cell-free gene expression mixture totally reconstructed *in vitro*, and makes use of purified recombinant protein factors.[94]

For optimal biological function, some proteins require post-translational modification. In order to fulfill this requirement, extracts from mammalian cells, vegetable cells and insect cells

have been shown to be an appropriate environment for the expression of eukaryotic proteins from cell lysate.

Alternative cell-free systems are in very early stages of development and continue to evolve. An example is the original strategy of polypeptide synthesis directed by DNA as a template in cell-free extracts of *Thermus thermophilus* HB27.[95] Finally, Kigawa and Yokoyama[96] have demonstrated the tremendous potential of cell-free protein synthesis in a high-throughput expression system in which several hundreds of molecules a day have been expressed without prior cloning.[97]

6.2 Trypanosome (*Leishmania tarentolae*)

The microorganism *Leishmania tarentolae* is a nonpathogenic protozoan parasite of the gecko (*Tarentolae anularis*), and a potential host for recombinant protein production. The rationale behind such a curious choice includes the fact that *Trypanosomatidae* are rich in glycoproteins, with a pattern of glycosylation closely related to those of mammals and higher vertebrates, and thus provide an alternative to mammalian cells in culture.

According to Breitling et al.[98] up to 30 mg/L of culture can be obtained using green florescence protein (GFP) as a reporter when genes of interest are integrated into the small-unit ribosomal RNA gene. The intrinsic feature of the system is the bypass of RNA polymerase II, making it less likely to be subjected to multiple downregulation stages. The proof of principle of this system was established through expression of human erythropoietin; a molecule which requires glycosylation and appropriate modifications for activity.

7. Conclusion & Perspectives

The process of compiling a comprehensive list of expression systems is an ongoing process, and identification of an ideal host/vector combination is the ultimate quest. Expression system development is a dynamic field that is constantly evolving with the emergence of new hosts, and the implementation of new genetic elements. In selecting the most suitable expression system, the following critical determinants need to be considered:

- protein characteristics;
- quantity;
- time required for set-up and development;
- purity;
- cost-effectiveness;
- compliance with safety requirements;
- intellectual property status.

Based on these determinants and the current literature, it is safe to conclude that a universal expression system does not exist, and it is difficult to imagine that it ever will. Every protein is unique and thus requires a dedicated strategy for its optimal expression.

Among other considerations relevant to the pharmaceutical industry, an important issue is the fact that once a product enters clinical trials it is generally too late to modify the expression system being used. This emphasizes the need for a certain degree of anticipation in the process of optimising expression systems in the early phases of development.

The development of expression systems is a dynamic and challenging field in which current knowledge provides the platform for further evolution.

Acknowledgements

No sources of funding were used to assist in the preparation of this review. The author has no conflicts of interest that are directly relevant to the content of this review.

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