Optimizing the Production of Recombinant Proteins in Microorganisms

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Introduction

Living cells can be viewed as chemical reactors of very high complexity. A typical cell contains more than 1,000 different proteins that participate in an even larger number of reactions. Intracellular reactions are coordinated in a remarkably precise manner, allowing the cell to survive and proliferate in diverse environments. The cell's physiology is determined by its DNA. It has been known for more than 40 years that changes in the DNA alter the metabolic capabilities of living organisms. Mutations (changes in the DNA) are generated in a random fashion when an organism is exposed to a stressful chemical environment. Mutations alter some biochemical processes within the cell causing an identifiable change in the growth pattern. Mutants that exhibit increased biocatalytic specificity—i.e., convert a large fraction of the limiting nutrient into the desired product—are very useful in industrial fermentations. Such cells are often called overproducing mutants since they synthesize larger amounts of product compared to the naturally occurring organism. In the past, the development of useful mutants relied on macroscopic methods. These methods are indirectly aimed at generating and identifying changes at the DNA level. Within the last 15 years, the manipulation of DNA at the molecular level has become fairly straightforward due to the development of genetic engineering. The relevant techniques are collectively termed "recombinant DNA technology" and rely on the *in-vitro* (i.e., outside the cell in an artificial environment) rearrangement of nucleic acid sequences. Currently, recombinant DNA methods have been greatly simplified and at the same time have become the single most powerful tool available for the understanding of biological mechanisms.

The practical objective of genetic engineering is to force the cell to synthesize an optimal level of one or more desirable proteins. More specifically, genetically-engineered cells are used for two purposes:

1. Production of large amounts of a recombinant proteins
2. Production of metabolites (other than proteins)

In the latter case, the organism produces recombinant proteins that facilitate the synthesis of the desired product. For example, consider a compound such as ethanol which is normally made by fermentation. A number of enzymes are involved in the conversion of carbohydrates to ethanol. Normally, the intracellular concentration of one of these enzymes limits the overall rate of ethanol production. Genetic engineering techniques can be employed to force the cell to produce a higher amount of the limiting enzyme. Better still, the cell can be made to synthesize a moderate amount of a somewhat different protein that catalyzes the limiting reaction more efficiently. In either case, the result will be to accelerate the overall reaction rate and force the cell to convert a higher fraction of the raw materials into ethanol. Using this approach, Ingram et al. (1987) have developed a genetically-engineered bacterium that synthesizes appreciable amounts of ethanol. Another alternative is to engineer a cell to synthesize enzymes which catalyze reactions that do not normally take place in that cell. The presence of these enzymes causes a rearrangement of the cellular metabolism and the establishment of a new biochemical pathway. Some of the normal cellular metabolites are diverted to the synthesis of a desired compound. This kind of redesign of metabolic processes is generating great excitement and is the subject of a new field called metabolic engineering. Potential applications of metabolic engineering include:

- Production of antibiotics and vitamins by *fermentation* rather than by organic synthesis (e.g., ascorbic acid)
- Development of bacteria with enhanced biodegradative capabilities for waste detoxification
- Design of improved strains and processes for the production of bulk chemicals such as organic solvents and acids

However, at present, metabolic engineering is still in the development stage. Currently, the main application of genetically-engineered cells is the production of high-value proteins. Several recombinant proteins including human insulin and growth hormones are already available commercially while the production of many more is being developed, Table 1. As the number and volume of recombinant proteins continue to increase, the role of chemical engineers in this new industry is expanding. It must be emphasized that the problems encountered in the overproduc-

Table 1. Examples of Proteins Produced by Genetically Engineered Cells

Hormones: growth hormones (human, bovine, porcine), endorphin, erythropoietin, insullin, factor VIII
Enzymes: proteases, cellulases, pullulanases, prochymosin, elastase, urokinase, streptokinase, tissue plasminogen activator, superoxide dismutase
Physiologically active agents: interferons, interleukins, tumor necrosis factor, colony stimulating factor
Toxins: ricin toxin A, diptheria toxin, *Pseudomonas* exotoxin A
Vaccines: hepatatis B surface antigen, foot and mouth disease antigen

tion of recombinant proteins differ from those of metabolic engineering. Although this review concentrates specifically on the former, many of the biological processes described here are also important for metabolic engineering.

The information that determines the amino acid sequence of a protein is encoded in a gene, as explained in the Appendix. For the production of a recombinant protein the gene corresponding to the desired protein product is first isolated from a donor cell. Subsequently, this gene is inserted and stably propagated within a suitable host organism (gene cloning). The host cell is used to synthesize the protein more efficiently than the donor organism. In a commercial process, the overall objective is to maximize the production of a desired protein. Often the cell is forced to accumulate very high concentrations of product (in excess of 10% of the cell's dry weight). The overproduction of a particular protein does not serve the organism in any way. Instead, it disrupts the coordination of metabolic processes and may ultimately result in cell death. To optimize production, the culture has to be maintained in this abnormal state. For this reason, the special characteristics and physiology of genetically-engineered cells must be taken into account in the design of optimal bioreactors. The scale-up of processes involving recombinant organisms is currently one of the central problems in biochemical engineering. Related research is being conducted in cellular physiology, fermentations, reactor design, mathematical modelling, instrumentation, and product recovery.

Selection of Host Organism

The first, and in many ways the most crucial, decision in the production of genetically-engineered proteins is the choice of a suitable host organism. For large-scale production, it is desirable to use fast-growing cells with simple nutritional requirements. The host must not be pathogenic, nor should it produce toxic substances. From a biochemical engineering perspective, it is also preferable that the cells be grown to high densities and that the protein product be excreted into the growth medium. The nature of the protein product sometimes imposes additional constraints on the selection of the host. Proteins consist of one or more polypeptides (sequences of amino acids). After synthesis has been completed, some polypeptides are modified by the attachment of carbohydrates, lipids, or metal complexes on the amino acid sequence. These components are synthesized and attached to the protein by special cellular components that are present only in some cells. In general, microorganisms are unable to perform all the intricate modifications that normally take place in higher cells (i.e., cells from plants or animals). When the gene for a protein from a higher cell is introduced into a microbe, the genetically-engineered organism produces the correct amino acid sequence but cannot conduct the proper modifications. For the production of extensively modified recombinant proteins, it is necessary to employ genetically-engineered higher cells. However, higher cells are characterized by slow growth rates, sensitivity to contamination, and complex nutritional requirements. As a result, fermentation costs are increased substantially. Processes involving higher cells are competitive only for the production of very valuable proteins.

Many important proteins are either not modified in their natural state, or function very well without the additional lipid or carbohydrate components. For these proteins, lower cells, i.e, microorganisms, are the most suitable hosts for large-scale production. Many microorganisms grow quickly by utilizing simple and inexpensive substrates. With most microorganisms, high cell densities and high volumetric productivities are obtained relatively easily. In addition, the scale-up of microbial cultures is much simpler than that of higher cells. Since a large number of microbes have been employed successfully in industrial fermentations, one would expect there is a wide selection of potential hosts for genetic engineering. In practice, the choice of the host is limited by the amount of biological information available for a particular species. Except for a few microbes that have been adopted as biological models, there is little understanding of the biology and genetics for the vast majority of microorganisms. In addition, the molecular genetic systems necessary for recombinant DNA manipulations must be developed from the beginning for each potentially useful host (Imanaka, 1986). This task is not easy and requires considerable work. In this area, the efforts of biochemical engineers in Japan are particularly noteworthy. The group of Imanaka, Aiba, and their colleagues have developed recombinant DNA tools for a bacterium that can grow at elevated temperatures and offers several advantages for genetic engineering (*Bacillus stearothermophilus,* for review see Imanaka and Aiba, 1986). Recently they have exploited this organism to develop new enzymes with increased stability to thermal deactivation (Matsumura and Aiba, 1985; Matsumura et al., 1986).

At present, the most widely used hosts for genetic engineering are the bacterium *Escherichia coli,* certain *Bacillus* species, and the yeast *Saccharomyces cerevisiae,* see box. The wealth of fundamental information on the physiology and genetics of these microbes greatly facilitates commercial exploitation. *Escherichia coli* is by far the most commonly used bacterium for genetic engineering. The major advantage of *E. coli* is that it is the best

Escherichia coli is a bacterium found in the intestinal tract of mammals. It is a gram-negative bacterium: i.e., it is protected from the external environment by the presence of two membranes and a relatively thin cell wall. *Bacilli* sp. are gram-positive soil bacteria that produce many useful enzymes and are widely used industrially. Gram-positive bacteria pose only one membrane and have a much thicker cell wall. Yeasts, the best studied of which is *Saccharomyces cerevisiae,* have been used for years in several large-scale fermentations (e.g., ethanol production, bakers yeast). Yeasts are lower eucaryotic organisms: i.e., they are more complex than bacteria and their cellular organization resembles the structure of mammalian cells.

studied microorganism. In fact, most of our understanding of protein synthesis has been based on studies with *E. coli*. There are some practical limitations with this microorganism, such as its inability to excrete proteins under normal growth conditions and the production of a mildly toxic substance (endotoxin). Some recombinant proteins synthesized in *E. coli* do not assume their correct three-dimensional structure that is essential for biological activity. Instead, they precipitate inside the cell forming inactive protein aggregates, as will be discussed later. In spite of these problems, *E. coli* has been employed extensively for the large-scale production of many recombinant proteins (McGregor, 1984). Because of the commercial and scientific importance of *Escherichia coli*, it will be used as a reference in the following sections to illustrate some of the important principles in the production of recombinant proteins.

Gene Amplification

The synthesis of functional proteins proceeds through a series of steps that can be described by the following general scheme. (See Appendix as well as Bailey and Ollis (1986) for more details.):

$$\text{DNA—}m\text{RNA—Polypeptide—Functional Protein} \quad (1)$$

Briefly, the gene coding for the desired protein acts as a template for the synthesis of a corresponding sequence of ribonucleotides. The latter is the messenger RNA (*m*RNA), which is translated into a sequence of amino acids (a polypeptide) that constitutes the nascent form of the protein product. The amino acid sequence can be subsequently modified by the removal of one or more amino acids or the addition of other chemical groups and finally folds to attain the native three-dimensional conformation. The latter corresponds to the desired biologically-active protein.

Protein synthesis is kinetically-controlled. An increase in the synthesis of a desired protein can be obtained by increasing the formation of rate-limiting intermediates. Usually, the rate-determining step is the synthesis of *m*RNA. The rate of *m*RNA synthesis is roughly proportional to the number of copies of the corresponding gene. Therefore, one way to increase the rate of product formation is by maintaining a higher number of gene copies in the cell. For this, the gene must be inserted into a relatively small DNA molecule called plasmid (Appendix). Plasmids are propagated from one generation to the next and exist in several copies per cell. Each plasmid molecule normally contains one copy of the desired gene so that the higher the number of plasmids per cell the higher the rate of protein synthesis. Selection of the right plasmid for large-scale production requires careful consideration (Imanaka, 1986). Plasmids containing a desired foreign gene and designed specifically to effect a high level of product synthesis are called expression vectors.

In the absence of any other complications and at constant culture conditions, the *average* number of plasmid molecules per cell (the plasmid copy number) is determined by the origin of replication. The origin of replication is a specific segment of plasmid DNA containing the essential information that regulates the number of plasmid copies in the cell. Many *E. coli* expression vectors with different copy numbers are available. For the high-level expression of foreign proteins in *E. coli*, the most widely used plasmids contain a well-characterized origin of replication denoted ColE1 and are derivatives of a plasmid denoted pBR322. Under normal circumstances such plasmids are present at a level of about 15–50 copies per cell. The molecular mechanism, which controls the number of plasmid molecules with a ColE1 origin of replication, is understood fairly well (reviewed by Davison, 1984; Polisky, 1986; Thomas, 1988).

Plasmid Stability

Although the average number of plasmid molecules per cell is determined by the replication origin, individual cells in a culture differ in their plasmid content. The partition of commonly-used plasmids to the daughter cells is an essentially random event. When a cell containing n plasmid molecules divides, each of the daughter cells can inherit from 0 to n plasmid molecules. Cells containing no plasmid molecules will frequently arise. The appearance of plasmid-free cells leads to the rapid deterioration of the culture's performance. Plasmid-containing cells are obligated to synthesize more DNA, *m*-RNA, and proteins than the plasmid-free cells. Therefore, they consume more energy per cell (Da Silva and Bailey, 1986). In addition, the plasmid and chromosomally-encoded genes must compete directly for the use of precursors (e.g., nucleotides and amino acids) and catalysts—i.e., enzymes involved in DNA, RNA, and protein biosynthesis. These enzymes are present in limiting amounts within the cell (Peretti and Bailey, 1987). As a result, the growth rate of plasmid-containing cells is decreased relative to plasmid-free cells. The difference in growth rates depends on the level of synthesis of plasmid-encoded macromolecules. Cells which have lost their plasmid are not only unproductive, but compete and eventually outgrow the plasmid-containing cell population. This phenomenon, known as segregational plasmid instability, causes a rapid deterioration in the culture's productivity, especially in continuous fermentations. The severity of segregational instability depends on two parameters: the probability of generating cells that do not contain a plasmid and the ratio of the growth rate of plasmid-free (−) over plasmid-containing (+) cells, i.e., μ^-/μ^+. The higher the value of μ^-/μ^+, the greater the growth advantage of the plasmid-free cells and the shorter the time before they completely dominate the culture. Clearly these are macroscopic parameters that refer to population averages. In some cases, the relation between these parameters and microscopic events at the molecular level has been established.

For plasmids that are randomly partitioned between the daughter cells, the probability of generating a plasmid-free cell, $P(0)$, is given by a binomial distribution:

$$P(0) = 2_{(1-x)} \quad (2)$$

where x = number of plasmid molecules per cell.

Theoretically if there is an average of 40 plasmid copies per parent cell, the probability that a plasmid-free daughter cell will arise during cell division is about 2×10^{-12}. Consequently, the generation of plasmid-free cells should be very difficult to detect. In reality, plasmid-free cells are generated much faster and often dominate the culture after only a few reproductive cycles. In fact, the concentration of plasmid-containing cells in a continuous stirred tank reactor is time-dependent; steady state is reached only when the fraction of plasmid-carrying cells approaches zero. There are several reasons for this:

i) Experimentally-determined plasmid copy numbers represent population averages. There is a distribution in the number of plasmid molecules per cell in the culture. Some cells may

contain fewer plasmids or even no plasmids at all. Therefore, plasmid-free cells are generated at a higher frequency than predicted by Eq. 2. Very little information is available on the population distribution of plasmids because relevant experimental measurements are very difficult to obtain (Seo and Bailey, 1987).

ii) It is not necessary that all plasmid copies be physically distinct. Often several plasmid molecules are joined together in a multimeric form (Summers and Sherratt, 1984). Thus, the effective number of physically-independent species to be partitioned during cell division is decreased and the probability of generating plasmid-free cells is higher.

iii) In some cases, the synthesis of high amounts of plasmid-encoded proteins has been shown to lower the average copy number (Adams and Hatfield, 1984). As before, a lower copy number will result in increased segregation frequency and faster generation of plasmid-free cells.

As was mentioned earlier, the synthesis of plasmid-encoded macromolecules consumes energy and metabolic precursors. Therefore, as the synthesis rate of plasmid-encoded DNA, *m*RNA, and protein is increased, the culture becomes more unstable. For this reason, increasing the size of the plasmid is disruptive for the host cell because more DNA must be synthesized per plasmid molecule. Plasmids with small DNA inserts (approximately 1.3×10^6 daltons) are stable while large inserts cause a decrease in the copy number and result in complete plasmid instability (Warnes and Stephenson, 1986; Cheah et al., 1987). The burden imposed on the cell from the synthesis of proteins encoded by plasmid genes can be even more severe. In some cases, the protein product is toxic for the host and inhibits the growth of the plasmid-carrying cells (Nakahama et al., 1986). Since the nonproductive, plasmid-free cells are able to grow, they quickly dominate the culture. In other words, when the protein is toxic, the growth rate ratio (μ^-/μ^+) becomes very large. Similarly, plasmids directing the overproduction of foreign or even native proteins which themselves are not toxic also exhibit extreme plasmid instability (Siegel and Ryu, 1984; von Meyenburg et al., 1984). The cell is forced to synthesize high levels of both the respective messenger RNA and the protein. The formation of the polypeptide consumes more energy and is probably more taxing for the cell than the synthesis of *m*RNA (Brosius, 1984).

The relation between growth conditions and plasmid stability has been studied extensively. The effects of the growth-limiting nutrient on stability are quite complex and depend on both the plasmid and the host strain. For example, μ^-/μ^+ increases when the growth of the cells is limited by phosphate or magnesium in shake flask cultures (Klemperer et al., 1979). However, these growth conditions have an adverse effect on the stability of some plasmids, but not others (Jones and Melling, 1984). Other environmental conditions affecting plasmid stability include:

- Dissolved oxygen concentration (Hopkins et al., 1987).
- Growth temperature. Temperature effects on plasmid stability have been demonstrated both in *Bacillus* (Aiba and Koizumi, 1986; Kim and Ryu, 1984) and *E. coli* (Primbrose et al., 1984).
- Starvation for essential amino acids causes an increase in the copy number (Adams and Hatfield, 1984).
- The plasmid stability of continuous cultures decreases with decreasing dilution rates (Wouters et al., 1980; Weber and San 1987). This effect was surprising because the amount of plasmid DNA per unit cell mass is higher at low dilution rates (Seo and Bailey, 1986). Consequently, a higher copy number and increased plasmid stability would be expected. An interesting explanation of this phenomenon was recently proposed by Ataai and Shuler (1987). It has been observed that the cell volume decreases with dilution rate. As a result, the number of cells per unit mass is increased. Although there is more total plasmid DNA at low dilution rates, mathematical simulations have shown that the copy number per cell must be essentially independent of the dilution rate. However, the plasmid-encoded proteins represent a larger fraction of the total cellular protein of the smaller cells. For these cells, the metabolic burden due to the synthesis of plasmid-encoded proteins is more severe. The μ^-/μ^+ ratio increases at lower dilution rates and the plasmid-free cells dominate the culture faster.

In addition to the complete elimination of the plasmid-carrying cells from the culture, there exists another destablizing phenomenon called structural instability (Tsunekawa et al., 1981). In this case, the plasmid is present in the host cell, but the desired gene is inactivated. As a result, synthesis of the product stops and the culture becomes unproductive. In *E. coli,* this phenomenon depends on the genetic make-up of the host strain (for example, the presence of transposable genetic elements. (These so-called "movable" genes have the ability to physically insert within other chromosomal or plasmid genes. When a transposable element is inserted in a given gene, the production of the corresponding protein is disrupted.) It is possible to eliminate structural instability in *E. coli* by judicious selection of an appropriate strain. However, structural instability is a major problem in *Bacillus subtilis* (Ehrlich et al., 1986). Instability in *Bacillus* results from rearrangements, insertions or deletions in the plasmid DNA. These events occur more frequently towards the end of the fermentation when the nutrients have been exhausted and cell growth has decreased.

Mathematical Models of Plasmid Stability

To understand a process as complex as plasmid stability, the available experimental information must be incorporated into a mathematical formulation. Mathematical models can be used to test mechanistic hypotheses, suggest new experiments, or at a different level for process design and optimization. Several models of recombinant cells have been proposed (Zabriskie and Arcuri, 1986). Models of low mathematical complexity do not account for detailed biochemical processes (e.g., Imanaka and Aiba 1981, Kim and Ryu 1984). In these models, the biomass is usually divided into two subpopulations, the plasmid-containing and plasmid-free cells. The two subpopulations are assigned different growth rates and their competition is simulated. In contrast, more complex mathematical models include detailed biochemical information for protein synthesis, cell growth and plasmid replication (Ataai and Shuler 1986, 1987; Peretti and Bailey, 1987). These models have been used to investigate and refine the mechanism that determines the number of plasmid copies per cell (Thomas, 1988). In addition, they have provided guidelines for more efficient plasmid constructions and optimal biorector operation.

Stabilization of Plasmids

Ensuring the ability of a culture to synthesize the desired protein is a very important consideration in large-scale fermenta-

tions of recombinant organisms. There are several methods to prevent plasmid-free segregants from dominating the culture but at present none of these methods is generally accepted. The most common method is to use plasmids containing selection marker genes. These genes code for proteins that are required for the survival of the organism in an environment unfavourable for growth. In this environment, loss of the plasmid should result in cell death. For example, many expression vectors contain genes that confer resistance to antibiotics. Cells containing the plasmid can grow in antibiotic-containing media because they synthesize proteins that inactivate or expel the antibiotic. However, plasmid-free cells are susceptible to the antibiotic's action. Antibiotic selection, although widely used, can be ineffective in large-scale fermentations. Pierce and Gutteridge (1985) have found that all the antibiotic in the culture can be inactivated by a small fraction of plasmid-carrying cells which coexist and protect a much larger population of cells that have shed the plasmid. Furthermore, the antibiotic must be removed at the end of the fermentation, especially if the protein is destined for pharmaceutical purposes. The use of plasmids containing genes for bacterial toxins that kill plasmid-free cells has been proposed (Lauffenburger, 1987). However, synthesis of the toxin also has a deleterious effect on the productive (plasmid-containing) cells. A more effective method of killing the plasmid-free segregants without the use of toxins was recently developed by Gerdes et al. (1986). In this system, the cell produces a "suicide" protein whose synthesis is prevented in the presence of a plasmid. Using this host/plasmid combination, the experimental frequency of plasmid segregation has been decreased by three orders of magnitude (Boe et al., 1987). Finally, the stability of a culture can be increased when a plasmid-encoded protein is essential for cellular metabolism and the host cell is unable to synthesize this protein based on its chromosomal DNA. This system has been employed successfully to confer 100% plasmid retention of an otherwise unstable plasmid for more than 150 generations (Nilsson and Skogman, 1986). No information on the general applicability of this stabilization method is available at present.

An alternative strategy for plasmid stabilization is to decrease the frequency with which plasmid-free cells are generated. As was discussed earlier, the partition of most plasmids is essentially random. When certain partition genes are inserted in the plasmid, the probability of equal distribution of the plasmid molecules in the daughter cells is significantly increased (Gerdes and Molin, 1986; Ogura and Hiraga, 1983; Martin et al., 1987). As a result, plasmids containing partition genes exhibit considerably higher stability. Finally, the instability problem can be avoided altogether if, instead of using plasmids, the desired gene is inserted into the bacterial chromosome. However, the increased stability comes at the expense of lower protein synthesis because genes inserted in the chromosome are maintained in much fewer copies than genes present in expression vectors.

Transcription

The rate of protein synthesis is roughly proportional to the steady-state concentration of the corresponding messenger RNA. To maximize the formation of a protein product, the rate of transcription (i.e., the synthesis of *m*RNA) must be high. The synthesis of *m*RNA is catalyzed by the enzyme RNA polymerase. One way of increasing the level of *m*RNA is through a proportional increase in the number of gene copies, as was discussed earlier. The level of a specific *m*RNA also depends on the rate of transcription per gene copy. The frequency at which RNA polymerase initiates mRNA synthesis depends on how efficiently it interacts with the promoter, a regulatory sequence in the DNA preceding the structural gene, Figure 1. Once an *m*RNA molecule has been initiated, *m*RNA synthesis proceeds until the entire gene has been transcribed. The rate of transcription is determined by the rate at which mRNA synthesis is initiated and consequently on the characteristics of the promoter. Promoters can be classified as either weak or strong depending on the rate at which they are transcribed. The relative strength of natural *E. coli* promoters varies over four orders of magnitude. Using strong promoters, a specific protein may accumulate at a level of 10% or more of all the protein synthesized by the cell. A foreign protein cannot be synthesized by the host cell unless a functional promoter is placed in front of the corresponding foreign gene. Promoters and other regulatory sequences from one organism do not usually function correctly in other hosts.

The molecular events that take place during the initiation of *m*RNA synthesis in *E. coli* are known in considerable detail (Reznikoff and McClure, 1986; McClure, 1985). Studies of many promoters of varying strength have defined the structural features that determine the intrinsic maximum rate of transcription (Hawley and McClure, 1983; Nishi and Itoh, 1986). Specifically, two short DNA sequences present at a distance of 10 and 35 nucleotides behind the transcription initiation site determine the maximum rate of *m*RNA synthesis, Figure 1. There are two kinds of promoters: constitutive and regulated. Constitutive promoters are always turned on and the rate of *m*RNA synthesis is not modulated by other cellular components. In contrast, the level of *m*RNA synthesis initiated from regulated promoters is controlled by the cell and can vary from the intrinsic maximum rate to nearly zero. Regulated promoters

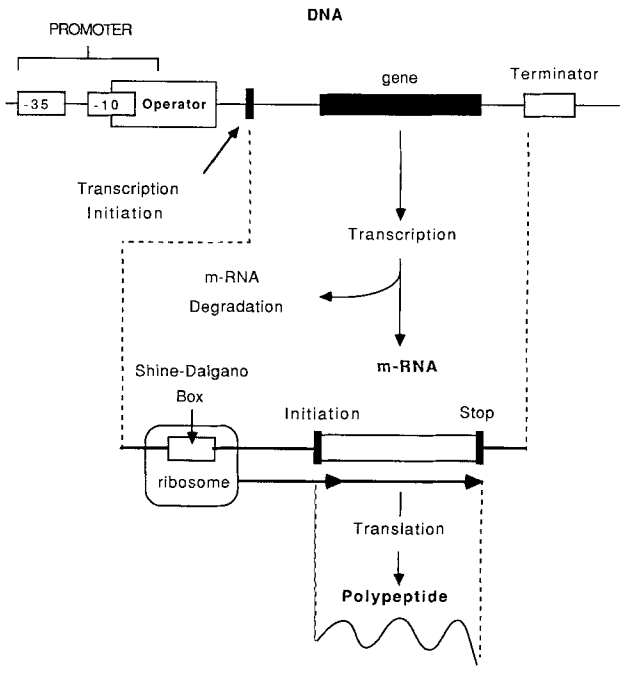

Figure 1. Transcriptional and translational events in protein synthesis in *Escherichia coli*.

are very important for the efficient production of genetically-engineered proteins and will be discussed in more detail. In bacteria, regulated promoters are controlled mainly by repressor proteins. These proteins bind to the operator, a DNA sequence overlapping the promoter. Each repressor protein is specific for the operator of a particular family of promoters. The bound repressor protein inhibits the interaction of RNA polymerase with the promotor and transcription is decreased. The affinity of the repressor protein for DNA is modulated by the concentration of certain small molecules, e.g., simple sugars, nucleotides, and amino acids. Promoters where a small molecule (*inducer*) binds to the repressor protein to decrease its affinity for DNA and enhance *m*RNA synthesis are called *inducible*. The opposite effect occurs in *repressible* promoters where an elevated concentration of a small molecule (*corepressor*) decreases transcription. Similar principles apply to promoters that are induced/repressed directly by physical changes in the growth conditions, e.g., temperature and osmotic pressure.

The molecular manipulations required for the insertion of an appropriate promoter in front of the desired gene can be accomplished rather easily *in vitro*. Many strong promoters have been constructed, Table 2, and the right choice has to be made based on fermentation criteria. In large-scale fermentations, it is important that protein synthesis is not initiated until a high cell concentration has been attained. Premature synthesis of recombinant proteins causes plasmid instability and decreases the culture productivity. In summary, promoters suitable for the large-scale production of recombinant proteins should have the following characteristics:

- High maximum level of transcription.
- The promoter must be regulated very tightly so that *m*RNA synthesis is completely inhibited until the promoter is intentionally switched on.
- The method used to initiate protein synthesis should not have side effects on the physiology of the cell.

Strong promoters

A wide selection of strong, regulated promoters is at present available only for *Escherichia coli* (Brosius, 1988). Selecting the right promoter is an important decision especially for large-scale fermentations where heterogeneous growth media and very high cell densities are normally employed. The following discussion focuses on the practical considerations in selecting frequently-used promoters.

Both inducible and repressible promoters have been used for large-scale production of proteins in *E. coli*. Protein synthesis from repressible promoters is initiated when the concentration of the corepressor is decreased. The widely-used, strong, repressible promoters *trp* and *PhoA* are switched on when the cells are starved for tryptophan or phosphate respectively. (The *trp* promoter is also inducible by addition of β-indolyl acetic acid.) However, the use of repressible promoters presents a serious practical difficulty. The cells must be grown in the presence of a high concentration of the corepressor to suppress premature protein synthesis. Product formation is initiated by transferring the cells to a low corepressor medium. Media changes are not easy to implement in large scale and can have adverse effects on the productivity of the culture. For these reasons, inducible promoters are usually preferable for large-scale production.

The most common inducible promoters are the *lacUV5,* the *tac,* and the λp_L. (For details on the chemical structure of these promoters, see Brosius 1988 and references listed in Table 2.) The last two are of comparable strength resulting in a maximum accumulation of the protein product to a level corresponding to more than 30% of the total cellular protein (Remaut et al., 1981; Amann et al., 1983). The *lacUV5* and the *tac* promoters are induced by adding small concentrations ($\sim 10^{-4}$ M) of a suitable inducer such as IPTG (isopropyl-β-D-thiogalactopyranoside) in the culture medium. However, IPTG is expensive (about \$15/g) resulting in increased fermentation costs. The very widely used λp_L promoter is a representative thermoinducible promoter. Synthesis of *m*RNA from the λp_L promoter is initiated by raising the fermentation temperature to 42°C. This mode of induction is inexpensive but can pose some other problems. First of all, to suppress protein production, the culture is grown at a low temperature instead of 37°C which is the optimal growth temperature for *E. coli*. Consequently, the length of the fermentation is increased. A more serious disadvantage is that incubation at 42°C also induces the nonspecific synthesis of other *E. coli* proteins. Some of these proteins are proteolytic enzymes (Baker et al., 1984; Goff and Goldberg, 1985; Buell et al., 1985) and accelerate the rate of degradation of the product. Finally, the profile of the temperature shift has a strong effect

Table 2. Strong *Escherichia coli* Promoters

Promoter	Induction Method	Characteristics*	Reference
lacUV5	Addition of IPTG	(about 5%)	Brosius, 1987
tac	As above	Induction results in cell death (>30%)	Amman et al., 1983; De Boer et al., 1983
lpp-OmpA	As above	Suitable for secreted proteins (20%)	Ghrayeb et al., 1983
lpp^p-5	As above	Strongest *E. coli* promoter (47%)	Inouye and Inouye, 1985
trp	Tryptophan starvation	Relatively weak (around 10%)	Brosius, 1987
λp_L	Growth at 42°C	See text (>30%)	Remaut et al., 1981
$\lambda p_L/cI_{trp}$	Addition of tryptophan	Easily inducible in large scale (24%)	Mieschendahl et al., 1986
att-nutL-p-att-N	10-min incubation at 42°C	No product is synthesized before induction (on/off promoter)	Podhajska et al., 1986
T7 Promoter	Addition of IPTG or viral infection	As above (>35%)	Studier and Moffatt, 1986
T4 Promoter	Viral infection	Method of induction inhibits product degradation	Duvoisin et al., 1986
phoA	Phosphate starvation	Induction in large scale complicated	Oka et al., 1985

*Typical values of accumulated product as percent of the total protein of induced cells are given in parenthesis.

on product formation and must be optimized carefully (Dipasquantonio et al., 1987). As the scale of the fermentation increases, it becomes more difficult to impose the desirable temperature profile. These problems have led to the development of promoters that offer the high strength of λp_L but are not thermoinducible. Mieschendahl et al. (1986) constructed one such promoter that can be induced inexpensively by adding tryptophan in the growth medium. Other recently-developed *E. coli* promoters are listed in Table 2. Perhaps the strongest *E. coli* promoter known is the synthetic promoter lpp^p-5 (Inouye and Inouye, 1985) resulting in product accumulation corresponding to 47% of the total cellular protein. One problem with most promoters is that there is some residual protein synthesis even when they are not induced. This can be a problem for the production of toxic proteins that cause cell death even at very low levels. An innovative on/off promoter system, where no protein synthesis takes place before induction, was developed recently (Podhajska et al., 1985; Hasan and Szybalski, 1987). In this system, the promoter is physically separated from the gene of interest. No *m*RNA synthesis takes place until the culture is subjected to a short heat pulse. The temperature increase causes a specific rearrangement in the DNA, the promoter is spontaneously inserted in an optimal position in front of the gene, and protein synthesis commences. Absolute on/off control is also possible with the very strong *T7* promoter, but induction may be difficult to accomplish in a large scale (Studier and Moffatt, 1986).

A limited number of strong promoters for other microorganisms are available. Some *E. coli* promoters are functional in several gram-negative bacteria and have been used for protein overproduction in these organisms (Bagdasarian et al., 1983; Leemans et al., 1987). On the other hand, many *E. coli* promoters are usually not functional in *Bacillus subtilis* or other gram-positive bacteria. The strong α-amylase promoter from *Bacillus* has been characterized and used extensively for the overproduction of recombinant proteins in this organism (Palva, 1982). Some inducible *Bacillus* promoters have also been constructed (Yansura and Henner, 1984; Joyet et al., 1986).

Our understanding of *m*RNA synthesis initiation in *Saccharomyces cerevisiae* is incomplete. Nevertheless, several strong promoters have been identified and are currently used for large-scale protein production. For example, the very strong unregulated promoter *GAP* (Bitter and Egan, 1984; Bitter, 1987) has been used to produce a protein vaccine to a level corresponding to more than 40% of the total cell protein (Kniskern et al., 1986). An inducible variant of this promoter was developed recently and has been used for large-scale production (Fieschko et al., 1987).

To complete this discussion on transcription initiation, it must be emphasized that irrespective of the promoter, the fermentation conditions during the initiation of product synthesis have a profound effect on the final yield. For example, it has been observed that the growth phase of the culture at which protein synthesis is initiated affects the productivity of human interferon-producing *E. coli* by one order of magnitude (Mizukami et al. 1986a, b).

mRNA Degradation and Transcription Termination

Protein synthesis depends on the steady-state concentration of *m*RNA, which in turn is affected not only by the rate of synthesis but also the rate of degradation. It has been shown that the level of human interferon α-5 synthesized in *E. coli* is proportional to the half life of its *m*RNA (Panayotatos and Truong, 1985). *m*RNA is a very short-lived molecule in *E. coli* with an average half life of around 2–3 minutes (Higgins and Smith, 1986). The rate of *m*RNA degradation is first order and decreases with temperature (Kennell, 1986). Thus, it is possible that lowering the fermentation temperature may be favorable for protein production. In a different approach, Panayotatos and Truong (1985) were able to increase the half life of interferon *m*RNA four times using genetic techniques. However, the general effectiveness of these techniques for *m*RNA's from other genes has not been ascertained.

Normally, when RNA polymerase reaches the end of a gene, it recognizes a special DNA segment, the transcription terminator, Figure 1. This sequence interacts with *m*RNA polymerase and acts as a stop signal for *m*RNA synthesis. Like promoters, transcription terminators are of different strength. A strong promoter must be accompanied by a strong transcription terminator. In the absence of a strong terminator, RNA polymerase proceeds beyond the end of the gene and a much longer *m*RNA molecule is formed. This longer *m*RNA interferes with the origin of replication causing extreme plasmid instability (Stueber and Bujard, 1982). Insertion of a strong transcription terminator at the end of the target gene has been used to avoid this complication (Brosius, 1984).

Translation

The next step in protein synthesis is the translation of the ribonucleotide sequence of *m*RNA into the corresponding

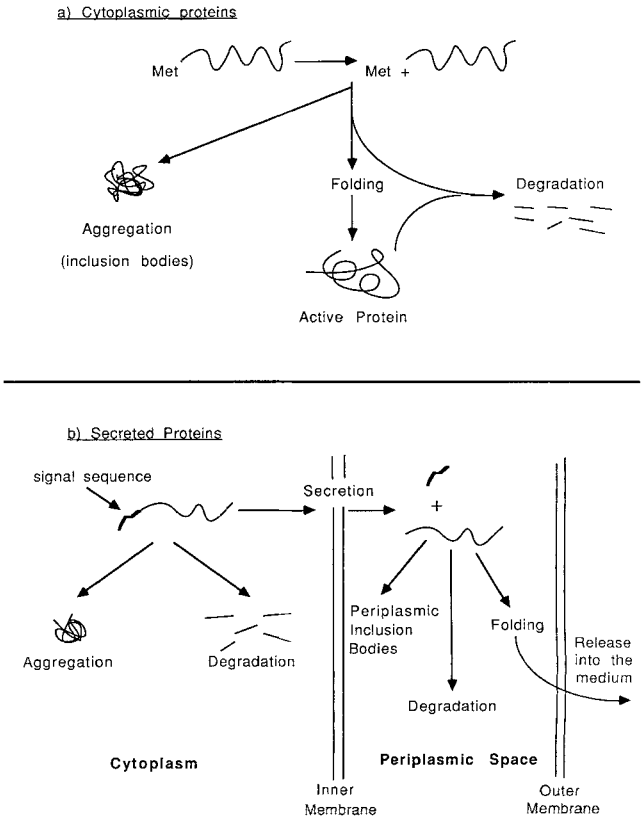

Figure 2. Post-translational events in protein synthesis in *Escherichia coli:* a) cytoplasmic proteins b) secreted proteins.

amino acid sequence of the protein product. This process is catalyzed by the ribosome. The ribosome recognizes and binds to the ribosomal binding site, a specific sequence of ribonucleotides within the *m*RNA molecule. Without it, protein synthesis is not initiated regardless of the amount of *m*RNA present. The structure of the ribosomal binding site is very important because it determines the efficiency of protein synthesis per *m*RNA molecule (Gold et al., 1981; Stormo, 1986). The nucleotide sequence surrounding the ribosomal binding site also affects the interaction of the ribosome with the *m*RNA and exerts an important effect on the rate of protein synthesis (Matteucci and Heyneker, 1983; Itoh et al., 1984; Schoner et al., 1984; Whitehorn et al., 1985; Stanssens et al., 1985; Lee, 1987). Other factors that affect the rate of translation include:

1) The nucleotide sequence immediately preceding and following the first triplet of nucleotides that is translated into an amino acid (Hui et al., 1984; Buell et al., 1985). Often, it is possible to modify some critical nucleotide sequences in the beginning of a gene and increase the rate of protein synthesis by more than 20-fold without changing the level of *m*RNA (for example, Hui et al., 1984; Looman, 1987). Such molecular modifications require considerable developmental work.

2) The nucleotide sequence of the gene of interest or, more specifically, the relative abundance of different nucleotide triplets within a gene (codon usage) (De Boer and Kastelein, 1986). The latter probably determines the speed with which a ribosome completes the synthesis of a protein molecule. However, the importance of the nucleotide sequence of a gene on the rate of translation was recently questioned (Ernst and Kawashima, 1988).

Post-Translational Effects

So far we have focused on the events that determine the rate of polypeptide assembly. It must be emphasized that this is not the terminal point in the formation of the complete protein product. There are several ways the cell can process the nascent polypeptide after translation has been completed. More specifically, the polypeptide may be: 1) modified covalently by the attachment of chemical groups; 2) degraded; 3) can precipitate in the cell to form a protein aggregate; or 4) secreted through the cellular membrane. The first three processes depend on the final location of the protein, i.e., whether it is secreted or remains in the cytoplasm. As mentioned earlier, the extent of chemical modification of the complete polypeptide varies from organism to organism. It is always desirable and sometimes essential that the recombinant protein resembles the authentic natural protein as closely as possible. The intracellular location and form (aggregate or soluble) of the polypeptide are also very important because they determine the purification strategy. For these reasons, the post-translational events of protein synthesis often determine the overall yield and economics of recombinant protein production.

Cytoplasmic Proteins

All proteins (with the exception of a few mitochondrial proteins in higher cells) are synthesized in the cytoplasm. The possible fates of foreign proteins in the cytoplasm of microorganisms are shown in Figure 2a. Protein synthesis is always initiated with the amino acid methionine or its derivative *N*-formylmethionine. Normally, in most cytoplasmic proteins the initiating methionine is removed post-translationally by the enzyme methionine amino peptidase (Ben-Bassat et al., 1987a). However, when several foreign polypeptides such as bovine growth hormone (Schoner et al., 1984), interleukin-2 (Ben-Bassat et al., 1987a) are overproduced in *E. coli,* the removal of this first amino acid is incomplete. Two forms of the product with and without the first methionine are produced. This extra amino acid results in a slightly different protein and may affect its physical and catalytic properties. The production of proteins that are exactly identical to the authentic product is particularly desirable in the pharmaceutical industry. For pharmaceutical proteins, a difference of a single amino acid may provoke an immune response in the patient (and also complicates the approval of the product by FDA). It was recently shown that removal of the terminal methionine can be accomplished either *in vitro* by enzymatic action on the purified protein or *in vivo* by using a host that produces high levels of methionine aminopeptidase (Ben-Bassat et al., 1987a). The reverse problem of the *in vivo* removal of several amino acids from the amino terminal has also been observed (Kronheim et al., 1986). In any case, regardless of whether the polypeptide has been trimmed or not, it can be subsequently processed in three ways. Cytoplasmic proteins can be degraded by the action of proteolytic enzymes, aggregate to form large particles known as inclusion bodies or they can attain their active conformation and remain soluble inside the cell. The later corresponds to the desired active protein.

Proteolytic degradation

Proteolytic degradation of the product is a serious problem in the overproduction of genetically-engineered proteins. Although proteolysis is less of a problem in *E. coli* compared to other microbial hosts like *Bacillus,* it is well known that several foreign proteins in *E. coli* are very unstable. For example, Talmadge and Gilbert (1982) have shown that the half life of the important pharmaceutical protein preproinsulin (the precursor of insulin) in the *E. coli* cytoplasm is less than two minutes. Clearly, if degradation is rapid, it will determine the final protein yield and any other improvements in the process are likely to have only minor effect (Gerard et al., 1986). Proteins differ greatly in their susceptibility to degradation. Their relative stability depends on the amino acid sequence in a way that is not completely understood. The cell identifies some recombinant proteins as different or "abnormal" compared to the native proteins. Such abnormal proteins are quickly targeted for destruction. Among other things the sequence of the first amino acids of a protein may be an important signal in degradation (Bachmair et al., 1986). *E. coli* has at least eight enzymes that degrade proteins by attacking internal peptide bonds [known by the rather melodic names Do, Re, Mi, Fa, So, La, Ci and Pi (Goldberg and Goff, 1986)]. Protease La appears to play a central role in the degradation of abnormal proteins. In cells that do not produce La, protein degradation is decreased (Maurizi et al., 1985). On the other hand, it has been shown that overproduction of foreign proteins causes an increase in the level of La that accelerates product degradation (Goff and Goldberg, 1985).

Proteolysis can be reduced by optimizing the growth conditions. For example, starvation of the culture for carbon source or amino acids results in increased proteolysis (Goldberg and St. John, 1976; Tsai et al., 1987). Recombinant interferon αA synthesized in *E. coli* was degraded rapidly in cells starved for glu-

cose or under conditions of oxygen limitation (Kitano et al., 1987). This result is particularly significant since glucose and oxygen limitations can arise in large-scale, high-density fermentations that are employed for recombinant protein production. A simple and potentially important method for reducing protein degradation is to grow the cells at suboptimal temperatures, e.g., 30°C (Oka et al., 1985). However, more work is required to fully understand how the conditions encountered during high cell density fermentations affect product stability. Two more approaches have been employed to prevent protein degradation. First, it is possible to fuse the gene coding for an unstable protein either to itself or to a different gene coding for a native *E. coli* protein (Shen, 1984; Germino and Bastia, 1984; Silhavy and Beckwith, 1986). The protein resulting from such a gene fusion is a hybrid polypeptide. It consists of the amino acid sequence of the product preceded by the amino acid sequence of the other gene. The presence of the additional amino acid sequence somehow stabilizes the hybrid protein against degradation. The hybrid can then be produced in large amounts and is purified by conventional techniques. Often the additional sequence in the hybrid is selected to facilitate its recovery. After the hybrid has been purified, the part corresponding to the desired polypeptide is cleaved off by either enzymatic or chemical means. This technique has been very effective for the production of many small proteins (Sharma, 1986; Abramsen et al., 1986). In many cases, however, it is not possible to use hybrid proteins because of the difficulty in obtaining correct cleavage of the product from the hybrid protein. Alternatively, the product can be protected from degradation if it forms aggregates (inclusion bodies) inside the cell. These aggregates appear to be inaccessible to proteolytic enzymes (Cheng et al., 1981; Kitano et al., 1987).

Inclusion bodies

Williams et al. (1982) first reported that overproduction of recombinant human insulin in *E. coli* results in the aggregation of the protein product inside the cell. Similar aggregates have since been observed with many other proteins (for example, Kawaguchi et al., 1984; Winkler et al., 1985; Wetzel, 1986; Marston, 1986). It is now known that aggregation of recombinant proteins occurs not only in *E. coli* but also in gram-negative bacteria (Leemans et al., 1987), gram-positive bacteria (Schein et al., 1986; Ruppen et al., 1986), and yeasts (Cousens et al., 1987). The protein aggregates are called inclusion bodies or refractile bodies because they appear as dark, refractile areas when the cells are examined microscopically, Figure 3. It appears that any protein can form inclusion bodies when it is synthesized at an abnormally high rate (Sharma, 1986). In *E. coli,* some foreign proteins such as bovine growth hormone, urokinase, or viral coat proteins are found almost exclusively in inclusion bodies, while others such as interferon are partially soluble in the cytoplasm (Wetzel, 1986). The latter, however, precipitates as the fermentation proceeds and after 30 hours 99% of the interferon is present in aggregated form (Buell and Panayotatos, 1986).

The formation of inclusion bodies is an important phenomenon in recombinant protein production. Inclusion bodies consist primarily of the overproduced polypeptide together with small amounts of impurities. These include other proteins as well as nucleic acids (Hartley and Kane, 1987). The aggregated protein is present in a partially or more likely a completely denatured state and therefore it is catalytically inactive. In some cases, the

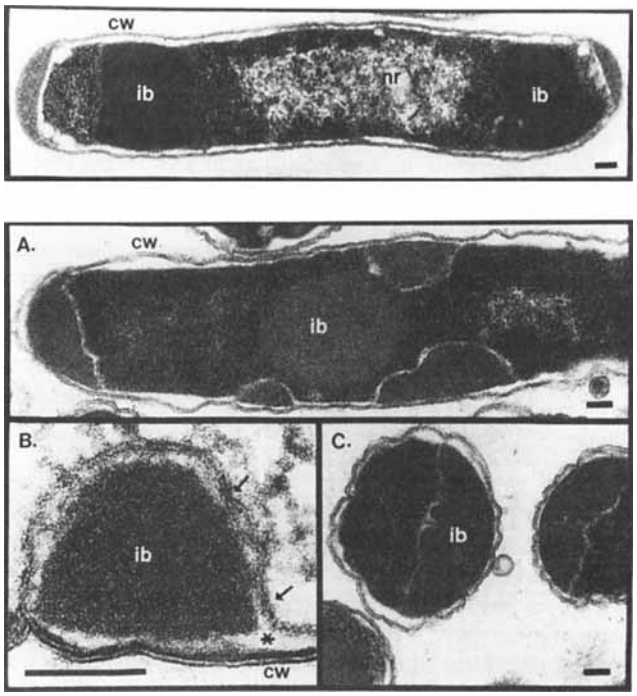

Figure 3. Electron micrographs showing inclusion bodies in *Escherichia coli.*

Inclusion bodies appear as darkly staining areas. Upper panel—overproduced alkaline phosphatase, a secreted protein that precipitates in precursor form inside the cell: *ib* = inclusion body; *cw* = cell wall; *nr* = nuclear region; bar = 100 nm. Lower panel—periplasmic inclusion bodies of the secreted protein β-lactamase: A) longitudinal section near one end of the cell; B) inclusion body in the periplasmic space (*) between the cell wall and inner membrane (arrows); and C) cross section of cells containing β-lactamase inclusion bodies

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inclusion bodies are composed of multimers of the protein product covalently linked together by intermolecular disulfide bonds (Shoemaker et al., 1985). Inclusion bodies can be solubilized completely by the addition of high concentrations of denaturants such as urea or guanidium-HCl in the presence of reducing agents. Under these conditions, the protein is completely denatured and its conformation approximates a random coil. When the denaturing agents are removed, relatively small polypeptides relax into their correct three-dimensional structure. However, larger proteins containing several intramolecular covalent linkages (i.e., disulfide bonds) very rarely refold to their native three-dimensional conformation. In general, if the protein can undergo reversible unfolding, the active protein can be recovered from inclusion bodies rather easily. The inclusion bodies are harvested from lysed cells, washed to remove impurities, and are resuspended in a denaturing solution. Subsequently, the protein is allowed to refold by removing the denaturant either by dilution or dialysis (McGregor, 1984; Kawaguchi et al., 1984; Georgiou et al., 1986). In many cases the recovery of the active protein is incomplete. Arakawa et al. (1986) have shown that refolding of recombinant interleukin 2 from inclusion bodies yields a mixture of the active protein together with aggregated forms. Reaggregation is in fact the major impediment in the recovery of the active protein during refolding. Despite these problems, the formation of inclusion bodies is not entirely undesirable. As was mentioned earlier, aggregation protects the

product from proteolytic degradation. Furthermore, the formation of inclusion bodies may in fact facilitate downstream processing. Because of the size and density of the protein aggregates, they can be separated from other cellular components by centrifugation (Taylor et al., 1986). The concentration of the protein of interest in the precipitate can be as high as 80% and therefore centrifugation alone provides considerable enrichment of the product. Removal of the remaining cellular impurities is straightforward (Langley et al., 1987). In contrast, more complex purification schemes have to be used to recover completely soluble proteins. Ultimately, the recovery of the product from inclusion bodies depends on the effectiveness and ease of the refolding process. At present, renaturation procedures have been developed for a relatively small number of commercially important proteins.

Protein export in Escherichia coli

The cytoplasm of *E. coli* is protected from the external environment by the presence of two hydrophobic lipid bilayer membranes, the inner and the outer membrane. Most proteins cannot normally transverse a cellular membrane. In *E. coli,* the inner membrane confines these proteins within the cytoplasm. However, there is an elaborate mechanism that allows certain proteins to be secreted through the membrane. Proteins destined for secretion are synthesized in precursor form: i.e., they contain an amino acid extension consisting of approximately 20–30 amino acids (the signal sequence) at their amino terminal (Oliver, 1985). The presence of a signal sequence is necessary although not sufficient for secretion. The precursor form of a protein is able to transverse the inner membrane. Once on the other side, the signal sequence is cleaved and the mature form of the protein folds to its native conformation. The possible destinations of overproduced secreted proteins are illustrated in Figure 2b. Overproduction of secreted proteins can result in accumulation of the protein in the cytoplasm, if the rate of synthesis exceeds the rate at which the protein can be secreted, Figure 3. In this case, the precursor form of the protein may aggregate to form cytoplasmic inclusion bodies (Georgiou et al., 1986) or it may be degraded by proteolysis. A fraction of the total amount of the protein precursor will be secreted through the inner membrane, an event which is followed by the removal of the signal sequence. The mature form of the protein can now fold into its correct three-dimensional conformation. If both the rate of synthesis and secretion are high, the concentration of the protein in the periplasmic space may be such that aggregation takes place resulting in the formation of periplasmic inclusion bodies, Figure 3. This phenomenon has been observed with the secreted proteins β-lactamase and staphylococcal nuclease (Georgiou et al., 1986; Takahara et al., 1985).

Secretion into the periplasmic space is desirable because it results in decreased product degradation (Talmadge and Gilbert, 1982), higher yields of correctly-folded proteins and precise trimming of the amino terminal of the protein. For these reasons, there have been many unsuccessful attempts to engineer the export of proteins that are normally found in the cytoplasm. Apparently, the structure of many cytoplasmic proteins seems to be incompatible with secretion (Oliver, 1985). Fortunately, many high-value pharmaceutical proteins such as insulin, human growth hormone, and interferon are naturally excreted proteins. When these proteins are synthesized by genetically-engineered *E. coli,* they are transported through the inner membrane and end up in the periplasmic space (Talmadge and Gilbert, 1982; Matteucci and Lipetsky, 1986; Barbero et al., 1986). Specially constructed plasmids are used to obtain high-level synthesis and efficient secretion (Ghrayeb et al., 1984). The production of recombinant secreted proteins in *E. coli* is affected by the culture conditions. The effects of the carbon source and the culture pH have been documented (Chang et al., 1986).

Excretion of recombinant proteins into the medium

Excretion of recombinant proteins into the culture fluid is highly desirable from a biotechnological point of view. (Secretion is defined here as the translocation of a protein through a single lipid membrane, whereas excretion is the complete release of a protein into the fermentation medium. An excreted protein must pass through at least one cellular membrane.) First, the recovery and purification of the protein is simplified. Excretion eliminates the need to rupture the cells to harvest the product. The cell mass is removed by centrifugation or filtration, and the product is purified from a relative dilute suspension of contaminating material. Excretion can also stabilize the product from proteolytic degradation, provided that the host organism does not release proteolytic enzymes into the culture fluid. This is true for *E. coli* and yeasts but not for *Bacillus.* The latter normally excretes many proteases that destroy the recombinant product, as will be discussed later. Finally, another advantage of protein excretion is that it allows to use continuous fermentation processes.

In *E coli,* the final location of secreted proteins is either the outer membrane or the space between the inner and the outer membranes, i.e., the periplasmic space. Normally *E. coli* does not excrete any proteins. The presence of the outer membrane prevents the complete release of the mature form of the protein into the extracellular fluid. The only way to effect the release of proteins into the medium is by altering the permeability of the outer membrane. Proteins secreted into the periplasmic space may subsequently diffuse into the medium through the altered outer membrane (Hancock, 1984; Pugsley and Schwartz, 1985). A number of chemical and physical methods have been used to effect the release of proteins from the periplasmic space, including osmotic shock (Heppel, 1971) and heat treatment (Tsuchido et al., 1985). These methods are difficult to reproduce on a large scale. Furthermore, they often result in the release of undesirable contaminants such as endotoxins that must be removed during downstream processing. Alternatively, the use of *E. coli* mutants with a defective membrane, i.e., "leaky cells" has been suggested (Lazzaroni and Portalier, 1981). However, these mutants are quite fragile and difficult to grow (Nicaud et al., 1986). Finally, several promising systems for protein excretion in *E. coli* have been developed recently using genetic engineering techniques (Abramsen et al., 1986; Kato et al., 1987; Georgiou et al., 1988).

Protein transport through the membrane(s) of other organisms follows the same principles that apply to the secretion of proteins across the inner membrane of *E. coli.* Unlike *E. coli,* however, many microorganisms readily excrete proteins into the culture medium. *Bacilus subtilis,* for example, does not have an outer membrane. Proteins that can pass through the cytoplasmic membrane are released directly into the medium. This is a very

attractive characteristic that has stimulated efforts to use this organism for protein excretion. Extracellular production of several recombinant proteins has been documented with the aid of a signal sequence derived from *Bacillus* proteins (Mezes and Lampen, 1985; Schein et al., 1986). More than 1 g/L of recombinant human growth hormone, an important pharmaceutical protein, has been obtained from the extracellular fluid of high cell density *Bacillus subtilis* cultures (Ruppen et al., 1986). However, two problems have been encountered. For reasons that are not clear, export of foreign proteins is less efficient than expected, that is, a large fraction of the protein remains inside the cell (Schein, 1986; Ulmanen, 1985). Even more important is the very low stability of proteins excreted from *Bacillus*. This organism releases many proteolytic enzymes in the medium, which cause rapid degradation of the product. In one case, the extracellular level of the desired protein was 10^4 times smaller than expected (Ulmanen et al., 1985). Although the complete elimination of proteolytic enzymes from the culture medium has proven difficult, significant progress in that direction has been accomplished (Wong et al., 1986).

Yeasts are more complex organisms than bacteria. For this reason, the mechanism by which proteins may be excreted into the medium is not as straightforward. Excreted proteins are inserted into intracellular vesicles that are transported into the cell's surface where they release their contents. *Saccharomyces cerevisiae* does not normally excrete many proteins into the medium. Nevertheless, excretion of foreign proteins has been documented (Nicaud et al., 1986; Das and Schultz, 1987). Only relatively small recombinant proteins have been excreted from yeast so far. It remains to be seen whether large proteins can be excreted as efficiently or if they are trapped in the complex cell wall surrounding the yeast cell. However, the excretion of foreign proteins from yeasts offers an important advantage. Due to the presence of a more complete system of post-translational processing excreted proteins undergo certain complicated modifications that cannot be performed by bacteria (e.g., disulfide bond isomerization and glycosylation). For this reason, the final product may resemble its natural, authentic form more closely.

Reactor Considerations

The objectives of fermentations involving recombinant organisms are very similar to the goals of other more traditional fermentations. They include high volumetric productivities and yields, as well as high product concentrations to facilitate downstream processing. These goals can be achieved by: i) high rates of cell growth and product formation; ii) high cell densities; and iii) increased product stability. At present, either batch or fed-batch (semicontinuous) processes are employed for large-scale production of recombinant proteins. In batch fermenters, all the nutrients are provided at the onset of the fermentation. Initially, the growth of the culture is unrestricted and the rate of biomass formation is high. However, high concentrations of glucose and unrestricted growth (i.e., no oxygen or other nutrient limitation) saturate some key cellular metabolic pathways (Holms, 1986). As a result, growth decelerates and inhibitory metabolic by-products begin to accumulate in the culture medium. In turn, both the final cell density and the overall yield of cell mass per unit weight of carbon source are decreased. This phenomenon (Crabtree effect) is a typical example of substrate inhibition and is observed in many different organisms. In *E. coli,* the most important inhibitory by-product is acetate although other metabolites including high concentrations of dissolved CO_2 may also become inhibitory (Zabriskie and Arcuri, 1986; Pan, 1987). Production of acetate by bacteria depends on the composition of the growth medium (Reiling et al., 1985). Yeasts, on the other hand, accumulate mainly ethanol that is equally inhibitory for growth. To prevent substrate inhibition and obtain high cell densities, a low concentration of glucose must be maintained throughout the fermentation. This can be accomplished by slow feeding of glucose in fed-batch fermenters. By controlling growth through glucose addition cell densities in excess of 80 g dry cell weight/L for *E. coli* and up to 200 g/L for *Saccharomyces cerevisiae* have been obtained (Zabriskie and Arcuri, 1986; Fieschko et al., 1987; Shay et al., 1987).

Another important consideration is the concentration of dissolved oxygen in the fermenter. When the rate of oxygen consumption exceeds the rate at which it is supplied by the mass transfer equipment, the culture becomes oxygen-limited. Both the growth rate and carbon yield are adversely affected by oxygen limitations. The problem is exacerbated by high cell densities, because oxygen consumption is proportional to biomass concentration and growth rate (Bailey and Ollis, 1986). Furthermore, the viscosity of the fermentation broth increases with cell concentration leading to a decrease in the mass transfer coefficient. The rate of oxygen transfer can be increased either by increasing agitation or by sparging pure oxygen. Mori et al. (1979) were able to attain very high concentrations of *E. coli,* using a batch fermenter where the dissolved oxygen level was kept at a constant value by manipulating the mass transfer variables (i.e., agitation, air, and pure oxygen flow rates). Alternatively, the rate of oxygen consumption in high cell density fermentations can be reduced by maintaining lower biomass growth rates. Reduced temperatures or low feeding of the limiting nutrient are used for growth control (Zabriskie and Arcuri, 1986). Decreasing the growth rate does not seem to affect the final concentration of the recombinant protein (Zabriskie et al., 1987). In general, mixing and supply of sparingly soluble gasses to a high cell density culture at a large scale remains a difficult challenge. Recently, Khosla and Bailey (1988) demonstrated a very innovative solution to the problem of oxygen transfer. The gene for an oxygen binding protein was inserted in *E. coli.* The engineered cells produced a functional protein product that allowed them to grow faster in an oxygen-limited environment.

The characteristics of the host strain sometimes determine the maximum cell density. Some of the strains that are commonly used in genetic engineering have been developed specifically for this purpose and are more susceptible to environmental influences. For reasons that are not clear, such strains often lyse when they are grown to moderate or high cell densities.

Obtaining high cell densities is only one of many parameters that determine the productivity of fermentations employing recombinant organisms. While progress in that direction has been impressive, there is little published information on the optimization of growth conditions for the synthesis of recombinant proteins. A detailed study of recombinant protein production in a high cell density fermentation was presented recently by Fieschko et al. (1987). In general, many issues need to be addressed, including plasmid stability, timing and conditions of induction and finally the effect of growth conditions on post-translational effects such as proteolysis and aggregation. Recent reports have demonstrated that protein aggregation in *E. coli*

depends on culture parameters such as the growth temperature (Schein and Noteborn, 1988). The composition of the culture medium during the production phase is also very important (Tsai et al., 1987). Many studies on the effect of environmental conditions on protein production have generated surprising results that could not have been predicted based on our current knowledge of cell physiology. For example, it was recently shown that small amounts of ethanol (which normally inhibits growth) or metal ions increase the formation of interferons and other recombinant proteins (Ben Bassat et al., 1987b; Kitano and Shigeru, 1987). These results illustrate the complexity of optimizing fermentations with recombinant organisms and underline the need for further investigations.

The use of continuous fermenters for the production of genetically engineered proteins is complicated by the instability of plasmid-containing cells (Imanaka and Aiba, 1981; Siegel and Ryu, 1985). Since they cannot compete effectively with plasmid-free cells they are eventually washed out of the fermenter. Plasmid stability in continuous fermenters can be increased substantially by immobilizing the recombinant cells (De Taxis du Poet et al., 1986, 1987). In immobilized cell bioreactors, the cells are confined either by a porous membrane or through entrapment within a solid phase. Liquid flows continuously through the reactor to provide nutrients to the cells and remove the product while the immobilized cells are retained within the reactor. The feasibility of constructing continuous immobilized bioreactors has been established with recombinant *Bacillus subtilis* (Mosbach et al., 1983), *Escherichia coli* (Inloes et al., 1983; Georgiou et al., 1985) and *Saccharomyces cerevisiae* (Karkare et al., 1986).

Monitoring Product Formation

The objective of fermentations employing recombinant cells is to maximize the formation of the active protein product. In principle, it is desirable to monitor the formation of the product on-line and use this information to optimize the fermentation environment. At present it is not possible to measure protein concentrations on-line. Extracellular proteins, for which spectrophotometric assays exist, can be quantified off-line easily. However, if a rapid assay is not available, fairly complex and time-consuming analytical procedures must be employed. For many pharmaceutical products, the time required for biological assays often exceeds the duration of the fermentation itself. To obtain a better representation of the overall cell productivity, the amount of protein present in inactive form as inclusion bodies must also be taken into account. Measurement of the total protein product requires several steps. First, the cells are harvested from the fermenter and are disrupted under conditions that solubilize all their proteins. Subsequently, the (denatured) proteins are separated by gel electrophoresis and the product is identified by its relative mobility. Sometimes immunochemical techniques must be used for quantifying the amount of the desired protein (Shatzman and Rosenberg, 1987). The whole analysis requires several hours. There is a great need for faster techniques of product monitoring in recombinant fermentations. Several methods that provide faster protein analyses are currently being developed, including Fast Protein Liquid Chromatography (FPLC) (Gustafsson et al., 1986) and solid-phase immunochemical sensors.

Concluding Remarks

The development of efficient techniques for the identification and *in vitro* manipulation of nucleic acids has generated exciting opportunities in all the industries that involve living organisms and their products. One of the first and most important applications of the new technology is the production of high-value proteins. Yet, the impact of genetic engineering is by no means limited to the efficient production of naturally occurring proteins. Rapid advances in closely-related areas, such as the *de novo* design of proteins with new catalytic characteristics (protein engineering), are generating exciting prospects for the production of efficient biocatalysts.

At present, many proteins are already being produced by genetically-engineered cells while many more are currently in the development or scale-up phase. *Escherichia coli* is the most commonly used microbial host for production purposes. Other microorganisms such as *Saccharomyces cerevisiae* and *Bacillus* were used to a limited extent in the past. Recently, however, they have gained wider acceptance as some of the earlier technical problems are being surpassed. In *Bacillus subtilis,* protein excretion occurs readily but the major problem has been the rapid degradation of the extracellular product. Efforts to eliminate product instability through the development of strains with reduced levels of proteases and manipulation of the growth conditions are currently under way. Earlier skepticism concerning the ability of *Saccharomyces cerevisiae* to produce high levels of foreign proteins (Nicaud et al., 1986), is dissipating as well (Bitter, 1987). In addition, it was recently shown that *Saccharomyces* can post-translationally modify foreign proteins more correctly than was previously anticipated (Hallewell et al., 1987; Ernst et al., 1987). Finally, several less characterized microorganisms have been proposed as potential hosts for recombinant protein production. Most promising for large-scale applications are different species of *Streptomyces* and mycellial fungi, notably *Aspergillus nidulans.* (Cullen et al., 1987; Upshall et al., 1987). However, at present, *E coli* remains the "workhorse" for genetic engineering both in the laboratory and in industry. Its importance is underscored by the fact that the development of alternative expression systems is usually based on analogies and paradigms found in *E. coli.*

Acknowledgment

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Appendix

The information that determines the amino acid sequence of a protein is contained within the DNA, a high molecular weight polymer of deoxyribonucleic acids. The remarkable ability of DNA to mediate the reproduction of an exact copy of itself is the basis of heredity. DNA consists of four different deoxynucleic acid building blocks arranged in a way that can be interpreted by other cellular components and used to synthesize proteins.

A gene is a segment of DNA containing all the information necessary for the synthesis of a protein. Part of the gene contains sequences of nucleotides involved in the regulation of the level of protein synthesis. Another part actually codes for the amino acid sequence of the protein. Within the latter region, every three residues in DNA code for a single amino acid. In the first step of protein synthesis, the DNA of the gene is transcribed into

a more flexible intermediate. This molecule is a polymer of ribonucleic acids and is called the messenger RNA.

There is a one-to-one correspondence between the sequence of DNA and the respective messenger RNA. Subsequently, the messenger RNA is translated into protein. This process is mediated by the ribosome, a large intracellular particle composed of several proteins and ribonucleic acids. The ribosome recognizes the nucleotide triplets in the *m*RNA, selects the corresponding amino acid, and catalyzes the formation of a peptide bond between the amino acid and the growing polypeptide chain. The complete protein molecule is released from the ribosome and attains its three-dimensional conformation.

All the genetic information necessary for the survival of an organism is contained in the chromosome(s). An exact copy of the chromosome is faithfully reproduced by the cell. When the cell divides, each of the daughter cells contains a copy of the chromosome ensuring the transmission of the genetic traits. Using recombinant DNA techniques, a gene can be isolated from the chromosome of a donor organism and may be manipulated *in-vitro,* for example by altering its regulatory regions. Subsequently the gene is introduced into a recipient organism, usually one that grows fast and can synthesize large amounts of the gene-encoded protein. However, unlike the chromosome, a random fragment of DNA cannot be reproduced by the cell because it does not contain the required regulatory sequences. As a result, it cannot be inherited into the daughter cells during cell fission. After a few generations cells containing the additional DNA fragment represent such a small proportion of the cell population that they cannot be detected. To ensure stable inheritance of a foreign gene it must be first inserted into a DNA vector. This is a DNA molecule that can be inserted in a cell and is stably transmitted to the progeny. There are three different kinds of vectors: viruses, cosmids, and plasmids. Plasmids are relatively small DNA molecules that exist autonomously of the chromosome. Many organisms have a wide repertoire of naturally existing plasmids but, as a rule, plasmids from one organism cannot function into another. Some plasmids exist in only one copy per cell; others may be present in several hundred copies. In contrast, there are normally one or two copies of the chromosome per cell. Certain plasmids have been manipulated to facilitate the introduction of foreign genes. These plasmids are called cloning vehicles or cloning vectors and are extremely useful for genetic engineering. These plasmids are present in multiple identical copies per cell. [More information on the fundamentals of molecular biology can be found in Bailey and Ollis (1986), and Watson et al. (1983, 1987).]

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