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Review: Optimizing inducer and culture conditions for expression of foreign proteins under the control of the *lac* promoter

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This review examines factors which influence the expression of foreign proteins in *Escherichia coli* under the transcriptional control of the *lac* and *tac* promoters, and discusses conditions for maximizing the production of a foreign protein using this system. Specifically, the influence of IPTG (isopropyl-β-D-thiogalactoside) concentration, temperature, composition of the growth medium, the point in the growth curve at which cells are induced with either IPTG or lactose, and the duration of the induction phase are discussed.

Keywords: *lac* promoter; *tac* promoter; recombinant DNA; protein overexpression; fermentation strategies; IPTG; lactose

Introduction

During the past two decades, research utilizing recombinant DNA technology has led to the development of a variety of products for use in fields as diverse as cancer treatment and pulp bleaching [29]. The majority of these products have been produced using *Escherichia coli* [17,31,36] since this bacterium has been well characterized at the molecular level and the introduction of foreign genes into it is easily accomplished. In addition, *E. coli* can be grown rapidly to a high cell density using inexpensive media and readily available fermentation equipment.

A wide variety of commercially important proteins has been expressed in recombinant *E. coli*, including xylose isomerase [8], xylanase [63], T4 DNA ligase [86], CD4 [70], and a range of different human immunomodulators, growth factors, hormones, blood proteins and viral antigens for use as vaccines [34]. The expression of these proteins in *E. coli* is generally accomplished by placing the foreign gene into a multi-copy plasmid vector under the transcriptional control of either a constitutive or regulatable strong promoter. Constitutive promoter systems provide a simple means of overproducing a foreign protein in a bacterial cell since the target protein is continually expressed. Unfortunately, continuous high level expression often causes a drain or metabolic burden on the cell's energy resources leading to a reduction or inhibition of cell growth [12,30,80]. Since the overall yield of the target protein is a function of both the cell yield (g of cells L^{-1}) and the yield of product per cell, the reduced cell growth that generally accompanies constitutive expression leads to lower yields of the target protein than is possible when using regulatable promoter systems.

Regulatable promoter systems provide the ability to 'turn on' the expression of a foreign gene by varying an environmental factor such as temperature [66,86,89], dissolved oxygen tension [41,61] or the concentration of a particular component in the growth medium [18,59]. For example, the bacteriophage lambda *p*L and *p*R promoters in conjunction with a temperature sensitive cI_{857} repressor gene can be thermally induced when the temperature of the fermentation broth is raised from the growth-optimum temperature of 30–35°C to 42°C [56,86]. The *E. coli trp* and *pho*A promoters are induced following depletion of tryptophan and phosphates, respectively, from the medium [18,70], while the *lac* promoter is induced in the presence of lactose or isopropyl-β-D-thiogalactoside (IPTG) [59]. The ability to induce foreign gene expression at will allows for the separation of cell growth from the product synthesis or induction phase of the fermentation. After obtaining a high concentration of cells in a fermenter during the growth phase, the foreign gene can then be induced resulting in higher total yields of the recombinant protein than otherwise could be achieved using constitutive expression. In some cases, the level of transcription of the foreign gene can be regulated by using an appropriate level of the inducing stimulus. This in turn can further improve yields by allowing the level of expression to be optimized during the induction phase. In order to take advantage of the flexibility of inducible promoter systems, the optimal point in the fermentation for inducing expression as well as the optimal level of inducer that needs to be used in the process must be determined.

The *lac* promoter from the *E. coli* lactose operon is one of the promoters most commonly used to regulate the expression of recombinant genes in bacteria. It is the most well understood of all bacterial promoters and has been extensively characterized at the molecular level [6,69]; however, only a small number of studies have optimized the production of recombinant proteins using this system. This article reviews factors that influence foreign protein expression from the *lac* (or *tac*) promoter(s) in *E. coli*, and thus provides a starting point for researchers attempting to optimize the conditions for expressing a target protein using this system.

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Transcriptional regulation of the *lac* promoter

The lactose (*lac*) operon in wild-type *E. coli* consists of three genes (*lac*Z, *lac*Y and *lac*A) which encode proteins responsible for metabolism of the sugar lactose. The *lac*Z protein, β-galactosidase, converts lactose to glucose and galactose; the *lac*Y gene codes for lactose permease which provides the active transport of lactose across the cytoplasmic membrane, while the *lac*A gene product has transacetylase activity and may function to detoxify lactose analogs which are harmful to the cell [91].

Transcription from the *lac* promoter is regulated by the *lac* repressor, the product of the *lac*I gene [9,69]. In the absence of inducer (ie lactose or IPTG), the *lac* repressor inhibits transcription from the *lac* promoter by binding to the operator region of the *lac* operon. When the repressor is bound to the operator, its presence prevents the proper binding of RNA polymerase to the promoter region so that transcription of the *lac* genes does not occur. However, since a chemical equilibrium exists between bound and unbound repressor molecules, the operator site is not continuously occupied by the *lac* repressor and thus there is generally a low basal level of transcription of the *lac* genes.

When the cell encounters lactose or other galactosides, transcription of the *lac* genes may increase up to 1000-fold [40]. The increased transcription occurs because, once inside the cell, a small portion of the lactose is converted, by the basal level of β-galactosidase, to an intermediate compound known as allolactose, which then binds to the repressor protein. This binding causes a conformational change in the repressor protein reducing its affinity for the *lac* operator. With no repressor bound at the operator region, significantly increased transcription of the *lac* genes occurs and expression is induced.

Transcription from the *lac* promoter is also regulated by the binding of the catabolite activator protein (CAP) to the promoter region [9,69]. When CAP binds to the promoter, it increases the affinity of the promoter for RNA polymerase, thereby increasing transcription of the *lac* genes. The affinity of CAP for the promoter is enhanced by its association with cyclic adenosine monophosphate (cAMP). Cellular levels of cAMP are highest when the amount of glucose in the medium is lowest. Thus, provided that no repressor is bound to the operator, high intracellular concentrations of cAMP lead to high levels of cAMP-CAP complex at the promoter site and cause a high level of transcription of the *lac* genes. These interactions are illustrated schematically in Figure 1 [1].

Catabolite repression allows *E. coli* to metabolize glucose preferentially prior to lactose (and a variety of other sugars) when a mixture of sugars is present in the growth medium. When the cell encounters high concentrations of glucose, transport of glucose into the cell reduces the intracellular level of cAMP. Thus, even in the presence of inducer, a high concentration of glucose leads to a low level of transcription from the *lac* promoter (Figure 1). In small scale culture, the effects of catabolite repression however, may be reduced by adding cAMP to the growth medium when inducing the *lac* promoter [10]. Glucose also indirectly modulates expression from the *lac* promoter by a process known as carbohydrate-mediated inducer exclusion

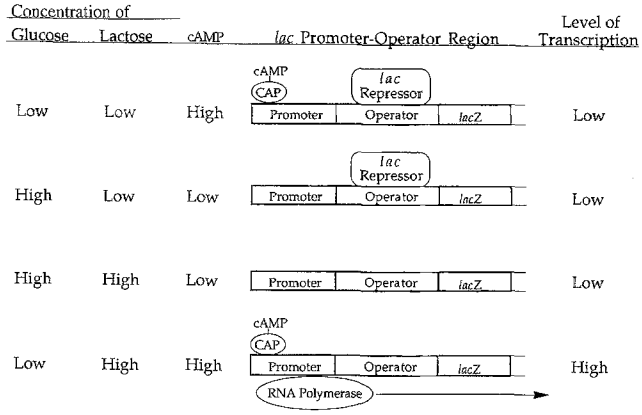

Figure 1 Effect of glucose and lactose concentration in the growth medium on the level of transcription from the *lac* promoter in *E. coli*. Adapted from [1].

[65]. Glucose (and certain other sugars, such as fructose and mannitol) are transported across the cytoplasmic membrane via the phosphotransferase system (PTS). The transport of glucose into the cell results in dephosphorylation of the glucose specific-PTS enzyme III^{Glc}, which in its non-phosphorylated form interacts with lactose permease and inhibits the transport of lactose, and hence the *lac* inducer, into the cell.

IPTG is commonly used for inducing expression from the *lac* promoter and offers a number of distinct advantages, especially in small scale experiments. Unlike lactose and other galactosides, IPTG is a metabolic-free, or gratuitous inducer, because it is not metabolized by the cell. This ensures that the level of induction remains constant following the addition of IPTG to the growth medium. IPTG is transported into the cell by methods other than *lac* permease making this compound less susceptible to inducer exclusion by glucose and sugars whose assimilation is mediated by the PTS system.

A number of variations on the *lac* promoter have been reported. For example, the *lac*UV5 promoter contains a mutation in the consensus region of the *lac* promoter that increases the promoter strength [69], and therefore has been used in place of the wild type *lac* promoter in some high expression plasmid vectors [28,82]. The *tac* promoter, a hybrid of the tryptophan (*trp*) and *lac*UV5 promoters [4,23], is reported to be 5–10 times stronger than the *lac*UV5 promoter [23,24] and is induced using IPTG and other galactosides while not being subject to catabolite repression.

Expression of foreign proteins

There are a wide variety of factors which influence the expression of foreign proteins in *E. coli*. As mentioned previously, the presence of recombinant plasmid DNA and the expression of a recombinant protein generally imposes a metabolic drain on the cells' energy resources [13,30]. This added metabolic burden often reduces cellular growth rates, causes segregational and structural plasmid instability and causes metabolic, genetic and physiological changes in the host cell which, in turn, may substantially affect the total and functional yields of a target product. While in some

cases problems of metabolic burden and low yields of recombinant protein have been solved by altering codon usage [38] or host cell metabolism through genetic manipulation [21], functional yields have also been improved by modifying culture conditions and the level of recombinant protein expression during the induction phase [73,86]. Thus, it is important to understand the influence of conditions such as temperature, growth medium composition, inducer concentration as well as the point of induction and the duration of the induction phase on the expression of the target protein in *E. coli*.

The effect of IPTG concentration

Despite the popularity of the *lac* expression system for producing recombinant products, few studies in the literature have reported optimizing the IPTG concentration for inducing recombinant gene expression, and in most cases the reasons for using a particular level of IPTG are rarely stated. While a wide range of IPTG concentrations, ranging from 0.005 to 5 mmol L^{-1}, have been reported [19,32,62,82,87], expression is commonly induced using 1 mmol L^{-1} IPTG.

As a result of metabolic burden (sometimes called metabolic load), the high concentrations of inducer that are often used in an effort to fully induce the *lac* promoter do not necessarily lead to maximal expression of a target protein [30]. In many cases, the optimal inducer concentration is chosen to balance the decreasing yields of recombinant cells following induction with increasing cellular levels of target protein [12]. For example, in studying the dynamics of chloramphenicol-acetyl-transferase (CAT) expression using the *tac* promoter, Bentley *et al* [12] showed that the increased specific CAT productivity (g CAT synthesized g^{-1} biomass), following mid-log phase induction with IPTG reduced the specific growth rate of the cells in batch culture. Moreover, the severity of the reduction in specific growth rate increased with increasing levels of CAT protein expression. At high inducer concentrations (3.4 mmol IPTG L^{-1}), the reduction in specific growth rate was so severe (ie 80%) that it led to the early onset of the stationary phase and cell death, while at slightly lower levels (2.1 mmol L^{-1}), the cells partially recovered from the initial shock following induction and established a constant specific growth rate that was 60% lower than pre-induction levels. Thus, the overall level of CAT expression in batch culture involved a tradeoff between increasing product expression in the cell and decreasing cell yields resulting from a lower specific growth rate following induction with IPTG. As a result, use of an intermediate IPTG concentration of approximately 0.8–1.0 mmol IPTG L^{-1} balanced the opposing phenomena of increasing target protein expression and decreasing cell yields to maximize the overall expression of the recombinant product [12].

To understand the transcriptional and translational responses to varying levels of induction with IPTG, Wood and Peretti [88] used continuous culture to study the steady-state responses of cells to increasing levels of metabolic load when producing recombinant β-galactosidase. The β-galactosidase was under the transcriptional control of the *tac* promoter and the metabolic load was modulated by inducing with 0.01 to 7.5 mmol IPTG L^{-1}. Under these conditions, the synthesis rate of β-galactosidase mRNA and the steady state activity of this enzyme increased linearly with IPTG concentration up to 1 mmol IPTG L^{-1}. Further increases in IPTG concentration did not lead to stoichiometric increases in *lac*Z transcription. While it was possible that most of the *lac* promoters were fully induced above 1 mmol IPTG L^{-1} (due to the complete titration of repressor molecules by IPTG), in this case it appears that degradation of the β-galactosidase mRNA played an important role in limiting protein expression. The degradation rate of the β-galactosidase mRNA increased with increasing levels of induction such that the 42-fold increase in the β-galactoside mRNA synthesis rate when the inducer concentration was increased over the range studied led to only a 4-fold increase in the steady state level of β-galactosidase mRNA. The fact that raising the IPTG concentration from 1 to 7.5 mmol L^{-1} increased the *lac*Z transcription rate by 75%, but increased the steady state levels of β-galactosidase mRNA and activity by only 32% and 22%, respectively, suggested that mRNA stability rather than transcription limited product expression.

The study of Wood and Peretti [88] also suggested ways in which the induction of recombinant mRNA synthesis can alter the cellular processes and redirect energy resources. At low levels of inducer (<0.1 mmol IPTG L^{-1}), increased levels of transcription reduced the synthesis of ribosomal RNA (rRNA). However, when IPTG concentrations greater than 0.1 mmol L^{-1} were used, the cells responded to induction by increasing the synthesis rate and steady state levels of ribosomal RNA. It was theorized that induction of the *tac* promoters with IPTG diverted RNA polymerase away from ribosome operons, leading to the observed decrease in rRNA synthesis. At high levels of induction, the increased steady state levels of mRNA synthesis may have severely depleted the pool of free non-translating ribosomes in the cell. Under these conditions, the recombinant cells may have diverted energy into producing rRNA to increase translational capacity in order to deal with increased translational demands. Thus, under transient conditions, this diversion of energy (metabolic load) to increase translational capacity observed by Wood and Peretti [88], may account for at least a temporary reduction in growth rate and plasmid stability as the cell attempts to keep pace with the added burden of the recombinant gene expression.

Induction with IPTG also appears to cause a variety of stress responses in the cell. In non-transformed strains with a single *lac* promoter on the chromosome, increased synthesis of the heat shock proteins DnaK, GroEL, GroES was observed 30 min after the addition of 0.5 mmol IPTG L^{-1} [45]. A temporary inhibition of H35 protein—normally present only during exponential growth—following induction was also encountered, suggesting that cells must adapt to even low levels of expression induced with IPTG. As well, proteolytic degradation of abnormal proteins can be influenced by the level of induction with IPTG, with degradation by some pathways increasing or decreasing with increasing IPTG levels [44]. High level recombinant protein expression using IPTG has also been shown to induce the expression of a variety of proteases in media where the concentrations of certain amino acids may be limiting [32].

The location of the *lac* repressor as well as the quantity

of *lac* repressor molecules produced in the cell will greatly affect the level of IPTG needed to fully induce expression from the *lac* promoter. For example, in cases with high plasmid copy number, plasmid-borne *lac* or *tac* promoter/operators may partially or completely titrate repressor molecules expressed from the chromosomal *lacI* gene. As a result, full induction may be achieved with as little as 0.001–0.1 mmol IPTG L^{-1}, depending on plasmid copy number during the induction phase [48]. In addition, in those instances in which the region of the plasmid DNA that controls the plasmid copy number is just downstream from a *lac* or *tac* promoter, and there is no strong transcription terminator between these elements, induction of the promoter may artificially increase the plasmid copy number (ie cause a phenomenon known as runaway replication). This may result in a higher level of gene expression as a consequence of the increased gene copy number [84]. While this strategy might seem to be an attractive way to increase the level of foreign gene expression, the potential for plasmid instability and other metabolic alterations as a consequence is likely to be problematic especially in large scale fermentations.

In other cases, where the gene for *lacI* is present on the recombinant plasmid to prevent promoter leakiness (ie high levels of non-induced background expression), the level of *lac* repressor present will depend on the plasmid copy number in the cell, and IPTG concentrations required for a particular level of induction will vary accordingly [88]. Thus, the optimal concentration of IPTG appears to be highly system-dependent, and the types of metabolic changes found at the particular IPTG concentrations used by Wood and Peretti [88] may occur at different concentrations of IPTG in other systems. This may explain why the optimal induction of glutathione-*S*-transferase using the *tac* promoter in the *lacI*-containing strain DH5α required only 0.062 mmol IPTG L^{-1} [87]. The mutant $lacI^q$ gene which produces 10-fold more repressor than *lacI*, is often used in recombinant strains to provide better repression of recombinant gene expression in the absence of inducer. Thus, the $lacI^q$ phenotype is generally optimally induced using greater concentrations of IPTG compared to wild type *lacI* strains.

Recombinant protein expression in strains containing either $lacI^q$ or plasmid borne *lacI* may be lower than that of wild-type *lacI* strains, even when fully induced with high levels of IPTG [48]. While plasmid-borne *lacI* genes provide more complete repression of the *lac* promoter, the high level of repressor protein adds to the metabolic burden of recombinant cells and may affect the cells' translational capacity [88]. Mathematical models have been developed to evaluate the effects of different promoter/repressor configurations on recombinant gene expression [48,52,53] and translational capacity [49] in *E. coli*. These models, however, do not account for situations such as inclusion body formation or for other limitations such as protein folding and secretion to the periplasm.

The characteristics of a particular protein, its desired form and the cellular location of the expressed protein may also significantly influence the level of IPTG used for optimal induction. For example, the activity of secreted proteins is often enhanced when expression is only partially induced. Reducing IPTG concentration from 1 to 0.005 mmol L^{-1} greatly enhanced the activity of subtilisin E secreted to the periplasm, while reducing cell lysis [82]. Replacing the *lac* promoter with the stronger *lac*UV5 promoter to increase the expression of secreted F_{ab} fragments, provided a 5- to 10-fold increase in target protein yield, but gave little to no improvement in the yield of functional F_{ab} molecules [76]. Similarly, a 2- to 10-fold increase in the yield of functional secreted F_{ab} fragments was observed when the *lac* promoter was induced with 0.01–0.1 mmol IPTG L^{-1}, rather than 1.0 mmol L^{-1} [73]. Efficient production of periplasmic β-lactamase and human epidermal growth factor from the *tac* promoter also utilized 0.1 mmol IPTG L^{-1} [19]. In all of these cases, it appears that the solubility and the proper folding of secreted proteins benefited greatly from the lower transcription rates when less than the 'standard' inducer concentration of 1 mmol IPTG L^{-1} was used.

In contrast to the above examples, higher levels of transcription have been used to enhance the expression and/or localization of some secreted proteins. For example, high level expression from a *tac* promoter, in overnight cultures, was used to improve the expression of periplasmic β-lactamase activity and enhance outer membrane permeability, so that 90% of the β-lactamase activity was released to the culture medium [28]. Leakage of the periplasmic proteins, however, required high levels of target protein expression, since leakage of β-lactamase activity to the medium was not observed when a weaker *tac*II mutant promoter was used. This periplasmic leakage, at high levels of β-lactamase expression, may occur because the secretion of the foreign protein interferes with the expression of the outer membrane proteins and, therefore, weakens the integrity of the outer membrane [28].

Differences in the level of periplasmic expression with increasing promoter strength may be due in part to the nature of the recombinant product itself. β-lactamase is a native *E. coli* protein with a very efficient secretion signal sequence. High transcription and secretion rates for this protein may, therefore, be possible since evolution has likely 'optimized' its structure for secretion into the periplasm. β-lactamase may also fold more efficiently than many foreign proteins in the periplasm, so that expression limitations of activity due to proper folding and/or aggregation may be less significant. In the case of antibody fragments, the solubility and level of secretion of single chain F_vs may be significantly affected by the order in which the V_H and V_L domains are secreted [5]. For F_{ab} and F_v fragments, the individual light and heavy chains must fold and associate properly to form functional proteins. Thus, the expression of F_{ab} and F_v fragments may become limited at higher induction levels by the folding of each protein chain as well as the homodimerization or incorrect chain associations leading to aggregation [42]. Since point mutations in F_{ab} and F_v fragments have been found to significantly improve their folding and soluble expression in the periplasm [43], the amino acid sequence of a protein can significantly influence its soluble and functional expression when secreted to the periplasmic space.

The level of inducer required for optimal expression depends on the strength of the promoter, the presence or absence of repressor genes on a plasmid, the cellular

location of product expression, the response of the cell to recombinant protein expression, the solubility of the target protein and the characteristics of the protein itself. For inducing the expression of an intercellular recombinant protein, the use of 1 mmol IPTG L^{-1} is a reasonable starting point since maximal induction is predicted to occur for both *lac*I and *lac*I^q strains at this level [48]. For secreted proteins, however, IPTG concentrations of 0.01 to 0.1 mmol L^{-1} IPTG may be the optimal range when using the *lac* or *tac* promoters, to minimize potential problems due to product insolubility, growth inhibition and cell lysis. One approach to optimizing the level of foreign gene expression and reducing costs includes the development of a simple mathematical model and the use of optimization theory to describe the effect of variations in IPTG concentration and time of induction on β-galactosidase production [51].

The effect of temperature

The maximum specific growth rate of *E. coli* occurs at a temperature of 37–39°C [35]. While this temperature also coincides with the maximal activity of the *lac* and *tac* promoters [6], the use of suboptimal growth temperatures in some cases can reduce unwanted metabolic responses to the synthesis of a foreign protein and as a consequence improve the yield and/or solubility of the target protein product. For example, induction of either β-galactosidase or recombinant single-chain urokinase-like plasminogen activator (rscuPA) expression with IPTG at 30°C rather than 37°C minimized or eliminated the concomitant synthesis of stress or heat shock proteins in *E coli* [45,81]. Reducing culture temperatures from 37°C to 25–30°C also significantly reduced proteolytic degradation and improved the stability of expressed rscuPA, β-galactosidase, interferon α-2 and periplasmic protein A-β-lactamase fusion proteins [7,20,45,81]. Also, ATP-dependent proteolytic activity was shown to decrease more than 2.5-fold as the temperature was decreased from 42°C to 30°C, while ATP-independent proteolysis varied by only 25% over this range [44]. Since it has been suggested that ATP-dependent proteolysis of abnormal or recombinant proteins may initiate further degradation by ATP-independent proteases, lower culture temperatures may minimize the activity of pathways that lead to product degradation [44]. Increased proteolytic degradation of recombinant products above 30°C may also result from conformational changes in the target protein that expose sites for proteolytic cleavage [20].

Precipitation of overexpressed protein in the form of insoluble aggregates (inclusion bodies) is also less prevalent at lower temperatures. For example, growth and induction at 21°C instead of 37°C enhanced the soluble yield of cytoplasmic F_{ab} fragments 10-fold [16], while the solubility of human interferon and murine protein Mx was greatly improved by using culture temperatures of 23–30°C. Similarly, reducing the temperature from 37°C to 20–30°C reduced the formation of periplasmic inclusion bodies for both β-lactamase and human epidermal growth factor, while improving the total activity and extracellular purity of these proteins [19]. Since formation of insoluble aggregates is believed to result from the interaction of hydrophobic regions of folding intermediates [33,55], lower temperatures may act to reduce the kinetics of off-pathway protein aggregation relative to the folding reactions, resulting in higher yields of properly folded product.

In a similar manner, lower culture temperatures may enhance the proper export, folding and assembly of functional recombinant proteins secreted to the periplasm. For example, total and functional yields of human epidermal growth factor as well as F_v and F_{ab} fragments, were consistently greater at 20–30°C compared to those at 37°C [19,76]. In other studies, functional yields of subtilisin E, and sF_v and F_{ab} fragments were also substantially improved by reducing the culture temperature [73,78,82,83].

As with reduced inducer concentration, lower culture temperatures may enhance functional protein formation by reducing the rate at which an overexpressed protein is formed. Reduced expression rates reduce the concentration of the unfolded (recombinant) intermediate in the cell. At lower concentrations, reduced interaction between unfolded intermediates may allow the proteins to react via folding pathways rather than those leading to aggregation [42]. Improved folding at lower temperatures may also reduce the level of interaction between improperly folded recombinant product and the inner and outer membranes. Since long-term exposure to improperly folded proteins may substantially reduce membrane integrity [75], this may explain why outer membrane leakiness, cell lysis and death observed at 37°C in response to expression of secreted proteins was reduced or eliminated at 20–30°C [19,75,78,82].

The effect of growth medium composition

Studies examining the expression of recombinant proteins under the transcriptional control of the *lac* promoter have utilized both complex (such as LB medium [72]) and minimal (such as M9 medium [72]) growth media. Complex media generally provide an abundance of amino acids, vitamins and trace elements to support growth and product expression in *E. coli*. However, their undefined nature makes it difficult to analyze and monitor the effect of a limiting substrate on cell and product yields during a fermentation. As a result, minimal media which contain defined amounts of carbon sources (sugars), nitrogen sources, minerals and trace elements are more commonly used for research purposes. While minimal media may be less expensive than complex media, the use of defined minimal media can lead to lower cell growth rates and product yields if the composition is sub-optimal [71].

The composition of the growth medium at or during the induction phase can significantly affect foreign protein expression. As previously mentioned, overexpression of a protein places an added metabolic burden on the cell's energy and carbon and amino acid pools, which may result in reduced cell growth rates and a stress response characterized by enhanced protease and heat shock protein synthesis [32,45,46,81]. Providing additional amino acids by supplementing the medium with casamino acids, peptone or yeast extract during induction has been shown to improve foreign protein expression [54,58,86] and stability [86]. In many cases, where the amino acid composition of a recombinant protein is significantly different from the native proteins of the host cell, stress responses may result from the cell's attempt to replenish amino acids that were depleted by the onset of the foreign protein expression

[32,67]. Supplementing the medium with particular amino acids to account for alterations in amino acid requirements when expressing a recombinant product reduces protease levels and increases metabolic efficiency to improve yields of recombinant CAT under transcriptional control of the *tac* promoter [67]. However, the metabolic consequences of amino acid supplementation must be carefully considered when developing a feeding strategy since increasing the concentration of some amino acids (eg phenylalanine and histidine) can significantly repress or inhibit the biosynthesis of other amino acids and/or essential metabolites [67]. In addition, it has been suggested that 'amino acid supplementation, combined with moderately strong plasmid-encoded protein expression, results in a depletion of low-molecular-weight organics compared to plasmid-free cells' [74]. In this case, supplementing a fermentation with particular amino acids leads to a situation in which other metabolites limit growth, thereby leading to plasmid instability.

It has been reported that addition of yeast extract increases non-induced background expression from the *lac* promoter [25,77] and that yeast extract is more effective than various other amino acid and complex protein supplements (ie peptone, casamino acids, etc) for improving cell yields [54]. In addition to enhancing cell yields, supplementing complex LB medium with a 0.5–1.5% (w/v) yeast extract during induction enhanced specific and/or overall recombinant protein expression in some strains of *E. coli* [54]. While the reasons for these effects are unknown, the presence of transcription enhancers such as cAMP or other compounds in yeast extract may provide improved yields over other complex supplements.

The effect of glucose concentration

The expression of foreign proteins under the transcriptional control of the *lac* promoter is influenced by the level of glucose that is present during the induction phase. While glucose is easily metabolized by the cell and is therefore an ideal substrate for cell growth, the presence of glucose in the cell represses transcription from the *lac* promoter via catabolite repression. Since catabolite repression results from low levels of cAMP in the cell, supplementing the medium with cAMP has been effective in overcoming this suppression and improving foreign protein synthesis [10,11].

Glucose can be used effectively as a carbon source when expressing foreign proteins from the *lac* promoter provided that the concentration is sufficiently low, ie below 0.1% (w/v), during the induction phase [22,54]. Fermentation strategies whereby the glucose concentration was depleted (and/or maintained) below the level causing catabolite repression prior to the start of the induction phase have been successfully used to achieve high yields of a variety of recombinant proteins [22,37,59,60,62].

The presence of glucose is often advantageous under conditions where expression of the foreign product must be limited. For example, maintaining catabolite repression during the growth phase using appropriate levels of glucose, can reduce promoter leakiness, or non-induced background expression, from the *lac* promoter. This may be particularly useful in preparing an inoculum for a fermenter. As well, in cases where background expression may be deleterious to the growth rate or viability of the host cell, catabolite repression has been used to protect the cell during the growth phase and substantially improve cell yields prior to induction [22].

Glucose has also been used to enhance plasmid stability and improve Protein A synthesis under the control of the *lac* promoter in continuous culture of *E. coli* [85]. By using glucose rather than glycerol under carbon-limited conditions, low levels of catabolite repression appeared to partially limit the constitutive expression of Protein A from the *lac* promoter so that plasmid stability could be maintained. Thus, when used under appropriate conditions, catabolite repression caused by glucose can substantially improve the overall yields of recombinant protein regulated by the *lac* promoter.

When cells are grown on glucose as a carbon source, harmful by-products such as acetate may accumulate, thereby inhibiting cell growth and protein production [21]. In one study, it was shown that it is possible to modulate the glucose uptake rate genetically and, as a result, decrease some of the inhibition that results from acetate production [21]. In addition, strategies which employ fed-batch addition of glucose and limit the growth rate of the culture would also be expected to produce lower levels of acetate.

The use of lactose as an inducer

Despite the fact that lactose is readily available in large quantities as a by-product of the dairy industry, only a limited number of laboratory studies have examined conditions for using lactose to induce foreign protein expression from the *lac* or *tac* promoters. This reflects the fact that optimizing conditions using lactose as the inducer may be a more complex task than for IPTG due to the physiological response of the cell to the presence of the sugar and the fact that it is metabolized by the cell. However, because of the high cost of IPTG compared to lactose, the use of the latter may provide an inexpensive alternative means of inducing foreign protein expression from the *lac* and *tac* promoters. IPTG is not only costly, but it may also be toxic to humans so that its presence as a contaminant of the final purified protein product is problematic.

The genetic background of the host strain must be considered when using lactose to induce expression from the *lac* promoter. Unlike IPTG, which binds directly with the *lac* repressor without modification, lactose must be converted by β-galactosidase to allolactose in order for induction to occur [9]. Since *E. coli* strains deficient in β-galactosidase activity are often used when first cloning and expressing a gene from the *lac* promoter, many recombinant strains can not be induced with lactose nor utilize lactose as a carbon/energy source to support growth. Thus, it is important to use *E. coli* strains that contain β-galactosidase activity when planning to induce transcription from the *lac* promoter using lactose.

Lactose is as effective as IPTG for inducing recombinant calf prochymosin and tyrosine phenol lyase using the *tac* promoter [26,39]; F_{ab} fragments using the *lac* promoter (Donovan, Robinson and Glick, unpublished observations); and hoof and mouth disease viral protein 1 (VP1) under the control of the T7 phage promoter/polymerase system

controlled by the *lac* promoter [59,60]. For the expression of calf prochymosin, simultaneous growth and induction in batch culture using lactose with glycerol as a carbon source yielded greater amounts of recombinant protein than was produced using 1 mmol IPTG L^{-1} [39]. While recombinant protein was produced during the log phase of batch growth with IPTG, high levels of product were produced with lactose during the late log and stationary phases [26,39]. Similarly, while VP1 synthesis was observed immediately following the addition of IPTG to the growth medium in late-log phase, product synthesis was delayed by approximately 1–2 h when lactose was added under these conditions in both batch and fed-batch cultures grown on glucose [59,60]. In addition, delayed expression of VP1 protein occurred in fed-batch culture despite depletion of lactose from the growth medium during this period [60].

The delayed response to induction with lactose in these studies may have resulted from the need to fully induce or activate the *lac* permease for lactose transport into the cell [26,39]. Given that non-PTS sugars (such as glycerol and lactose) compete for the same pool of III^{Glc} enzymes to activate their membrane transport proteins [65], preferential binding of III^{Glc} to glycerol permease over lactose permease may have resulted in higher levels of transport of glycerol into the cell compared to lactose. If so, high level induction may not occur until glycerol levels are reduced later in the growth phase. Alternatively, since lactose transport causes a temporary depletion of the membrane proton motive force [3] and may compete for the cell's energy resources [79], the energy required for recombinant protein synthesis may not be available in the cell until sufficient energy is acquired from the metabolism of lactose. This may explain why recombinant VP1 synthesis was delayed following the addition of lactose by approximately 1–2 h despite the depletion of lactose from the growth medium during this period [59,66].

The effect of induction time and duration

As mentioned previously, foreign protein expression generally causes a metabolic burden on the cell which can result in reduced growth rates, cell yields, product expression, and plasmid stability [12,13,30]. While the level of IPTG inducer used can be varied to adjust the extent of the metabolic burden imposed on the cell, the maximum yield of foreign protein from a fermentation will also depend on the point in the growth cycle at which expression is induced. For strains whose growth and/or viability is drastically reduced following induction, induction in late-log or stationary phase provides high cell densities for increased product formation. However, as shown for CAT expression under the control of the *tac* promoter [12], low growth rates and protease activity brought on by depleted nutrient levels in the stationary phase can reduce the yield of foreign protein. In this case, optimal induction in the mid-log phase provided sufficient levels of CAT protein within the cell while achieving a high cell density to produce the maximal yield. When product expression is low and/or does not significantly influence cell growth, overall foreign protein yield is maximized by inducing expression throughout the entire growth phase. This was seen for production of secreted penicillin acrylase (PA) in *E. coli* under the control of the *lac* promoter [68], where early-log induction was optimal because PA transport to the periplasm rather than the energy level available to the cell was the limiting factor.

Optimal induction with lactose is also dependent on the time at which the inducer is added. For example, cellular levels of VP1 protein were greatest when lactose was added just before the glucose was depleted from the medium in late log phase [59]. The addition of lactose 1 h later in the stationary phase produced poor yields of the target protein. Thus, the point in the culture where glucose is nearly depleted may be an optimal time for inducing recombinant protein expression with lactose. Therefore, the use of on-line analyses for quantifying the growth phase carbon-source would be an effective control and optimization strategy for the chemically induced production of foreign proteins in *E. coli*.

A number of studies have reported that secreted foreign proteins could be isolated from the culture medium [14,19,64,73,82,83]. While *E. coli* does not normally secrete proteins to the medium, increasing the duration of the induction phase enhances the release of periplasmic proteins to the surrounding environment. For example, the release of subtilisin E to the medium began 6 h after induction with IPTG [82], while periplasmic F_{ab} fragments leaked into the culture media as the induction phase was extended beyond 10 h [73]. For the production of a sF_v fragment, 10% of the target protein was found in the culture medium after 4 h, while the proportion of extracellular product increased to 40% after 8 h and to 90% following 20 h of induction [83]. In other studies, long induction periods (16–24 h) have been used to produce 2–10 mg L^{-1} yields of antibody fragments in the culture medium [14,83]. While in some cases, the release of recombinant proteins to the medium has been associated with cell lysis [78,83], periplasmic leakage has also been associated with actively growing cells [19,28]. Continual exposure to improperly folded periplasmic proteins [75], or high level secretion of the foreign protein that interferes with the synthesis of outer membrane proteins [28], may lead to increased membrane permeability with time. A weakened outer membrane may also result from high levels of foreign protein expression during extended stationary phase culture. Other studies have suggested that the uncontrolled environment of the shake flask [62] may contribute to periplasmic leakiness since protein release was found to be lower when the recombinant cells were grown in a fermenter [18,62].

Several systems have been reported in which foreign proteins synthesized in *E. coli* are deliberately released to the external medium [2,15,47,57]. A novel method of inducing recombinant cells to continuously release periplasmic proteins to the growth medium involves addition of both glycine to the medium and the concomitant expression of bacteriocin release protein [90]. It was recently reported that, while fusion proteins that included *E. coli* thioredoxin were localized in the cell cytoplasm, they could be released, in a soluble form, to the exterior of the cell by osmotic shock, thereby simplifying their purification [50].

Conclusions

The optimal conditions for inducing recombinant protein expression under the control of the *lac* promoter depend on

the promoter system being used (ie *lac* or *tac*), the level of *lac* repressor, the extent of the metabolic load caused by the recombinant product expression and the point in the fermentation when the inducer is added. Also, the characteristics of the target protein and its desired form, and the cellular location of target protein accumulation can significantly affect the optimal inducer concentration. For soluble cytoplasmic proteins, optimal expression may occur at IPTG levels of approximately 1.0 mmol L^{-1}. For secreted proteins or proteins which may form insoluble aggregates, reduced levels of expression using lower levels of IPTG (0.005–0.1 mmol L^{-1}) may be optimal.

Providing appropriate levels of amino acids for product expression during the induction phase can reduce target protein degradation and cell stress responses, increasing product expression. Yeast extract at concentrations of 0.5–1.5% (w/v) may provide nutrients, in addition to amino acids, which enhance foreign protein expression when using the *lac* promoter. Careful control of the glucose concentration at the time of induction can also improve the yield of target protein from the *lac* promoter while minimizing the unwanted effects of catabolite repression and inducer exclusion. Lactose appears to be as effective as IPTG for inducing recombinant protein expression, however, it must be provided under the appropriate conditions.

Systems excreting proteins to the periplasm appear to benefit from the use of suboptimal growth temperatures (20–30°C) during the induction phase. Depending on the product being produced, induction at lower culture temperatures may improve the expression of a soluble target protein as well as reduce proteolytic degradation and heat shock-like responses in the cell.

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