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Protein Production by Auto-Induction in High-Density Shaking Cultures

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Abstract

Inducible expression systems in which T7 RNA polymerase transcribes coding sequences cloned under control of a T7*lac* promoter efficiently produce a wide variety of proteins in *Escherichia coli*. Investigation of factors that affect stability, growth and induction of T7 expression strains in shaking vessels led to the recognition that sporadic, unintended induction of expression in complex media, previously reported by others, is almost certainly caused by small amounts of lactose. Glucose prevents induction by lactose by well-studied mechanisms. Amino acids also inhibit induction by lactose during log-phase growth, and high rates of aeration inhibit induction at low lactose concentrations. These observations, and metabolic balancing of pH, allowed development of reliable non-inducing and auto-inducing media in which batch cultures grow to high densities. Expression strains grown to saturation in non-inducing media retain plasmids and remain fully viable for weeks in the refrigerator, making it easy to prepare many freezer stocks in parallel and use working stocks for an extended period. Auto-induction allows efficient screening of many clones in parallel for expression and solubility, as cultures have only to be inoculated and grown to saturation, and yields of target protein are typically several-fold higher than obtained by conventional IPTG induction. Auto-inducing media have been developed for labeling proteins with selenomethionine, ^{15}N or ^{13}C, and for production of target proteins by arabinose induction of T7 RNA polymerase from the pBAD promoter in BL21-AI. Selenomethionine labeling was equally efficient in the commonly used methionine auxotroph B834(DE3) (found to be *metE*) or the prototroph BL21(DE3).

Keywords

auto-induction; T7 expression system; lactose; pBAD promoter; arabinose; protein production; high-density batch cultures; metabolic control of pH; selemomethionine labeling; isotopic labeling

Background and Introduction

DNA sequencing projects have provided coding sequences for hundreds of thousands of proteins from organisms across the evolutionary spectrum. Recombinant DNA technology makes it possible to clone these coding sequences into expression vectors that can direct the production of the corresponding proteins in suitable host cells. An inducible T7 expression system is highly effective and widely used to produce RNAs and proteins from cloned coding sequences in the bacterium *Escherichia coli* [1, 2]. The coding sequence for T7 RNA polymerase is present in the chromosome under control of the inducible *lacUV5* promoter in hosts such as BL21(DE3). The coding sequence for the desired protein (referred to as the target protein) is placed in a plasmid under control of a T7 promoter, that is, a promoter recognized specifically by T7 RNA polymerase. In the absence of induction of the *lacUV5* promoter, little

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T7 RNA polymerase or target protein should be present and the cells should grow well. However, upon addition of an inducer, typically isopropyl-β-D-thiogalactoside (IPTG)[1], T7 RNA polymerase will be made and will transcribe almost any DNA controlled by the T7 promoter. T7 RNA polymerase is so specific, active and processive that the amount of target RNA produced can be comparable to the amount of ribosomal RNA in a cell. If the target RNA contains a coding sequence with appropriate translation initiation signals (such as the sequence upstream of the start codon for the T7 major capsid protein), most protein synthesis will be directed toward target protein, which usually accumulates to become a substantial fraction of total cell protein.

A problem in using inducible T7 expression systems is that T7 RNA polymerase is so active that a small basal level can lead to substantial expression of target protein even in the absence of added inducer. If the target protein is sufficiently toxic to the host cell, establishment of the target plasmid in the expression host may be difficult or impossible, or the expression strain may be unstable or accumulate mutations [3-6]. An effective means to reduce basal expression is to place the *lac* operator sequence (the binding site for *lac* repressor) just downstream of the start site of a T7 promoter, creating a T7*lac* promoter [2, 4]. *Lac* repressor bound at the operator sequence interferes with establishment of an elongation complex by T7 RNA polymerase at a T7*lac* promoter and substantially reduces the level of target mRNA produced [4, 7, 8]. If sufficient *lac* repressor is present to saturate all of its binding sites in the cell, the basal level of target protein in uninduced cells is substantially reduced, but induction unblocks both the *lacUV5* and T7*lac* promoters and leads to the typical high levels of expression. Thus, the T7*lac* promoter increases the convenience and applicability of the T7 system for expressing a wide range of proteins.

Structural genomics is an area where multi-milligram amounts of many widely different proteins are sought for determination of protein structures by X-ray crystallography or nuclear magnetic resonance (NMR) [9]. Not all target proteins will be well expressed and soluble, so it is desirable to screen in parallel many small cultures expressing different target proteins to identify those useful for scaling up. A significant difficulty in large-scale screening is to obtain all of the cultures in a comparable state of growth, so that they can be induced simultaneously. Differences in lag time or growth rate typically generate a situation where different cultures will be ready for induction at different times. Even if cultures were grown in a multi-well plate and densities could be read simultaneously in a plate reader, considerable effort would be required to follow growth and add inducer to each culture at the proper time. If all of the cultures were collected at once, choosing a collection time when all had been induced to optimal levels and none had suffered overgrowth by cells incapable of expressing target protein might be difficult or impossible.

One strategy for obtaining fairly uniform induction is to incubate a plate until all of the cultures have grown to saturation, add fresh medium, grow for an appropriate time, and add inducer to all wells at the same time. If all cultures in a plate saturate at comparable density and grow after dilution with similar enough kinetics, the culture-to-culture variation in density at the time of induction might be low enough that most cultures will be optimally induced. However, in a test of this strategy, I encountered the unintended induction described by Grossman et al. [6], who found that cultures growing in certain complex media induce substantial amounts of target protein upon approach to saturation, in the absence of added inducer. Induction at saturation would stress cells to different extents, depending on the levels of induction and relative toxicity of target proteins to the host cells, making a strategy of saturation followed by dilution unworkable in media that have such inducing activity. Grossman et al. [6] concluded

[1]*Abbreviations used:* IPTG, isopropyl-β-D-thiogalactoside; PDB, Protein Data Bank; SSAT, human spermidine/spermine acetyltransferase; SeMet, selenomethionine; TRB, terrific broth; PTS, phosphoenolpyruvate:carbohydrate phosphotransferase system

that the known inducer lactose was not responsible for unintended induction but that cyclic AMP is required, and they found that using a host mutant unable to make cyclic AMP improved plasmid stability and protein production. Consistent with a role for catabolite repression, they also found that addition of 1% glucose to the complex medium prevented unintended induction. However, I observed that addition of 1% glucose also caused saturated cultures to become very acidic, which limits saturation density and again makes it difficult to get uniform growth upon dilution.

Upon further investigation, I found that media made with N-Z-amine AS from a 100-pound barrel recently acquired for structural genomics work showed induction at saturation whereas otherwise identical media made from the previous (almost exhausted) barrel from the same supplier did not. Screening different lots of N-Z-amine or other enzymatic digests of casein for those without the inducing behavior did not seem to be an attractive solution: besides the obvious inefficiency, such lots might not always be available. To address the problem of sporadic, unwanted induction, I undertook a systematic analysis of the components of both complex and defined media and their effects on growth and induction. The goal was to develop formulations for reliable growth of cultures of T7 expression strains to saturation with little or no induction and to define conditions suitable for growth and induction of many cultures in parallel.

Materials and methods

Bacterial strains and plasmids

E. coli strains used for testing growth and expression were primarily BL21(DE3) and B834 (DE3). B834 is a restriction-modification defective, galactose-negative, methionine auxotroph of *E. coli* B [10]. BL21 is a Met^+ derivative of B834 obtained by P1 transduction [1]. DE3 lysogens contain a derivative of phage lambda that supplies T7 RNA polymerase by transcription from the *lacUV5* promoter in the chromosome [1]. BL21-AI (Invitrogen) is a derivative of BL21 that supplies T7 RNA polymerase by transcription from the arabinose-inducible pBAD promoter in the chromosome.

Coding sequences for target proteins were cloned under control of the T7*lac* promoter and the upstream translation initiation signals of the T7 major capsid protein [2, 4, 11] by placing the initiation codon at the position of the *Nde*I site of pET-13a [12] or pET-24b (Novagen), or the *Nco*I site of pREX vectors (equivalent to the *Nco*I site of pET-11d [2]; to be described elsewhere), all of which confer resistance to kanamycin. Plasmids containing the T7*lac* promoter also contain a copy of the *lacI* gene to provide enough *lac* repressor to saturate all of its binding sites.

A variety of different target proteins were used in developing and testing non-inducing and auto-inducing media, including a set of about 100 yeast proteins cloned for a structural genomics project (http://proteome.bnl.gov/targets.html). For convenience, specific yeast proteins mentioned in the text are referred to by their target numbers: P07 refers to yeast protein YBL036C, Protein Data Bank (PDB) 1B54, structurally similar to the N-terminal domain of an amino-acid racemase [13]; P19 refers to yeast protein YBR022W, of unknown function; P21 refers to the protein specified by yeast gene *sup45*, a translation release factor; P35 refers to the protein specified by yeast gene *hem13*, PDB 1TXN, coproporphyrinogen III oxidase; and P89 refers to yeast protein YMR087W, PDB 1NJR, proposed from its structure to be an ADP-ribose-1″-monophosphatase [14]. The coding sequence for human spermidine/spermine acetyltransferase (SSAT) was amplified by reverse transcriptase and PCR from total RNA from a human cell line (the kind gift of Paul Freimuth) and cloned in pET-13a. Bacteriophage T7 proteins specified by genes *10A* (the well-expressed major capsid protein), *5.3* and *7.7*, (highly toxic proteins of unknown function) [3, 4] were expressed from pREX vectors.

The expression host for cloned yeast proteins was B834(DE3), in the mistaken belief that a methionine-requiring host would be better for labeling proteins with selenomethionine (SeMet) for crystallography (see section on *Auto-induction for labeling proteins with SeMet for crystallography*). The RIL plasmid from BL21-Gold(DE3)RIL (Stratagene) increases the expression of some yeast target proteins by supplying tRNAs for codons used frequently in yeast but not *E. coli*. T7 proteins and some other proteins were expressed in BL21(DE3) or BL21-Gold(DE3)RIL (into which Stratagene introduced the Hte phenotype for high transformation efficiency and an *endA* mutation to reduce endonuclease activity). The RIL plasmid is derived from a pACYC plasmid and confers resistance to chloramphenicol.

Freezer stocks for long-term storage of expression strains are made by adding 0.1 ml of 100% (w/v) glycerol to 1 ml of culture in log phase or grown to saturation in non-inducing media such as PG, LSG or MDG (Table 1), mixing well, and placing in a −70°C freezer. Subcultures for use as working stocks are made by scraping up a small amount of frozen culture with a sterile plastic pipettor tip without melting the rest of the stock and inoculating into non-inducing media. After growth to saturation, such working stocks are typically stable for weeks in the refrigerator.

Growth media

N-Z-amine AS, a soluble enzymatic digest of casein (in 100-pound barrels), and yeast extract (HY-YEST 444 in a 55-pound barrel) were obtained from Quest International, 5515 Sedge Blvd., Hoffman Estates, IL 60192, telephone 800-833-8308. For convenience, the designation N-Z-amine will refer to N-Z-amine AS, which could be substituted for by other enzymatic digests of casein, such as tryptone, in the media described here. Smaller quantities of enzymatic digests of casein or yeast extract as well as sugars, salts, amino acids, vitamins and other components of growth media were obtained from Difco, Sigma, Fisher or other biochemical and chemical suppliers. Media previously described [1] for growth of *E. coli* and production of target proteins with the T7 expression system include ZB (10 g N-Z-amine and 5 g NaCl per liter), ZYB (previously ZY) (10 g N-Z-amine, 5 g yeast extract and 5 g NaCl per liter), M9 (1 g NH_4Cl, 3 g KH_2PO_4, 6g Na_2HPO_4, 4 g glucose and 1 ml of 1 M $MgSO_4$ per liter) and M9ZB, the combination of M9 and ZB. For convenience, concentrations of certain media components are given in percent (w/v). The previously named ZY medium will here be called ZYB medium to indicate the presence of 0.5% NaCl, analogous to ZB medium. The name ZY will be reserved for 1% N-Z-amine, 0.5% yeast extract with no salt added.

The compositions of some of the newly developed media for growing cultures to high density without induction and for auto-induction are given in Table 1. Media are conveniently assembled from sterile concentrated stock solutions added to sterile water or ZY just before use. Standard stock solutions of mixtures include **20xP** (1 M Na_2HPO_4, 1 M KH_2PO_4, 0.5 M $(NH_4)_2SO_4$); **50xL** (0.625 M Na_2HPO_4, 0.625 M KH_2PO_4, 2.5 M NH_4Cl, 0.25 M Na_2SO_4); **50xM** (1.25 M Na_2HPO_4, 1.25 M KH_2PO_4, 2.5 M NH_4Cl, 0.25 M Na_2SO_4); **50x5052** (25% glycerol, 2.5% glucose, 10% α-lactose monohydrate); **100x505** (50% glycerol, 5% glucose). The term lactose will refer to α-lactose throughout the paper. Stock solutions of individual compounds include 40% (w/v) glucose; 5% (w/v) aspartic acid neutralized with NaOH; 2.5% methionine; 1 M disodium succinate; and 1M $MgSO_4$. Heating in a microwave oven is helpful for dissolving concentrated stock solutions that are slow to dissolve. These stock solutions are sterilized by autoclaving 15 min and stored at room temperature. The 50xM solution may be close to saturation or supersaturated; although bottles remained clear for long periods, occasionally a sample showered crystals, which redissolved readily upon heating in a microwave oven.

An amino-acid mixture containing 1% of each of 17 of the 20 natural L-amino acids, lacking methionine, tyrosine and cysteine, was sterilized by filtration and stored in the refrigerator. Methionine was omitted for convenience in labeling, tyrosine because it is not soluble enough to include at this concentration, and cysteine because slow oxidation to the much less soluble cystine causes precipitate to form. The mixture of 18 amino acids (including methionine but lacking tyrosine and cysteine) was as effective in promoting growth of BL21(DE3) as a mixture of all 20 amino acids (an example is given in Table 7). Free amino acids were used to make the mixture, except for monosodium glutamate, asparagine monohydrate, arginine monohydrochloride, lysine monohydrochloride and histidine monohydrochloride monohydrate. The molarity of 0.5% of each amino acid used in the mixture is given in Table 4. When concentrations of amino-acid mixture greater than about 200 μg/ml of each are used, the amino acids may have to be neutralized with NaOH to keep the pH of the final medium near neutral.

A stock solution of 0.1 M $FeCl_3$ was dissolved in a 100-fold dilution of concentrated HCl (final concentration ~0.12 M HCl). This solution was combined with autoclaved stock solutions of other metals to make a 1000x trace metal mixture containing 50 mM $FeCl_3$, 20 mM $CaCl_2$, 10 mM each of $MnCl_2$ and $ZnSO_4$, and 2 mM each of $CoCl_2$, $CuCl_2$, $NiCl_2$, Na_2MoO_4, Na_2SeO_3 and H_3BO_3 in ~60 mM HCl. These solutions were stored at room temperature. Upon prolonged storage, small amounts of precipitate formed in the mixture.

Antibiotic stock solutions were kanamycin (25 mg/ml), chloramphenicol (25 mg/ml in ethanol) and ampicillin (50 mg/ml). Kanamycin was initially used at 25 μg/ml and subsequently at 100 μg/ml (see section *High phosphate promotes kanamycin resistance*). Chloramphenicol was used at 25 μg/ml and ampicillin at 50 μg/ml.

The naming convention for media listed in Table 1 and related media is to give a letter designation to each uniquely different composition of the salts that supply phosphate, ammonium and sulfate ions (other than $MgSO_4$). P, M and L identify sets of media that supply 100, 50 and 25 mM phosphate, respectively; N and C identify variants used for isotopic labeling with ^{15}N or ^{13}C. All media contain 2 mM $MgSO_4$ and trace metal mix (although trace metal mix can be omitted in media containing N-Z-amine and yeast extract). Abbreviations for complex components, if any, are placed ahead of the letter designation, and abbreviations for amino acids, glycerol, glucose and lactose are placed after. Thus, Z indicates 1% N-Z-amine, Y indicates 0.5% yeast extract, and P indicates the salts composition in ZYP medium. The designation 505 refers to 0.5% glycerol, 0.05% glucose (as in ZYM-505); 5052 refers to 0.5% glycerol, 0.05% glucose, 0.2% lactose (as in ZYP-5052); and 750501 refers to 0.75% glycerol, 0.05% glucose, 0.01% lactose (in C-750501). G indicates 0.5% glucose, as in PG; D indicates 0.25% aspartate, as in MDG; and A indicates 200 μg/ml of each of 18 different amino acids (0.36% total amino acids), as in PAG. The S in LSG represents 20 mM succinate and the SM in PASM is for selenomethionine (SeMet). The names of some media have been shortened from designations in previously distributed recipes, as indicated in Table 1.

Culture conditions

Cultures were grown in sterile glass vessels in an incubator shaker (New Brunswick G25 series), usually at 300-350 rpm, as indicated on the meter. The incubation temperature was 37• [unk], unless stated otherwise. Target proteins were expressed at temperatures as low as 18• [unk]. The standard configuration for growing cultures in parallel was to place 0.5 ml of culture in 13×100 mm culture tubes with plastic caps. When more than about 0.2 ml of culture was to be removed for following the time course of growth, pH or induction, 1.5 ml of culture was grown in 18×150 mm culture tubes or 5-10 ml of culture in 125-ml Erlenmeyer flasks. These configurations provided sufficient aeration to sustain logarithmic growth to an A_{600}

approaching 10 in appropriate media, and expression results seemed to translate well to growth in 400-500 ml culture volumes in 1.8- or 2.8-liter baffled Fernbach flasks (Bellco), convenient for producing multi-milligram amounts of proteins in an incubator shaker. Higher rates of aeration could be obtained with smaller volumes of culture per vessel.

The standard measure of culture growth was optical density at 600 nm (A_{600}) after dilution in water to concentrations that gave readings below 0.25 in a 1-cm path-length cuvette in a Beckman DU 640 spectrophotometer. The pH of cultures was measured after 10-fold dilution in water. Viability and stability of cultures grown under different conditions were tested by plating on 1% agar plates containing ZB, except as noted. Viable cultures of BL21(DE3) produced approximately 2×10^9 colonies per ml per A_{600} over a rather wide range, from log phase through dense saturated cultures.

Plaque assay for induction of T7 RNA polymerase

To test induction of T7 RNA polymerase in expression hosts in the absence of a target plasmid, the bacteriophage T7 deletion mutant 4107 was used [1]. This mutant lacks the entire coding sequence for T7 RNA polymerase and is unable to form a plaque on a lawn of cells unless the host supplies T7 RNA polymerase. When BL21(DE3) is grown and plated on media that have no inducing activity, the basal level of T7 RNA polymerase is low enough that only small plaques develop at low efficiency, and they typically take more than 3 hours to become visible. In contrast, when BL21(DE3) is induced by including 0.4 mM IPTG in the plate, 4107 efficiently forms the large plaques typical of wild-type T7, which become apparent in less than 2 hours. This 4107 plaque assay was used to test whether T7 polymerase was induced in cultures of BL21(DE3) grown in different media.

Analysis of proteins on slab gels

Production of target protein was followed by gel electrophoresis of total cell proteins in the presence of sodium dodecyl sulfate on precast 4-20% polyacrylamide gels (Cambrex). Cells were lysed in Bugbuster plus Benzonase (Novagen) in 50 mM Tris-Cl, pH 8.0, and containing egg white lysozyme at 20 μg/ml. Lysozyme improves the release of large proteins into the soluble fraction but was omitted when it might interfere with identification of proteins of about the same size in the gel electrophoresis pattern. Benzonase is a DNase that reduces viscosity that could otherwise interfere with loading samples or cause bands to smear on the gel. Either a 5x lysis mixture was added directly to an appropriate dilution of culture, or cells were pelleted by centrifuging 1 min in a micro centrifuge (1.5 ml tubes), the supernatant aspirated, and the pellet suspended in 1x lysis mixture. The final volume of cell suspension was 40 μl, usually at a concentration corresponding to a culture density of A_{600} ~5, but sometimes half or twice this concentration. Immediately after mixing, 20 μl of cell suspension was transferred to a second tube, and both tubes were left for approximately 30 min at room temperature for lysis. One of the tubes was used as the sample of total cells, to which was added 10 μl of 3x loading buffer (containing sodium dodecyl sulfate). The other tube was centrifuged 1 min and the supernatant removed with a pipetter and mixed with another 10 μl of 3x loading buffer to constitute the soluble fraction. The pellet (insoluble fraction) was suspended in 30 μl of loading buffer. All three tubes were heated for 1 min in a boiling water bath and 10 μl of each loaded on the gel for electrophoresis.

Rapid staining of the gel after electrophoresis uses the following protocol. The gel is suspended in ~50 ml of 50% ethanol, 10% acetic acid in a covered plastic box, heated almost to boiling in a microwave oven (with the lid ajar), and then placed on a rocker for at least 5 min at room temperature, during which the gel shrinks. The liquid is discarded and the gel is suspended in ~50 ml of 5% ethanol, 7.5% acetic acid plus 200 μl of a 0.25% solution of Coomassie brilliant

blue in 95% ethanol. After gentle rocking to disperse the stain, the gel is again heated almost to boiling in a microwave oven and placed on the rocker. The protein pattern usually becomes visible within a few minutes and continues to intensify over a few hours. The result can usually be visualized in less than 30 min but the gel is usually rocked overnight before scanning an image into the computer. A Kimwipe placed in the solution and rocked for a few minutes can rapidly take up the slight amount of excess stain in the solvent.

Results

Growth of shaking cultures to high density

Shaking cultures are convenient for growing many cultures in parallel, and rapid growth to high densities is desirable for maximizing the yield and efficiency of producing target proteins. Complex media containing enzymatic digests of casein and yeast extract are extensively used because they support growth of a wide range of *E. coli* strains with different nutritional requirements, and cultures typically grow 2-3 times faster than in simple mineral salts media with glucose as the sole carbon source. However, complex media can vary from lot to lot in ability to support growth, and some complex media have been found to induce high-level production of target protein in the T7 expression system upon approach to saturation without added inducer [6]. To determine what factors might limit growth to high density, and to try to understand and manage unintended induction, the effects of different components of growth media on saturation density, growth rate and induction were analyzed.

Results typical of exploratory experiments are shown in Table 2. Cultures of BL21(DE3) grown overnight in ZB, where 1% N-Z-amine is the sole source of nutrition, saturated at A_{600} ~1.2 and pH ~7.9-8.2. Addition of 0.5% yeast extract (to give ZYB) more than doubled the saturation density to A_{600} ~2.8. Saturation density increased approximately in proportion to concentration of N-Z-amine up to about 4%, reaching A_{600} ~6.9 at 8%. Tripling the concentration of ZYB almost tripled the saturation density to A_{600} ~7.6. Addition of 1% glucose to ZB, ZYB, 4xZB or 8xZB had little effect on saturation density, apparently because the acid generated by glucose metabolism overwhelmed the limited buffering capacity of these media and decreased pH sufficiently to stop growth. Although growth rate was slower in M9 (mineral salts plus 0.4% glucose), the saturation density of A_{600} ~2.5 was comparable to that in ZYB. Adding ZB to M9 tripled the saturation density to A_{600} ~7.5, but increasing the glucose concentration of M9ZB to 2% overwhelmed the buffering capacity of the 66 mM phosphate buffer in M9 and stopped growth at a lower density, A_{600} ~5.8 and pH ~4.6.

Inducing activity was also analyzed by the ability of BL21(DE3) grown to saturation to support plaque formation by 4107, a T7 deletion mutant completely unable to form plaques in the absence of T7 RNA polymerase supplied by the host. Media made with N-Z-amine from our old barrel (Old ZB in Table 2) had little if any inducing activity. Media made with N-Z-amine from the new barrel (from which all media were made unless specified otherwise) had appreciable inducing activity, and higher concentrations of N-Z-amine had higher inducing activity, as judged by plaque size and time of appearance. Addition of 1% glucose strongly suppressed inducing activity, as found previously by Grossman *et al.* [6], but 0.1% glucose had little effect, presumably because it was depleted well before saturation. This inducing activity is discussed further in the sections on **Non-inducing media** and **Auto-induction**.

Increasing the concentration of N-Z-amine and/or yeast extract can increase saturation density but can also increase inducing activity and is expensive relative to determining and supplying precisely what is needed for growth to high density. Simply adding 1 mM $MgSO_4$ to either ZB or ZY approximately doubled the saturation density (Table 2). Although excess glucose prevented induction, cultures could become acidic enough to stop growth. Determining and

supplying what is needed for growth to high density in batch cultures and understanding and managing unintended induction has been an iterative process. The following sections summarize first the growth media that resulted and then the experiments and rationale that led to them.

High-phosphate P media—Fully defined and complex P media (Table 1) can support the growth of BL21(DE3) and other *E. coli* strains to saturation densities of A_{600} ~10 or greater in reasonably well aerated cultures. In P media, an equi-molar mixture of Na_2HPO_4 and KH_2PO_4 provides buffering against metabolically generated changes in pH in both directions and is a source of sodium, potassium and phosphate ions. A phosphate concentration of 100 mM was chosen to provide as much buffering capacity as possible without stressing the cells. Higher phosphate levels can be tolerated but growth begins to slow, presumably because of the high ionic strength. An adequate supply of nitrogen and sulfur is supplied by 25 mM NH_4SO_4. The requirement for magnesium ions is satisfied by 1 mM $MgSO_4$, the concentration given in recipes previously distributed, but the recipes given in Table 1 call for 2 mM $MgSO_4$, to provide a larger cushion for growth to very high densities. Trace metals are required for maximal growth in fully defined media. The combined concentration of glucose, glycerol and other sugars in the recipes given in Table 1 is low enough that they should be depleted before cultures become irreversibly acidic, and saturated cultures usually have a pH greater than 6.0. In fully defined media such as PAG and PA-5052, a mixture of 18 purified amino acids increases growth rate as well as helping to attain approximately neutral pH at saturation. The standard 200 μg/ml of each amino-acid supported a smooth growth curve to saturation at densities of at least A_{600} ~10, whereas discontinuities were apparent at concentrations of 100 μg/ml or less, presumably because depletion of one or more amino acids required the induction of synthesis pathways. The doubling time of BL21(DE3) in log-phase growth at 37°C ranged from about 60-70 min in minimal media to about 30-35 min in media containing ZY or the mixture of 18 purified amino acids. The recipes for P media have been widely distributed and used successfully to grow stable stock cultures of T7 expression strains and to produce target proteins by auto-induction.

High phosphate promotes kanamycin resistance—Expression vectors that confer resistance to kanamycin were selected for our structural genomics work, to avoid possible overgrowth of induced cultures by cells that have lost plasmid. Such overgrowth can occur when expression vectors confer resistance to ampicillin, because secreted β-lactamase can degrade all of the ampicillin in the medium [1, 2]. However, I was surprised to find that BL21 (DE3) without any plasmid grew to high density overnight at 37°C in auto-inducing ZYP-5052 medium containing 25 μg/ml of kanamycin, a concentration that efficiently kills them in ZB or ZYB cultures or plates. The cultures that grew had typical plating efficiencies and remained sensitive to 25 μg/ml of kanamycin in ZYB plates. Furthermore, BL21(DE3) plated directly on ZP or ZPG plates containing 25 μg/ml of kanamycin formed smaller but uniform colonies at normal efficiency, indicating that all cells survived and grew in these media.

Systematic tests revealed that the increased resistance to kanamycin is due to high concentrations of phosphate combined with amino acids and perhaps other nutrients in rich media. At a kanamycin concentration of 25 μg/ml, BL21(DE3) did not grow in ZYB, which has no added phosphate, nor in the minimal PG, which contains 100 mM phosphate, but it grew quite well in the fully defined PAG, which contains both 100 mM phosphate and purified amino acids. Growth at 25 μg/ml was also observed in other media that contain relatively high concentrations of phosphate and amino acids, such as M9ZB (64 mM phosphate) and terrific broth (89 mM phosphate) [15] (here abbreviated TRB to avoid confusion with tryptone broth (TB)). In rich media, the higher the concentration of phosphate, the higher the concentration of kanamycin needed to prevent growth and kill cells: BL21(DE3) failed to grow in M9ZB

and TRB at 50 μg/ml and was killed effectively at 100 μg/ml; PAG cultures still became turbid at a kanamycin concentration of 50 μg/ml, killing was somewhat faster than growth at 100 μg/ml and killing was effective at 200 μg/ml; ZYP-5052 cultures still became turbid at 100 μg/ml, killing was slightly faster than growth at 200μg/ml, and killing was effective at 400 μg/ml. Although many uninduced expression strains are relatively stable even in the absence of selective antibiotic, having rich media in which BL21(DE3) is more sensitive to kanamycin seemed preferable to resorting to concentrations as high as 400 μg/ml when selection is needed. A few attempts to develop an amino-acid mixture that would promote rapid growth without substantially increasing kanamycin resistance were not successful. Reducing the phosphate concentration in growth media seemed the most attractive way of increasing the sensitivity to kanamycin.

Lower phosphate M and L media—As described in the next section, cultures can be grown to high densities with only minimal buffering of pH by phosphate or other buffers. The M and L sets of media (Table 1) have phosphate concentrations of 50 mM and 25 mM respectively. Their salt composition was modified from that used in P media to allow independent variation of phosphate, sulfate and ammonium ions, which is useful for testing nutritional requirements and for isotopic labeling. Non-inducing and auto-inducing L media (25 mM phosphate) have been tested extensively and are satisfactory for most purposes, but the M media (50 mM phosphate) have smaller variations in pH during growth and are currently used for routine work. BL21(DE3) is killed about as fast as it divides in ZYM-5052 containing kanamycin at 50 μg/ml and is killed fairly effectively at 100 μg/ml. A kanamycin concentration of 100 μg/ml was adopted for routine work.

Metabolic control of pH—Cultures growing in media containing glucose (and in which no other nutrient is limiting) will continue to grow until the glucose becomes depleted or the acid generated by the metabolism of glucose exceeds the buffering capacity of the medium and causes the pH to drop to a level that stops growth. As long as sufficient glucose is present in the growth medium, catabolism of other carbon and energy sources that could balance the acid generated by metabolism of glucose is prevented by the phosphenolpyruvate:carbohydrate phosphotransferase system (PTS), acting through catabolite repression and inducer exclusion [16-20]. In the absence of glucose, glycerol can support growth about as effectively but suppresses the use of other carbon sources less dramatically than glucose by a mechanism affecting cyclic AMP production [21]. Excess glycerol can also generate enough acid to stop growth, but, in contrast to glucose, the presence of glycerol does not suppress the inducing activity found in complex media.

Another factor with a profound influence on growth is the availability of oxygen. If the culture becomes dense enough that oxygen consumption exceeds the rate of aeration in the shaking vessel, oxygen limitation triggers complex regulatory responses that attempt to adjust the metabolic capacities of the cell to the availability of oxygen and the carbon and energy sources in the medium [22]. The higher the rate of aeration (or oxygenation) the higher the culture density attained before oxygen limitation triggers these responses. Acid production from glucose or glycerol seems to increase as a result of the metabolic changes as oxygen becomes limiting.

Imbalances in needs for energy and carbon in growth with glucose as carbon and energy source are typically rectified by excretion of acetate and other compounds into the medium [23-25]. If glucose is depleted before the medium gets too far out of balance, the excreted acetate and other carbon and energy sources that may be present in the medium can then be metabolized, which can return the pH of the medium to the neutral or alkaline range. The decrease of pH upon metabolism of glycerol can also be reversed by metabolism of other carbon and energy

sources in the medium. Excursions of external pH outside the neutral range on either the acid or alkaline side also induce complex regulatory responses [26].

The stringent control of the order of catabolism of different carbon and energy sources in the growth medium, together with the complex regulatory responses to other environmental conditions, make it challenging to develop media in which the pH remains in a range that supports growth to high cell densities in shaking vessels. The 100 mM phosphate in P media provides enough buffering capacity to allow growth to depletion of 0.5% glucose with a saturation density greater than A_{600} ~5 while maintaining pH above 6.0. However, significant increases in glucose concentration or decreases in phosphate concentration usually produced cultures that saturated at low pH and lost viability within hours or days. In an attempt to provide a stronger buffer against decreasing pH, which would allow the use of higher glucose and glycerol concentrations or lower phosphate concentrations, organic acids with relatively high pKa were tested for their ability to buffer the medium and thereby allow growth to higher density.

Organic acids Succinate was found to be effective in countering the acid generated by glucose in minimal L medium (which has only 25 mM phosphate). It is apparent from results shown in Table 3 that, rather than acting simply as a buffer, succinate is metabolized as glucose nears depletion during growth: cultures reach a higher saturation density and a higher pH than in the absence of succinate. The growth rate and the changes in pH during growth (not shown) are consistent with glucose being metabolized first and then succinate, as glucose is depleted. Approximately 20 mM succinate seems optimal for balancing 0.5% (28 mM) glucose, usually producing saturated cultures with a pH close to neutral. Substantially higher concentrations of succinate can cause the pH to rise well beyond 8.0, which can stress the cells and reduce viability. The presence of succinate does not cause detectable induction of T7 RNA polymerase, as measured by the 4107 plaque assay and as indicated by the viability and stability of saturated cultures of strains that express highly toxic target proteins. Cultures that saturate between pH ~6 and ~7.5 are stable for weeks in the refrigerator with little loss of viability or increase in lag time when growing subcultures. Fumarate, DL-malate and citrate were also able to balance the acid produced by glucose in much the same way as succinate. Added acetate was effective to a lesser extent. Maleate provided some buffering against the drop in pH but was toxic to BL21(DE3) at low pH, at least in some media.

Amino acids N-Z-amine, yeast extract or a mixture of 18 pure amino acids (no Y, C) increase both growth rate and saturation density of glucose- or glycerol-containing media. Uptake of amino acids from the medium and incorporation directly into proteins spares the cells from having to make enzymes for entire metabolic pathways and divert carbon from glucose into synthesis of proteins rather than production of energy or other metabolites. If the concentration of amino acids is high enough, at least some of them will remain to be catabolized for carbon and energy after glucose is depleted, causing pH to rise and potentially balancing acid generated from glucose. In contrast to N-Z-amine, purified amino acids contributed no inducing activity when added to defined media.

To determine which amino acids are most effective in balancing pH, each of the 18 pure amino acids used in the mixture was tested individually at a concentration of 0.25% for ability to balance the acid generated by 0.5% glucose in L medium (25 mM phosphate) (Table 4). The most effective single amino acid was aspartate, followed by serine, asparagine, glycine and glutamate, all of which increased the saturation density 60% to115% and produced a pH >6.2 at saturation (compared with pH ~4.1 in glucose itself). By comparison, 20 mM succinate (0.24%) increased saturation density by 90% and produced a pH of 6.8 at saturation, and the mixture of 18 amino acids (0.27%) increased saturation density 75% and produced a pH ~5.7.

Of the other amino acids, only glutamine and proline produced as much as a 25% increase in saturation density and only alanine produced a pH >4.0 at saturation. Of the amino acids that failed to balance pH at a concentration of 0.25%, only alanine was effective in balancing pH when tested at 0.5%. Several amino acids substantially increased the lag or decreased the growth rate in minimal LG medium, most notably serine, alanine, leucine and valine, presumably by repressing overlapping metabolic pathways [27, 28]. Addition of 0.01% each of leucine, isoleucine and valine restored normal growth in the presence of 0.25% serine. (The slow growth in histidine may reflect a low pH of the medium.)

To determine which amino acids are most effectively utilized as a carbon and energy source for BL21(DE3), cultures were grown in L medium with amino acids as sole carbon source (Table 4). A mixture of the 18 amino acids, each at 100 μg/ml (0.18% total amino acids) was provided to promote some growth and to alleviate possible inhibitory effects of individual amino acids, which were added at a concentration of 0.5%. The mixture of 18 amino acids by itself supported growth to A_{600} ~1.4 with a final pH ~6.8. Of the individual amino acids, proline was the most effective carbon and energy source, supporting growth to A_{600} ~9.6 and pH ~7.0, comparable to A_{600} ~9.0 and pH ~5.7 supported by 0.5% glycerol. Other amino acids that substantially increased the saturation density were serine, glutamate, alanine and aspartate, with smaller increases by threonine and asparagine. Each of these amino acids also increased the final pH at least somewhat, indicating that they were metabolized. The final pH ~5.2 of the histidine-containing culture represented a substantial decrease from an initial pH estimated to be ~6.0 by reconstitution (versus ~ 6.6 for the other amino acids), suggesting that metabolism of histidine decreases the pH of the culture. The remaining individual amino acids did not significantly affect either A_{600} or pH, suggesting that they were not significantly catabolized. A credible test of tryptophan was not done.

Minimum nutritional requirements for growth to high density—Metabolic balancing of pH made it possible to test the requirement for any nutrient including phosphate to support the growth of BL21(DE3), without the complication of the culture becoming too acidic or basic for optimal growth. A series of tests of mineral salts media with glucose or glycerol as primary carbon source established nutrient concentrations that limit growth to low densities, which could be extrapolated to determine approximate minimum concentrations needed for growth to high saturation densities. Table 3 shows results of one series of tests of minimal requirements for sulfur, nitrogen, phosphate and magnesium in modified LG medium, in the absence or presence of 25 mM succinate. The cultures were inoculated with a thousand-fold dilution of BL21(DE3) that had been grown to saturation in PG, and 0.5 ml cultures were grown in 13×100 mm tubes in a shaking incubator for 14-15 hr at 37°C. Conclusions from these and similar experiments are summarized in the following sections.

Sulfur Carryover of 0.026 mM sulfate in the inoculum supported growth to A_{600} ~0.7 with pH ~6.7. The need for sulfate saturated at approximately 0.5 mM, in which BL21(DE3) grew to A_{600} ~6.1 at pH ~6.7. A sulfate concentration of 0.5 mM or greater at near neutral pH was also enough to produce very stable cultures, as measured by viability after three weeks in the refrigerator. The 5 mM Na_2SO_4 in L and M media and 25 mM $(NH_4)_2SO_4$ in P media should supply enough sulfur to support growth to very high densities in shake flasks.

Nitrogen Saturation density continued to increase with NH_4Cl concentration until at least 50 mM, which supported growth to A_{600} ~5.5 at pH ~7.1. Cultures retained high viability for at least three weeks in the refrigerator at NH_4Cl concentrations of 20 mM or higher and pH near neutral. In minimal media in which pH was maintained near neutral, 50 mM NH_4^+ reproducibly supported growth to slightly higher density than 25 mM and is therefore the standard

concentration used in P, M and L media. However, 25 mM NH_4Cl is sufficient for most purposes, including labeling of proteins with ^{15}N for NMR studies.

Phosphate Carryover of 0.1 mM phosphate in the inoculum supported growth to A_{600} ~0.8 at pH ~6.7. The presence of 1 mM phosphate in the medium supported growth to A_{600} ~3.8 at pH ~6.4 but the need for phosphate did not appear to saturate until 10-15 mM at A_{600} ~5.9 and pH ~8.2. *E. coli* cells have complex regulatory responses when phosphate becomes limiting in the medium [29], and alternative uses of internal phosphate may account for the relatively slow increase in saturation density between 1 mM and 10 mM phosphate. The buffering capacity of phosphate in the medium did not significantly reduce the pH increase due to succinate metabolism until 35-50 mM phosphate. The minimum phosphate concentration in the media given in Table 1 is 25 mM, to try to avoid a phosphate limitation that would induce response mechanisms. Experiments in which saturation densities were pushed well above A_{600} ~10 have occasionally suggested that even 25 mM phosphate may become limiting at densities achievable in shaking vessels.

Magnesium No growth of BL21(DE3) was apparent in the absence of magnesium, but, interestingly, cultures containing only limiting amounts of magnesium grew to much higher densities (5- to 10-fold) when the growth medium contained succinate than when it did not. The need for magnesium appeared to saturate at 0.5 mM, which gave A_{600} ~6.4 and pH ~6.2. However, viability after three weeks in the refrigerator seemed to remain somewhat higher in cultures grown in 1-2 mM $MgSO_4$ than in those grown at lower concentrations. Magnesium levels as high as 10 mM (the highest concentration tested) showed no inhibition of growth. Previously distributed recipes for P medium contain 1 mM magnesium, but 2 mM (as given in Table 1) may provide a greater margin for growth to very high densities.

Trace metals Fully defined media made from purified components contain contaminating trace metals in amounts sufficient to support growth to moderate density but not sufficient for growth to high density with good expression of target proteins by auto-induction. Table 5 summarizes results from an auto-induction experiment to test the effects of trace metals. In this experiment, the expression strain saturated in ZYP-5052 at A_{600} ~18 with the target protein expressed at high level. In slightly modified PA-5052 without added trace metals, saturation was at A_{600} ~4.4 with little expression of target protein. Addition of trace metals about tripled the saturation density, to A_{600} ~13, and allowed high-level expression of target protein. Clearly, a deficiency of trace metals limited culture growth and auto-induction of target protein in this fully defined medium.

Individual metal ions were tested at concentrations of 1, 10 and 100 μM for ability to increase saturation density and for possible toxicity (Table 5). The trace metals were chosen as being likely to have a functional association with proteins or participate in some biological process. Iron ions at 10 and 100 μM increased saturation density to A_{600} ~13 but 1 μM increased the density only to A_{600} ~7.8. Manganese ions at 1, 10 and 100 μM also increased saturation density to A_{600} ~13, as did cobalt ions at 1 and 10 μM. However, 10 μM cobalt ions caused a lag of about an hour before attaining normal growth rate, and 100 μM cobalt prevented growth. Zinc ions appeared to have only a slight stimulatory effect, and nickel, molybdate, calcium, copper, selenate or borate even less. Selenate did not appear to be toxic at 10 μM but prevented growth at 100 μM.

Many proteins of unknown function are being produced in structural genomics projects, any one of which might have an unsuspected metal ligand. Target proteins of 50,000 Da produced at 100 mg/liter would have a concentration of 2 μM and proteins of 10,000 Da a concentration of 10 μM. The 1x concentration of metal mix supplies 50 μM iron, 20 μM calcium, 10 μM

manganese and zinc, and 2 μM cobalt, copper, nickel, molybdate, selenate and borate, amounts that are not toxic to growth but could saturate potential binding sites in many target proteins. Of course, if a target protein is known to have a metal ligand, the appropriate concentration of that specific metal can be added. Concentrations between about 0.1x and 2x metal mix supported maximum saturation density, 5x was slightly inhibitory and 10x markedly slowed growth but the culture still attained high density and a high level of auto-induction.

Iron Concentrations of 0.05x metal mix or lower did not support growth to high density in defined media and produced only low levels of target protein by auto-induction, primarily due to a deficiency in iron. In the presence of 0.02x metal mix, an iron concentration of 5 μM was sufficient for maximum growth and auto-induction in a defined medium without amino acids but 10 μM was needed in the presence of amino acids. The highest iron concentration tested, 500 μM, showed no evidence of toxicity. In a defined medium containing 100 μM $FeCl_3$, omission of the metal mix only slightly diminished the maximum density and the level of target protein produced by auto-induction, so 100 μM $FeCl_3$ may suffice for many purposes if a suitable metal mix is not available.

In contrast to the results summarized in Table 5, manganese or cobalt, alone or in combination, did not compensate for a deficiency in iron in subsequent experiments. A difference was that the media used in the tests reported in Table 5 contained seven different vitamins but subsequent experiments contained no added vitamins. Whether the presence of vitamins could account for the difference has not been tested.

Complex media Tests of nutritional requirements for growth of BL21(DE3) to high density in complex media indicate that media containing only ZY are deficient in magnesium, phosphate, carbon and energy sources, as well as the ability to buffer pH changes that occur during growth. The high concentrations of amino acids in ZY are almost guaranteed to provide sufficient nitrogen and sulfur, but the known variability from lot to lot makes it seem prudent to add 0.2x metal mix, or at least 10 μM of an iron salt, to ensure that trace metal requirements are met. The mineral salts components of P, M or L media are included in all formulations of complex media in Table 1 to ensure that minimal requirements for growth to high density and auto-induction are met.

Fully defined media have been formulated with well-metabolized amino acids at concentrations high enough to achieve saturation densities equal to or greater than those obtained in complex media. However, yeast extract appears to supply something that allows slightly more rapid initial growth than in those fully defined media. Addition of vitamins, purines and pyrimidines to the defined media had little effect on growth rate or saturation density. Yeast extract supplies a variety of metabolites, including fats and complex carbohydrates, any of which might be responsible for a slightly faster initial growth rate.

Non-inducing media

Besides our new barrel of N-Z-amine, a sample of Bacto tryptone (Difco) also had inducing activity, suggesting that inducing activity may be fairly common in enzymatic digests of casein. Addition of excess glucose to complex media that have inducing activity prevents induction of target protein [6], but cultures eventually become acid enough to stop growth and can lose viability. At intermediate glucose concentrations, cultures became induced if the pH rose at saturation, indicating that glucose was depleted, but not if the culture stayed acid, indicating that glucose remained in the culture. The rate of aeration also had a substantial effect on saturation density, acidity and induction. It seemed difficult or impossible to formulate complex media in which cultures reliably grow to saturation without induction and don't become so acid

as to reduce viability. Therefore, the non-inducing media given in Table 1 are fully defined, made with purified components that have no detectable inducing activity.

We currently use MDG medium for routine growth of non-induced cultures of BL21(DE3) expression strains but have previously used PG and LSG extensively for this purpose. These media support the growth of BL21(DE3) with a doubling time of approximately an hour. Being minimal media, they must be appropriately supplemented when growing strains with nutritional requirements, such as B834(DE3). Overnight cultures typically saturate at A_{600} ~5-9 and a pH near neutral without detectable induction of target protein. When grown to saturation in these media, even strains that express highly toxic target proteins remain stable and viable for weeks in the refrigerator, and subcultures grow with little or no lag. This makes it convenient to grow both freezer stocks and working cultures overnight to saturation, whereas previously we tried to collect cultures in log phase to minimize potential instabilities if the target protein is toxic to the host. The cells that settle out of working cultures stored in the refrigerator usually disperse readily, but occasionally they have been sticky and more difficult to disperse. The reason for this occasional stickiness has not been determined but may be associated with a slightly alkaline pH in the saturated culture.

Agar plates made with fully defined non-inducing media such as MDAG or PAG enabled the isolation of some BL21(DE3) transformants that were unable to form colonies on the ZYB plates we usually use for selection. Apparently the inducing activity in ZYB plates caused enough expression of highly toxic target protein to prevent colony formation, but the lack of inducing activity in the MDAG or PAG plates allowed colonies to form. MDAG or PAG plates are rich enough that innocuous clones form colonies on them almost as rapidly as on ZYB plates.

Auto-induction

Unintended induction is almost certainly due to lactose in the medium—Media made with N-Z-amine from the old barrel did not have inducing activity. Apparently, something in the new N-Z-amine was causing induction (rather than something in the old N-Z-amine preventing induction) because increasing the concentration of new N-Z-amine in the medium also increased the inducing activity, as judged by 4107 plaque size and time of appearance (Table 2). Grossman *et al.* [6] had concluded that unintended induction was not due to the presence of lactose in the medium. However, it seemed reasonable to test whether addition of lactose to media made with N-Z-amine from the old barrel would produce inducing behavior similar to that observed in media made from the new barrel. Indeed, the results summarized in Table 6 show that it does. As expected, no induction of B834(DE3)P35 was apparent in the absence of added lactose. The smallest concentration of lactose tested in this set, 0.005% (139 μM), gave a high level of induction of P35 protein, but the culture density, viability and maintenance of plasmid were all comparable to what was found in the absence of added lactose. Apparently, P35 protein is not very toxic to the cell. With increasing amounts of lactose, production of P35 protein remained high and the density of the saturated cultures decreased somewhat, but the viability decreased substantially, particularly at 0.05% lactose and higher. At these higher lactose concentrations, most of the surviving cells had lost the expression plasmid. High levels of induction are known to kill cells that carry a multi-copy plasmid with a T7 promoter, even if the target protein is innocuous [1, 3].

Other experiments (not shown) found that production of P35 protein was still appreciable with as little as 0.003% (83 μM) lactose, and detectable on stained gels at 0.001% (28 μM) but not at 0.0003% (8.3 μM). The limit of detection in the assay used by Grossman *et al.* [6] to test for possible lactose in their inducing medium was stated by them to be 0.002%, in the range where induction of P35 protein was observed. I conclude that the unintended induction described by

Grossman *et al.* and observed by us in media made with N-Z-amine from our new barrel is due to small amounts of lactose in the medium. This seems entirely reasonable, as N-Z-amine is an enzymatic digest of casein, a milk protein, and milk contains lactose. The casein would have been purified before digestion, but differing trace amounts of lactose remaining in the final product presumably account for the differences in inducing activity in different lots of N-Z-amine or tryptone. The finding that glucose prevents unintended induction is also consistent with a large body of work showing that the presence of glucose in the medium prevents the uptake and utilization of lactose [16-20]. In retrospect, we were lucky that the barrel of N-Z-amine used for most of our previous work in developing the T7 expression system had low enough levels of lactose to be free of unintended induction.

Amino acids suppress induction by lactose in log-phase growth—Although the presence of a small amount of lactose in the medium explains most observations related to unintended induction, it seemed curious that B834(DE3)P35 could grow to relatively high density in ZYP containing 0.05-1% lactose, even though high levels of induction kill the cells (Table 6). Indeed, the titer per A_{600} indicated that more than 90% of the cells in the saturated cultures were incapable of forming a colony. Similar results were obtained with B834(DE3) RIL producing yeast target protein P21, which was used for an extensive exploration of induction phenomena. Total proteins of cells growing in ZYP containing 0.5% lactose showed no detectable P21 protein in early log phase but rapid, high-level production as the growth rate slowed on approach to saturation (Figure 1A), similar to the timing observed by Grossman *et al.* [6]. The time course looked similar whether the medium contained 0.1, 0.2, 0.5, 1 or 1.5% lactose, with induction in each case beginning at A_{600} ~1 and reaching a maximum level of P21 protein per A_{600} at A_{600} ~3, which was maintained to A_{600} ~5-6. When incubation was continued for 15 hours overnight, further increases in culture density were greater the higher the lactose concentration, reaching as high as A_{600} ~14.8 in 1.5% lactose. However, the amount of target protein per A_{600} was much reduced (Figure 1A), and titers showed that the density increases were due primarily to overgrowth of the culture by cells that had lost plasmid. Such overgrowth can occur in ZYP medium even at the kanamycin concentration of 100 μg/ml used in these experiments (see section on *High phosphate promotes kanamycin resistance*, above).

Something in ZYP medium prevents induction by lactose during log-phase growth. Conceivably, small amounts of glucose or other PTS sugars could be responsible, but N-Z-amine and yeast extract are both rich in amino acids and it seemed possible that amino acids somehow prevent or modulate the lethal levels of expression that would otherwise be induced by lactose. P medium containing 1.25% glycerol as a carbon and energy source was used to test the ability of purified amino acids to allow growth in the presence of 0.1% lactose (Table 7). No growth was apparent in the absence of amino acids, consistent with the inability of glycerol to prevent lactose induction that is strong enough to prevent cell growth. However, addition of 18 amino acids, each at a concentration of 100 μg/ml, allowed growth to high density with full induction of P21 protein. Of three subgroups of amino acids, only the group containing serine supported overnight growth, as did serine itself but not other amino acids in that subgroup. Although serine seems to be the most effective amino acid in suppressing induction and allowing growth in the presence of lactose, the combination of 17 amino acids lacking serine promoted growth in the presence of lactose almost as well as 18 amino acids including serine. Apparently, something about the uptake and metabolism of amino acids during log-phase growth prevents or modulates lactose induction of target protein sufficiently to allow cells to grow, but this inhibition is relaxed and full-blown induction occurs upon approach to saturation.

Metabolic regulation enables auto-induction—The recognition that lactose can induce production of target protein but is prevented from doing so by compounds that can be depleted

during growth opened the possibility of developing media in which target protein is produced automatically, without the need to monitor growth and add inducer at the proper time. I call this auto-induction. Ideally, the expression strain would grow in the auto-inducing medium without expressing target protein until rather high density, when depletion of inhibitory factors would allow the lactose present in the medium to induce expression, producing high concentrations of target protein.

Factors that affect the efficiency and reliability of auto-induction in high-density cultures were examined systematically in B834(DE3) and BL21(DE3), initially testing expression of the yeast target protein P21 over a wide range of conditions and then expanding to other proteins, including bacteriophage T7 proteins that are known to be highly toxic to the host bacterium. The experiments and conclusions are summarized in the following sections.

Carbon and energy sources for high-level production of target protein by auto-induction—As described in the *Complex media* part of the section on *Minimum nutritional requirements for growth to high density*, growth in ZYP is limited by lack of a carbon and energy source. Glucose can support growth to high density, but too much glucose prevents induction by lactose. Lactose itself can support the growth of BL21(DE3), but the initial products of lactose catabolism are glucose and galactose, and, since BL21 and B834 cannot use galactose, half of the carbon and energy of lactose is not available. Perhaps more important, induced T7 RNA polymerase can be so active that most transcription and protein synthesis in the cell is directed toward target protein [1]. This competition may limit the production of β-galactosidase and lactose permease, thereby limiting the ability of lactose to serve as a carbon and energy source for continued production of target protein.

Glycerol supports growth about as well as glucose and does not prevent induction by lactose. Cultures supplemented with glycerol grow to much higher densities before and after induction than with lactose as carbon and energy source (for example, compare Figure 1A and B). BL21 (DE3) can grow on other economical carbon and energy sources, including fructose, maltose and sorbitol (but not sucrose). In limited tests, maltose and sorbitol gave somewhat inconsistent growth and induction, offering no apparent advantages over glycerol. Therefore, glycerol was chosen as a carbon and energy source for both fully defined and complex auto-inducing media. Many combinations of glycerol, glucose, lactose and purified amino acids were tested to optimize auto-induction and reliability in producing high concentrations of target protein per volume of culture.

The standard 5052 mixture of 0.5% glycerol, 0.05% glucose, 0.2% lactose has produced reliable auto-induction of a wide variety of proteins in a range of media and growth conditions (Table 1). ZYM-5052 or ZYP-5052 is a good choice for the first attempt to express almost any new target protein. Auto-induced cultures with highly expressed proteins, such as T7 capsid protein and yeast P21 protein, often attain densities greater than A_{600} ~20, more than twice the density of BL21(DE3) or B834(DE3) themselves grown in the same medium. Microscope observations of cells from such highly expressing cultures suggested that the induced cells continued to elongate fairly uniformly, presumably without dividing.

For some target proteins, higher glycerol and/or amino-acid concentrations can produce higher culture densities and target protein concentrations, if aeration and other media components are appropriate for maintaining pH. Auto-induced cultures expressing T7 capsid protein have reached culture densities of A_{600} >40 in less than 24 hours in ZYP-5052 supplemented to a total of 2% glycerol and 25 mM succinate in well-aerated cultures (Figure 1C). Comparably high densities have also been reached in fully defined media supplemented with purified amino acids that supply carbon and energy.

Effect of aeration on timing and level of auto-induction of target protein—In testing the effect of different concentrations of lactose and glycerol on induction of P21 protein in ZYP medium, a substantial difference was observed in the amount of protein produced in ZYP containing 1.875% glycerol but no added lactose on two different days. The only obvious difference between the cultures appeared to be the level of aeration: a standard 0.5 ml of culture in a 13×100 mm tube reached saturation at A_{600} ~13.9 and pH ~5.6 with a high level of target protein, but a 5-ml culture in a 125-ml Erlenmeyer flask, reduced to a highly aerated 1.5 ml by sampling, reached saturation at A_{600} ~20.0 and pH ~6.7 with barely detectable target protein.

To test more systematically how growth and protein production are affected by level of aeration, a thousand-fold dilution of B834(DE3)RIL/P21 in 80 ml of ZYP containing 0.625% glycerol but no added lactose was distributed as 0.25, 0.5, 1 or 2 ml samples in 13×100 mm tubes and 2.5, 5, 10, 20 or ~39 ml samples in 125-ml Erlenmeyer flasks, which were all grown at 37°C in the incubator shaker at 325 rpm to provide a fairly wide range of rates of aeration. The time course of growth and protein production in the Erlenmeyer flasks containing 5 ml or more of culture was followed by withdrawing approximately 12 samples totaling about 4 ml from each, which produced a very high aeration rate all the way to saturation for the 5-ml culture in the 125 ml flask. Two time points and a total volume of approximately 75-215 μl were sampled from the remaining cultures before saturation. The saturated cultures were also titered with and without kanamycin to test for viability and plasmid retention. Saturation densities and pH, relative target protein levels, and titers are given in Table 8.

As shown in Table 8, the level of target protein and viability of saturated cultures varied tremendously with the rate of aeration: the highest rates of aeration gave no apparent production of P21 protein or killing of cells and the lowest rates of aeration produced very high levels of P21 protein and substantial killing of cells. The different cultures whose densities were measured in the growth phase (not shown) had about the same growth rate to A_{600} ~1.0, where lack of oxygen started to limit growth rate in the cultures with the lowest rates of aeration. The most highly aerated culture whose growth rate was followed (5 ml reduced to ~1 ml in a 125-ml flask) maintained a gradually slowing but steady increase in density all the way to saturation at A_{600} ~14.3, with little induction of target protein. The least well-aerated culture whose growth rate was followed (~39 ml in a 125-ml flask) began significant production of target protein by A_{600} ~1.5 and had accumulated high levels by A_{600} ~3. The doubling time of the culture was ~33 min between A_{600} of 0.1 and 1 but slowed markedly to ~150 min between A_{600} of 3 and 5. In the next 13 hr after reaching A_{600} ~5.3, the culture density reached 10.2, with no apparent decrease in the amount of target protein per A_{600}. At this point, essentially no cells that carried plasmid were capable of forming a colony, and cells that had lost plasmid had not yet overgrown the culture. Intermediate rates of aeration gave growth and induction behavior intermediate between these two extremes. The standard 0.5 ml cultures in 13×100 mm tubes appeared to provide aeration comparable to about 5-10 ml cultures in 125-ml Erlenmeyer flasks, considering that ~4 ml of culture was removed from the 10 ml culture to follow growth rate in this experiment. In this set of cultures, glycerol probably became depleted at the higher levels of aeration, and, except for the lowest levels of aeration, most cultures ultimately reached about the same saturation density and pH even though the amounts of target protein differed markedly.

The failure to produce target protein at the highest rates of aeration in the above experiment was due to the low concentration of lactose contributed by the N-Z-amine. Table 9 shows the saturation densities, target protein levels and titers attained at saturation for two sets of cultures grown in ZYP containing 0.625% glycerol and different concentrations of lactose. In the first set, 0.5 ml cultures were grown in 13×100 mm tubes, providing the standard, reasonably good rate of aeration; in the second set, 1.5-ml cultures were grown in 125-ml Erlenmeyer flasks,

providing an even higher rate of aeration. In the first set, target protein was highly induced even in the absence of added lactose. In the more highly aerated second set, little induction of target protein or cell killing was apparent at 0.001% or less added lactose and only minimal amounts of target protein or cell killing were apparent between 0.002% and 0.01% lactose. The typical high levels of target protein and substantial cell killing seen with 0.5 ml in a 13×100 mm tube were produced only at 0.05% lactose or higher. Clearly, the higher the rate of aeration the more lactose is needed to induce high-level protein production in auto-inducing media. The concentration of 0.2% lactose chosen for auto-inducing media seems likely to be high enough to induce full expression of target protein at almost any rate of aeration likely to be encountered with shaking vessels.

Inclusion of glucose in auto-inducing media and expression of toxic proteins— Previous workers used lactose to induce the expression of target proteins in T7 expression strains in fermenters, adding lactose after glucose was depleted [30] or using a fed-batch fermentation with mixtures of lactose and glucose, which appeared to provide lower rates of induction and improved solubility of target protein [31]. However, in testing whether mixtures of glucose and lactose could produce intermediate rates of production in auto-inducing media, it was clear that the presence of glucose completely prevented induction by lactose and that production of target protein occurred only after the glucose was depleted. These observations are in accord with a wealth of previous literature showing that glucose in the medium prevents lactose from inducing the *lac* operon [16-20].

Because glucose both prevents lactose from inducing expression of target protein and is metabolized preferentially during growth, I expected that simply adjusting the concentration of glucose in media containing an inducing concentration of lactose could allow auto-induction at any desired density of an actively growing culture. However, the finding that amino acids and oxygen level modulate the lactose induction of target proteins meant that fine tuning the culture density at which auto-induction occurred was not straightforward, particularly in rich media. Media containing amino acids and lactose but no glucose often exhibited a relatively slow production of target protein well before a rapid, high-level induction that coincided with slowing growth due to oxygen limitation. However, the presence of glucose always strongly prevented production of target protein. Therefore, a good approach appeared to be to include glucose in auto-inducing media at a concentration that would not be depleted until the culture had grown to moderate density, preferably just before the oxygen depletion that appears to trigger high-level production of target protein. The effects of different concentrations of glucose on the level of target protein accumulated were tested in different fully defined and complex media, in standard 0.5-ml cultures in 13×100 tubes, in time courses with larger volumes of culture, and at different levels of aeration. A glucose concentration of 0.05% seemed to be effective over a range of conditions and was selected for inclusion in the auto-inducing media given in Table 1.

An important question is whether the presence of 0.05% glucose completely prevents lactose from increasing the basal level of target protein in the early stages of growth in auto-inducing media. When target proteins are highly toxic to the cell, even a small increase in basal expression over that maintained in non-inducing media might have a significant effect on the ability of an expression strain to grow and maintain inducible plasmids until auto-induction takes place. This was tested with clones capable of expressing T7 gene *5.3* and *7.7* proteins, whose functions are unknown but which are highly toxic to BL21(DE3) and difficult to maintain and express [3, 4]. Certain plasmid clones capable of expressing *7.7* protein were toxic enough that BL21(DE3) transformants were not obtained on ZYB plates, which had inducing activity, but they were obtained on fully defined PAG plates, which lack inducing activity. These expression strains were stably maintained in PG and MDG non-inducing media,

and could be grown and auto-induced in PA-5052, ZYP-5052 and ZYM-5052 media to produce a strong double band at the approximate position expected for *7.7* protein in electrophoretic patterns of total cell proteins.

The *5.3* protein is even more toxic to BL21(DE3), and clones capable of expressing it could be obtained only in vectors specifically modified to accept and express highly toxic proteins (to be described elsewhere). Again, these expression strains were stable in non-inducing media and could be grown and induced in auto-inducing media. Auto-induction of active *5.3* protein caused the culture to stop increasing in density beyond A_{600} ~0.5 -1.5, presumably because of the toxic effect of the target protein on the host. A mutant *5.3* protein having a single amino-acid substitution produced a relatively strong band at the approximate position expected for *5.3* protein in electrophoretic patterns of total cell proteins. However, wild-type *5.3* protein was not detected, presumably because protein synthesis stopped before enough *5.3* protein accumulated to become visible over the background. Clearly, basal expression of target protein in auto-inducing media containing 0.05% glucose is low enough in the initial stages of growth that strains capable of expressing target proteins that are highly toxic to BL21(DE3) can be grown and target protein expressed in auto-inducing media.

Auto-induction is widely applicable and generally superior to IPTG induction for protein production—Auto-induction is more convenient than IPTG induction because the expression strain is simply inoculated into auto-inducing medium and grown to saturation without the need to follow culture growth and add inducer at the proper time. Furthermore, the culture density and concentration of target protein per volume of culture are typically considerably higher than what we had been obtaining by IPTG induction. Therefore, even as the auto-induction phenomenon was being explored and media were being optimized, auto-induction was being applied with great success to the production of proteins of interest to us and to colleagues in our department. Nevertheless, a more systematic and wider comparison of auto-induction and IPTG induction was undertaken.

At hand were expression strains for the first hundred or so yeast proteins selected for our structural genomics pilot project. The coding sequences had been cloned in pET-13a or pET-28b, both of which transcribe the target from a T7*lac* promoter. The expression host was B834(DE3), with or without the RIL plasmid that supplies tRNAs for codons rarely used by *E. coli*. The presence of RIL substantially increased production of several of the yeast target proteins and did not decrease the production of any. All of these clones had already been tested for expression and solubility of target proteins by conventional IPTG induction in M9ZYB at both 37°C and 20°C.

In all, 72 of the yeast clones were tested for expression and solubility of the target protein by auto-induction, most of them at both 37°C and 20°C, and the results were compared with the previous IPTG inductions. For 14 clones, IPTG and auto-induction were compared directly in the same experiment. In general, the level of expression per A_{600} of culture density and the solubility of target proteins appeared to be comparable whether expression was induced by adding IPTG or by auto-induction. The auto-induced cultures typically had considerably higher densities and therefore also had considerably higher concentrations of target protein per ml of culture.

Continued incubation of auto-induced cultures for several hours after full induction usually seemed to have little effect on the solubility or level of target protein per A_{600} of culture density, whether the culture was in a medium where the density remained constant or continued to increase slowly after full induction. This stability of auto-induced cultures at or near saturation, together with the relative uniformity of the inoculating cultures grown to saturation in non-

inducing media, makes it convenient to screen many strains in parallel for expression and solubility (or larger cultures for purification) simply by incubating thousand-fold dilutions overnight at 37°C, or somewhat longer at 20°C. IPTG-induced cultures, on the other hand, were usually collected 3 hr after induction at 37°C to avoid overgrowth by unproductive cells. Occasionally, continued incubation for many hours at 37°C reduced the apparent solubility of the target protein in an IPTG-induced or auto-induced culture or both. In the rare cases where such behavior was observed, the target protein appeared soluble in a parallel 20°C induction.

It is important to note that auto-induction and saturation often occur at considerably higher density at 20°C than at 37°C (perhaps due to the higher solubility of oxygen at the lower temperature). Higher saturation densities combined with slower growth at 20°C means that cultures may be quite dense after overnight incubation but not yet be induced, so care must be taken not to collect low-temperature cultures before they have saturated. The incubation time can be shortened by incubating at 37°C for a few hours, until cultures become lightly turbid, and then transferring to 20°C for auto-induction.

Auto-induction has become the standard procedure in our laboratory for testing expression and solubility of proteins produced by T7 expression strains and for producing target proteins in large amounts for purification. IPTG induction is rarely if ever used anymore. Several yeast proteins were produced by auto-induction and purified for possible structure determination. Three that gave crystals suitable for structure determination were labeled with SeMet by auto-induction (as described in the next section) and yielded structures (P35, PDB 1TXN; P89, PDB 1NJR; and P96, PDB 1NKQ), as has the human SSAT protein (through collaboration with J. Flanagan and M. Bewley). Dax Fu of this department (personal communication and [32]) has found that auto-induction increased the yields of five different bacterial integral-membrane proteins about ten-fold over the previous IPTG induction, to approximately 30-50 mg/liter. So far, he has determined the structure of one of them. Recipes and protocols for auto-induction have been distributed to many other laboratories, including structural genomics centers, and are proving to be highly successful.

Auto-induction for labeling proteins with SeMet for crystallography—Labeling proteins with SeMet is a standard and useful way to obtain phases for structure determination by X-ray crystallography [33]. Auto-induction seemed promising as a way to produce SeMet-labeled target proteins simply and efficiently. Expecting that a methionine-requiring host would be needed for efficient incorporation of SeMet, I started with B834(DE3), which had a methionine requirement of unknown genotype [10]. However, it turned out that SeMet labeling is equally efficient in BL21(DE3).

The concentration of methionine required for growth and auto-induction of B834(DE3)RIL/P21 was tested in a fully defined auto-inducing medium comparable to PA-5052. Cultures grew at the normal rate until the methionine was depleted, when the culture density abruptly stopped increasing. Concentrations equal to or greater than ~100 μg/ml of methionine were saturating for growth to A_{600} ~10.6. Production of target protein was not apparent at methionine concentrations less than ~40 μg/ml (where density stopped increasing at A_{600} ~3.8) but increased with methionine concentration until the maximum amount of target protein per A_{600} was reached at approximately 90 μg/ml. As for complex components, N-Z-amine supplied saturating amounts of methionine but yeast extract did not. Growth in YP-5052 stopped at A_{600} ~3.9 without induction of target protein, equivalent to ~40 μg/ml of methionine; addition of 100 μg/ml of methionine to the medium allowed saturation at A_{600} ~9.5 with full induction. The concentration of methionine in this lot of yeast extract, although not enough to support good auto-induction, is probably too high to make it a useful supplement in auto-inducing media for labeling with SeMet.

Growth and auto-induction in SeMet is stimulated by methionine B834(DE3) expressing yeast target protein P07, which had previously been labeled with SeMet in the process of structure determination [13], was used to test the potential for labeling with SeMet in auto-inducing media comparable to PA-5052. Total replacement of methionine by SeMet was not effective: SeMet concentrations of 50 or 100 μg/ml supported growth relatively poorly, to A_{600} of less than 2, with no induction of target protein, and 150 and 200 μg/ml prevented growth entirely. To test whether small amounts of methionine might alleviate the toxic effects of SeMet, growth and auto-induction were tested in PA-5052 containing 5-30 μg/ml of methionine plus 50-200 μg/ml of SeMet. Indeed, cultures containing as much as 150 μg/ml of SeMet attained A_{600} ~5 to ~8 (considerably higher than with either methionine or SeMet alone) and induced large amounts of target protein. However, the toxic effects of 200 μg/ml of SeMet overcame even 30 μg/ml of methionine, reducing saturation density to A_{600} ~1.9 and preventing auto-induction. Both SeMet and methionine seem to be used at all stages of growth, because growth rate in 30 μg/ml methionine was reduced by the presence of 100 μg/ml SeMet, and the growth curve seemed not to have a discontinuity that might indicate a strong preferential use of methionine until depletion. The stimulatory effect of methionine on growth and auto-induction in 100 μg/ml of SeMet was comparable between 10 and 30 μg/ml of methionine but was significantly diminished in 5 μg/ml.

These results suggested that auto-induction might produce target protein with more than 90% replacement of methionine by SeMet, if the methionine needed to stimulate growth and auto-induction could be less than 10% of the amount of SeMet in the medium. To test the level of incorporation of SeMet into target protein, 100-ml cultures of B834(DE3) expressing His-tagged P07 were grown in defined media comparable to PA-5052, containing either 200 μg/ml of methionine or 10 μg/ml of methionine plus 100 μg/ml of SeMet. The cultures saturated at A_{600} ~8.8 and 6.7 and yielded 2.8 and 1.9 mg of purified P07 protein, respectively. Mass spectroscopy determined that the P07 protein from the SeMet-containing culture was more than 90% labeled with SeMet.

Yeast target protein P89 was only partially soluble but had been purified from auto-induced cultures and crystallized, so it was a good candidate for SeMet labeling by auto-induction. However, a test of 0.5 ml culture in the medium used for SeMet labeling of P07 produced rather small amounts of soluble P89. In an attempt to improve the yield, different concentrations of methionine and SeMet were tried along with different concentrations of the other 17 amino acids plus a mixture of 9 vitamins. Interestingly, the vitamins had no effect in PA-5052 itself but significantly increased both the saturation density and level of P89 produced by auto-induction in the presence of SeMet. Tests of the individual vitamins showed that vitamin B_{12} was the only one needed for the stimulation: a mixture of the other 8 vitamins provided no stimulation. As little as 3 nM vitamin B_{12} was sufficient to provide maximum stimulation. Growth and auto-induction of B834(DE3)RIL/P89 in 400 ml of PASM-5052 (which contains 10 μg/ml methionine, 125 μg/ml SeMet and 100 nM vitamin B_{12}) produced 4 mg of purified SeMet protein, sufficient for phasing and structure determination [14].

B834 is a metE mutant In control experiments, it was discovered that the presence of vitamin B_{12} in the medium allows normal growth of B834(DE3) in the absence of methionine. This unexpected result shows that the methionine deficiency of B834 is due to a mutation in *metE*, which specifies the vitamin B_{12}-independent homocysteine methylase of *E. coli*, which catalyzes the last step of methionine synthesis [34]. (Previous reports [35, 36] that B834 is a *metB* mutant provided no supporting data and must be incorrect.) *E. coli* also contains a vitamin B_{12}-dependent homocysteine methylase, specified by *metH*. However, since *E. coli* is incapable of synthesizing vitamin B_{12}, this enzyme is active only when vitamin B_{12} is present

in the growth medium. Concentrations of vitamin B_{12} greater than ~0.75 nM allowed maximal growth and auto-induction of B834(DE3)RIL/P21 in PA-5052 lacking methionine.

The discovery that B834 is a *metE* mutant suggests a possible explanation for the stimulation of growth and auto-induction by vitamin B_{12} in the presence but not the absence of SeMet. The presence of methionine in the growth medium represses the synthesis of all of the enzymes specific for methionine synthesis except for the *metH* enzyme [34]. SeMet seems likely to have the same effect, since its presence in the growth medium inhibits the growth of BL21(DE3) and B834(DE3) to about the same extent even though BL21(DE3) would be fully competent to synthesize methionine. An important role for methionine is incorporation into S-adenosylmethionine, a methyl donor in reactions that generate S-adenosylhomocysteine as a product, which is ultimately metabolized to homocysteine [37]. Since methionine is not toxic, concentrations in the growth medium can always be made high enough to supply all of the needs for methionine without recycling homocysteine. However, at the concentrations of SeMet that can be tolerated in the growth medium, a substantial fraction may ultimately end up in Se-homocysteine, and the remaining SeMet may be insufficient for continued growth and synthesis of target proteins. The stimulatory effect of vitamin B_{12} might be due to its activation of the *metH* homocysteine methyltransferase, which regenerates SeMet from Se-homocysteine. If this interpretation is correct, vitamin B_{12} might also be expected to stimulate growth and auto-induction of target proteins in BL21(DE3) growing in the presence of SeMet. Some stimulation of target protein by the presence of vitamin B_{12} in PASM-5052 was apparent in one test with BL21(DE3)P19 but not in a second test. Vitamin B_{12} is included in PASM-5052 at a concentration of 100 nM.

SeMet labeling in BL21(DE3) The efficient substitution of SeMet for methionine in B834 (DE3) in the presence of vitamin B_{12} reinforced the conclusion that the combination of 10 μg/ml of methionine and 125 μg/ml of SeMet in PASM-5052 must repress the endogenous synthesis of methionine. Therefore, SeMet labeling by auto-induction in BL21(DE3), which does not require methionine for growth, should be just as efficient as in B834(DE3). Indeed, auto-induction of human spermidine/spermine acetyl transferase (SSAT) in PASM-5052 produced greater than 90% substitution of SeMet for methionine whether produced from B834 (DE3)RIL or BL21-Gold(DE3)RIL. Thus, target proteins can be efficiently labeled with SeMet by auto-induction in BL21(DE3), and the use of B834(DE3) is not necessary.

Generality of SeMet labeling To explore the general utility of auto-induction for SeMet labeling, production and solubility of target proteins in PASM-5052 relative to ZYP-5052 and PA-5052 were tested for 10 different yeast proteins expressed in B834(DE3)RIL by auto-induction of cultures grown from 1000-fold dilutions at both 37°C and 20°C. (In this set, PASM-5052 contained 100 μg/ml of SeMet.) All of the 37°C cultures appeared to be saturated and were sampled for gel electrophoresis after an overnight incubation of 14 hours. The 20°C cultures in ZYP-5052 and PA-5052 were sampled after 22 hours, but SeMet appears to inhibit growth much more strongly at 20°C than at 37°C, and the 20°C PASM-5052 cultures were not sampled until 65 hours and again at 85 hours. All but one of these 10 target proteins appeared to be produced about as well and to have comparable solubility in PASM-5052 as in the other two media at both temperatures. These results indicate that auto-induction in PASM-5052 should be generally useful for SeMet labeling of target proteins.

A SeMet concentration of 125 μg/ml was chosen for PASM-5052 medium because it seems sufficient but not much in excess of the amount needed to support growth and auto-induction in the presence of 10 μg/ml of methionine and 100 nM vitamin B_{12}. Cultures grown in significantly higher concentrations of SeMet tended to become an orange brown color upon prolonged incubation at saturation. The yield of the few SeMet-labeled proteins we have

produced by auto-induction in PASM-5052 for structure determination has been comparable to the yield of the unlabeled proteins.

Auto-induction for labeling proteins with ^{15}N and ^{13}C for NMR—Determination of protein structures by NMR requires substantial amounts of protein labeled with ^{15}N or ^{13}C. Auto-induction in fully defined minimal media is potentially very efficient at incorporating isotopic labels into target protein. Target proteins can be uniformly labeled with ^{15}N simply by using ^{15}N-labeled $(NH_4)_2SO_4$ in P-5052 or NH_4Cl in N-5052 or LS-5052. Auto-induction with a well expressed target protein will almost deplete the 50 mM ammonium ion in these media, so use of the isotope should be very efficient. Reduction below about 25 mM ammonium will significantly reduce the amount of well-expressed target protein obtained.

Glycerol can be used as a source of ^{13}C for labeling target proteins produced by auto-induction. Glucose, the most economical source, cannot be used because it prevents auto-induction. Fortunately, ^{13}C glycerol is relatively economical and undoubtedly would become cheaper if usage increased. The glucose in the auto-inducing medium will have been depleted by the time target protein synthesis begins, but lactose metabolism is necessary for auto-induction and some carbon from lactose is likely to be available for incorporation into target protein, at least in the early stages of synthesis. The usual auto-induction media contain 0.2% lactose and 0.5% glycerol, concentrations chosen to ensure maximal production of target protein even at the highest rates of aeration and to make it unlikely that the culture will go irreversibly acid even at relatively low rates of aeration. For efficient ^{13}C labeling of target protein, the flow of carbon from glycerol into target protein should be maximized and the flow from lactose minimized.

As discussed in the section on *Effect of aeration on timing and level of auto-induction of target protein*, the concentration of lactose needed for maximal induction of target protein decreases with decreased rate of aeration. Using a minimal medium containing 100 mM phosphate for good buffering, auto-induction of T7 capsid protein was followed as a function of decreasing lactose concentration at the different rates of aeration provided by different volumes of culture in 13×100 mm tubes. Increasing concentrations of glycerol were also tested. Based on these tests, the auto-inducing medium C-750501 (Table 1) should provide good ^{13}C labeling of target protein from glycerol. This medium contains 0.75% glycerol and 0.01% lactose, so almost all of the carbon entering target protein should be derived from glycerol. High-level induction was obtained at the aeration rate delivered by 0.75 ml cultures in 13×100 ml tubes, a somewhat lower aeration rate than with the standard 0.5 ml per tube. Induced cultures saturated at A_{600} ~10 in less than 24 hours at 37°C with a pH usually above 6.0. These conditions seem likely to scale to approximately 200-400 ml of culture in an unbaffled 1-liter Erlenmyer flask or perhaps as much as 1 liter in a 1.8-liter baffled Fernbach flask. Since our structural genomics project does not involve NMR, I have not tested whether the efficiency of ^{13}C labeling in this medium is adequate for structure determination. However the recipe has been distributed to several NMR groups in hopes that it will prove useful. A test of ^{13}C incorporation as a function of lactose concentration might find that higher lactose concentrations and higher rates of aeration also provide satisfactory labeling.

Auto-induction with arabinose—Expression systems in which transcription is controlled by the pBAD promoter of the arabinose operon have relatively low basal expression, which can make them useful for maintaining and expressing toxic genes [38, 39]. The AraC protein regulates the pBAD promoter both positively and negatively, and basal expression is further reduced in the presence of glucose. Expression from the pBAD promoter is induced by arabinose and modulated by catabolite repression. At least two groups have reported expression systems in which the sequence for T7 RNA polymerase has been placed under control of the pBAD promoter [40, 41], and Invitrogen markets BL21-AI, in which the pBAD promoter can

express T7 RNA polymerase from the chromosome of BL21. Basal expression of T7 RNA polymerase from the pBAD promoter in BL21-AI is expected to be lower than that from the *lacUV5* promoter in BL21(DE3), which is probably intrinsically leaky because β-galactosidase activity is needed to convert lactose to allolactose, the natural inducer of the lactose operon [42]. Therefore clones expressing highly toxic target proteins from a T7 promoter might be maintained and expressed more readily in BL21-AI than in BL21(DE3). Indeed, several clones capable of expressing the highly toxic T7 gene *5.3* protein that could not be established in BL21 (DE3) were readily established in BL21-AI.

When transcription of the target gene is from a T7*lac* promoter, as in the expression clones we are using, full expression requires both induction of T7 RNA polymerase and release of *lac* repressor from its binding site in the T7*lac* promoter. In BL21(DE3), both events are triggered by release of the *lac* repressor, which is conventionally induced by IPTG or auto-induced by the presence of lactose in the medium. The combination of expressing T7 RNA polymerase from a pBAD promoter in the chromosome and the target gene from the T7*lac* promoter in a multi-copy plasmid provides enough control that auto-induction of target protein production is feasible in BL21-AI. Auto-induction of T7 capsid protein in BL21-AI in ZYM-5052 plus 0.05% L-arabinose showed barely detectable capsid protein at A_{600} ~2.7, a distinct band that increased steadily to high levels between A_{600} ~4 and ~10, and an approximately constant amount per A_{600} during continued increase in culture density to A_{600} ~26 (Figure 4D). Auto-induction of the toxic T7 gene *5.3* protein stopped growth at A_{600} ~1.7 with no *5.3* protein apparent in gel patterns, consistent with results obtained in BL21(DE3). The presence of 0.05% glucose in the auto-inducing medium was necessary to allow growth of the *5.3* clone in the presence of 0.05% arabinose, and the growth was about as rapid as in the absence of arabinose. Apparently, the presence of glucose is necessary and sufficient to prevent significant induction by arabinose in BL21-AI in the early stages of growth in the auto-inducing medium. In contrast to the results obtained in BL21(DE3), the T7 gene *7.7* protein was not apparent above the background upon auto-induction in BL21-AI.

L-Arabinose concentrations between 0.01% and 0.5% all induced high-level expression of T7 capsid protein in ZYM-5052 (which contains lactose to induce unblocking of the T7*lac* promoter). Capsid protein was detectable in the presence of arabinose and absence of lactose, but much less was produced than in the presence of both, providing a measure of how effectively bound *lac* repressor blocks transcription from the T7*lac* promoter (to be described elsewhere).

To get a broader comparison of auto-induction in BL21(DE3) and BL21-AI, 42 different clones of yeast coding sequences under control of the T7*lac* promoter, which were known to be well expressed in BL21(DE3)RIL, were also placed in BL21-AI/RIL. In parallel tubes, the BL21 (DE3)RIL clones were auto-induced in ZYM-5052 and the BL21-AI/RIL clones were auto-induced in ZYM-5052 containing 0.05% L-arabinose. Levels of target protein were generally comparable in the two hosts, although a few clones appeared to be expressed to a slightly higher level or to be slightly more soluble in one host or the other. Whether these differences represent experimental variation or are more significant has not been explored. However, it is clear that auto-induction from the pBAD and T7*lac* promoters is generally effective for producing target proteins in BL21-AI.

Other growth media

High-density cultures for preparation of plasmids—The high-density culture conditions developed for auto-induction also are convenient for preparation of plasmid DNAs. Rich media such as ZYM-505 support growth of the plasmid-containing strains we work with to culture densities of A_{600} ~10 or higher when 1.5-2.5 ml of culture is grown in an 18×150

mm tube shaken at 300-350 rpm. Lactose is omitted unless auto-induction is desired. The presence of 0.05% glucose ensures rapid initial growth with little lag. Typically, yields of plasmid DNA have been several fold greater than obtained in media previously used for this purpose, and a single 1.5-ml microfuge tube usually provides more plasmid DNA than needed for most purposes. Adequate aeration ensures growth to high densities, but even moderate aeration gives high yields. John Dunn of this department (personal communication) is using auto-inducing media to obtain high yields of a single-copy plasmid that carries an inducible replication origin under control of a *lac* promoter.

Commonly used complex media can be deficient in magnesium—As this paper was being written, I thought to compare culture densities attained in ZYM-505 with terrific broth (TRB) and 2xYT, rich media commonly used for high-density growths for preparing plasmid DNAs [15]. All three media contain an enzymatic digest of casein plus yeast extract, but in different concentrations: 2xYT contains 16 g tryptone, 10 g yeast extract and 5g NaCl per liter whereas TRB contains 12 g tryptone and 24 g yeast extract, 89 mM phosphate and 4 ml glycerol (= 0.5% w/v) per liter. TRB has several of the same components as ZYM-505 but a considerably higher concentration of yeast extract. Having previously found that ZY was deficient in magnesium, I tested 2xYT and TRB as described and also containing 2 mM $MgSO_4$. The results are shown in Table 10.

The most striking result was that adding magnesium to TRB made from a commercial product (Gibco/BRL) increased the culture density from A_{600} ~3.6 to ~18.6. The stimulation from adding magnesium to 2xYT (made from our own barrels of N-Z-amine and yeast extract) was not as large, increasing from A_{600} ~5.7 to ~8.3. The difference indicated that our N-Z-amine and yeast extract probably had higher levels of magnesium than the lots used to make the Gibco/BRL product, and indeed, TRB made from our components produced a considerably higher A_{600} ~12.5, increasing to ~18.1 with added magnesium. Titration of the TRB from Gibco/BRL indicated that 0.5 mM $MgSO_4$ was sufficient to saturate the growth requirement. These results confirm that enzymatic digests of casein or yeast extract are likely to be deficient in magnesium needed for maximum growth (to different degrees in different lots), and that 1-2 mM magnesium ion should be added to complex media made with these components to ensure maximum growth.

The 50% higher saturation density in TRB + magnesium (A_{600} ~18.5) relative to ZYM-505 (A_{600} ~12) is due the greater than 2-fold higher concentration of complex components in TRB. A similar boost could be achieved more economically by increasing the glycerol concentration of ZYM-505, perhaps balancing pH by adding succinate or an inexpensive and well-metabolized amino acid such as aspartate or glutamate.

Discussion

The phenomenon of unintended induction was sporadic, being found in some lots of complex media but not others [6]. Furthermore, different portions of the same culture might produce widely different levels of target protein, depending on the rate of aeration (Table 8). The realization that lactose is responsible for unintended induction made it possible to develop non-inducing media in which T7 expression strains remain stable and viable all the way to saturation, and reliable auto-inducing media that produce high-density, fully-induced cultures completely unattended. This was an iterative process, addressing factors that limit growth to high-density in batch mode, affect the viability or stability of expression strains, or influence the level of production of target protein. Interestingly, lack of magnesium limits growth in typical lots of traditional rich media such as tryptone broth or LB (and newer media such as terrific broth). With a sufficiency of nutrients, the main limiting factors become maintenance

of a pH near neutral and the availability of oxygen as the culture becomes dense enough that the rate of aeration becomes limiting. The media given in Table 1 have been formulated to give reliable non-induced cultures and good auto-induction over a range of conditions. We currently use MDG as the non-inducing medium for growing cultures for freezer stocks or working stocks, MDAG plates for selecting strains that express highly toxic target proteins, ZYM-5052 for auto-induction, and ZYM-505 for growing high-density cultures for preparing plasmids.

The surprising finding that high phosphate concentration in rich media provides substantial resistance to kanamycin led to the formulation of lower phosphate media in which metabolic balancing maintains the pH of the medium near neutral. Growth in glucose or glycerol produces acid whereas growth in amino acids or tricarboxylic acid cycle intermediates such as succinate, fumarate, malate or citrate increases the pH. Although the presence of glucose or glycerol limits the catabolism of these other carbon and energy sources [16-21], appropriate mixtures can support growth to high densities with only moderate excursions toward acid pH followed by saturation or extended slow growth close to neutral pH. By increasing the glycerol and amino-acid concentrations well above those given in Table 1, auto-induction of well-expressed target proteins has produced culture densities of A_{600} >50 in shaking batch cultures, comparable to what has been reported in a fermenter [31]. Potentially, auto-induction could produce proteins economically on a commercial scale, as high-density cultures fully induced for target protein can be obtained without complex process controls in media made entirely from inexpensive components such as mineral salts and mixtures of glucose, glycerol and lactose, supplemented with fumarate, succinate or glutamate.

Auto-induction depends on mechanisms bacteria use to regulate the use of carbon and energy sources present in the growth medium. If glucose is present, catabolite repression and inducer exclusion prevent the uptake of lactose by lactose permease, the product of *lacY*, thought to be the only means of lactose uptake in wild-type cells [16-20]. When glucose is depleted, lactose can be taken up by a small amount of *lacY* present in uninduced cells and converted to allolactose, the natural inducer, by β-galactosidase, the product of *lacZ* [42, 43]. Thus, induction of the *lac* operon by lactose should require the presence of at least a small amount of lactose permease and β-galactosidase in the uninduced cell, and auto-induction should not be effective with strains that lack either of these activities. Contrary to this expectation, Grossman *et al.* [6] observed expression of *lacZ* from the T7*lac* promoter in a multi-copy plasmid upon approach to saturation in BL26(DE3), a derivative of BL21 from which the *lac* operon has been deleted [4]. Perhaps changes that occur on approach to saturation make cells permeable to lactose by some other mechanism [6].

The presence of 0.05% glucose in auto-inducing media blocks induction by lactose in the early stage of growth so effectively that even strains capable of expressing target proteins highly toxic to the host cell can grow and maintain functional plasmid until induction. In fact, basal expression may be low enough that antibiotic might not be needed in the auto-inducing medium to obtain high-level production of many target proteins.

Having a carbon and energy source other than lactose to support continued growth and production of target protein after induction enhances high-level production of target proteins from T7 expression strains. T7 RNA polymerase is so active that induction can direct most transcription and translation to the target protein [1], which might interfere with full induction of the ability to metabolize lactose for energy. Glycerol does not interfere with induction of target protein, and its presence in auto-inducting media more than doubled the yield of target protein relative to what was obtained with equivalent amounts of lactose as the primary energy source.

In the absence of glucose, amino acids appear to modulate or prevent induction of target proteins by lactose in the early stages of growth, until growth slows as oxygen becomes limiting upon approach to saturation. Complex mechanisms change metabolism in response to amino acid availability and oxygen levels [22, 44], but I was not aware that they are known to prevent lactose utilization. Serine appears to be particularly effective in preventing induction of target proteins by lactose in log phase but even high concentrations of serine do not prevent induction as cultures approach saturation. Interestingly, serine is the first amino acid to be depleted during growth on the mixture of amino acids present in a tryptic digest of casein [24, 25]. Perhaps the ability to prevent induction is related to a need for higher levels of allolactose to induce expression from the T7*lac* promoter in a multi-copy plasmid, because higher than normal levels of *lac* repressor are present to ensure saturation of all of the repressor binding sites [2, 4]. Slowing of growth upon oxygen limitation might allow higher levels of allolactose to accumulate, because of increased uptake of lactose from the medium, decreased catabolism of allolactose or some other change that promotes induction.

Although developed for expressing target proteins in the IPTG-inducible T7 expression system, auto-induction could in principle be developed for any expression system in which the elements driving expression of the target protein are induced by a change in metabolic state that is brought about by growth of a culture. This could include not only promoters whose induction is prevented by catabolite repression or inducer exclusion, but also, for example, promoters activated by approach to saturation, oxygen limitation, or depletion of a compound (such as methionine) whose synthetic pathway is blocked by its presence in the medium. Simply adding 0.05% L-arabinose to the auto-induction media of Table 1 allows them to be used for producing target proteins in BL21-AI, where T7 RNA polymerase is expressed from the chromosome by the arabinoseinducible pBAD promoter. Consistent with the general view that the pBAD promoter has lower basal expression than the *lacUV5* promoter, plasmids expressing bacteriophage T7 proteins that are highly toxic to the host cell were more easily tolerated in BL21-AI than in BL21(DE3). Level of expression and solubility of most target proteins tested were comparable in the two hosts.

Auto-induction has proved to be generally useful for producing a wide range of proteins, including membrane proteins. The stability and viability of cultures grown in non-inducing media makes it possible to work with many strains in parallel over a period of weeks. Re-transformation or streaking out cultures for a “fresh” single colony, an unfortunate and tedious practice in many labs, is almost never necessary for reproducibly expressing high levels of target protein. Cultures for auto-induction are simply inoculated and grown to saturation, which is much more convenient than IPTG induction and especially convenient for high throughput testing of many different target proteins for expression and solubility. The high culture densities attained by auto-induction produce more target protein per volume of culture than IPTG induction and also make efficient use of expensive reagents when labeling with SeMet or isotopes. Auto-induction is convenient, efficient and economical for producing proteins at almost any scale, from analysis of individual proteins in small laboratories to production of many different proteins in large projects, and possibly even for production of proteins on a commercial scale.

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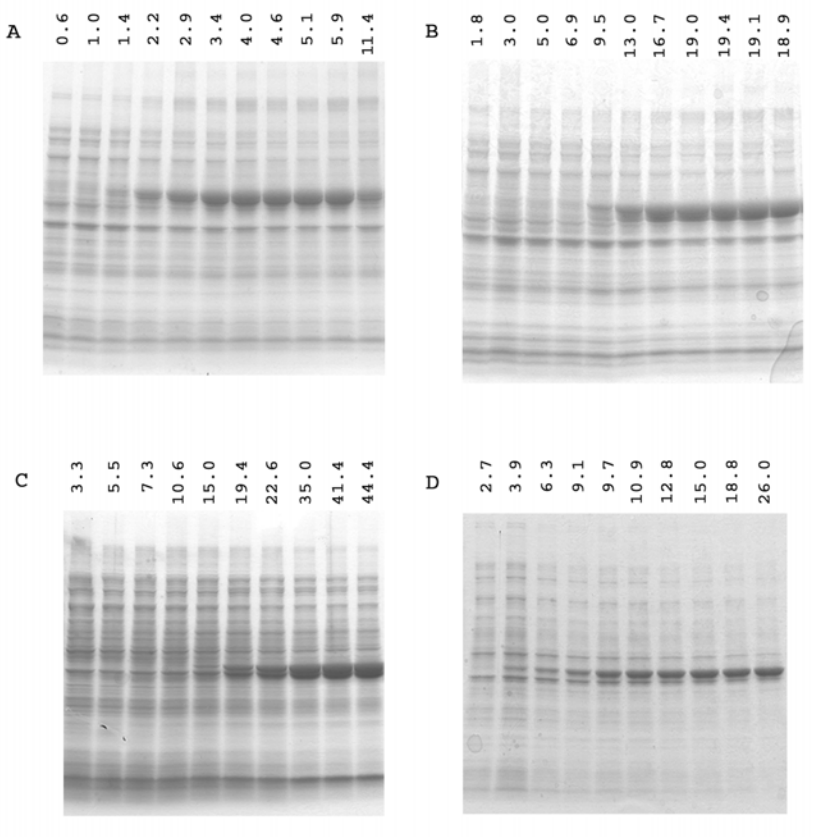

Figure 1.

Electrophoretic patterns of total cell proteins during growth of auto-inducing cultures at 37°C. Equal culture densities were analyzed in each lane of a set, and the A_{600} of the culture at the time of sampling is given above each lane. A) BL21(DE3)RIL/P21 was grown in 6 ml of ZYP + 0.5% lactose in a 125-ml Erlenmeyer flask. The culture was sampled every 30 min, except that the interval before the last sample was 15 hours. The cell suspensions before processing for electrophoresis were A_{600} ~10. B) BL21(DE3)RIL/P21 was grown in 5 ml of ZYP-5052 in a 125-ml flask (except that the glycerol concentration was 0.625% instead of 0.5%). The culture was sampled every 30 min. The cell suspensions were A_{600} ~10. C) BL21(DE3) T7-10A was grown in 2.5 ml of ZYP-20052 + 25 mM succinate in a 125-ml flask (the glycerol concentration was 2 %). The culture was sampled every 30-40 min until A_{600} ~22.6, and then intervals of 70 min, 55 min, and 13.5 hours. The cell suspensions were A_{600} ~5. D) BL21-AI/ T7-10A was grown in 2 ml of ZYM-5052 + 0.05% L-arabinose in an 18×150 mm culture tube. The culture was sampled every 30 min until A_{600} ~10.9, then three intervals of 60 min and a final interval of 16 hours. The cell suspensions were A_{600} ~2.5.

Table 1

d auto-inducing media. An explanation of the naming conventions is given under *Growth media* in
r growing freezer stocks and working cultures, ZYM-5052 for routine auto-induction, and ZYM-505

Comment	% N-Z-amine	% Yeast extract	mM Na_2HPO_4	mM KH_2PO_4	mM NH_4Cl	mM $(NH_4)_2SO_4$	mM Na_2SO_4	mM $MgSO_4$[a]	Trace metals[b]	% Glycerol	% Glucose	% Lactose	mM Succinate	% Aspartate	% 18 amino acids[c]
			50	50		25		2	0.2x		0.5				
			50	50		25		2	0.2x		0.5				0.36
^{15}N labeling			50	50		25		2	0.2x	0.5	0.05	0.2			
			50	50		25		2	0.2x	0.5	0.05	0.2			0.36
SeMet labeling			50	50		25		2	0.2x	0.5	0.05	0.2			SeMet[d]
		0.5	50	50		25		2	0.2x	0.5	0.05	0.2			
			12.5	12.5	50		5	2	0.2x		0.5		20		
^{15}N labeling			12.5	12.5	50		5	2	0.2x	0.5	0.05	0.2	20		
			25	25	50		5	2	0.2x		0.5			0.25	
			25	25	50		5	2	0.2x		0.5			0.25	0.36
			25	25	50		5	2	0.2x	0.5	0.05	0.2		0.25	0.36
Plasmid preps		0.5	25	25	50		5	2	0.2x	0.5	0.05				
		0.5	25	25	50		5	2	0.2x	0.5	0.05	0.2			
^{15}N labeling			50	50	50		5	2	0.2x	0.5	0.05	0.2			
^{13}C labeling			50	50	50		5	2	0.2x	0.75	0.05	0.01			

in previously distributed recipes

al but recommended in media containing ZY

ne)

(no C,Y,M), 10 μg/ml of methionine,125 μg/ml SeMet, and 100 nM vitamin B_{12}

Table 2

Saturation densities and induction of T7 RNA polymerase in different growth media. BL21 (DE3) was grown 17 hr, 37°C from 10^4 dilution, 10 ml in 125-ml Erlenmeyer flasks. N-Z-amine in growth media was from the new barrel, which had inducing activity, except that the "Old ZB" culture and the plates for testing 4107 plaque formation were made from the old barrel, which lacked inducing activity.

				T7 4107 plaques [a]		
Growth medium	**Addition**	**A_{600}**	**pH**	**Number**	**Size**	**Time of appearance**
ZB		1.2	8.25			
ZYB		2.8	7.65			
3xZYB		7.6	7.35			
Old ZB		1.0	7.90	39	small	4.5 hr
ZB	IPTG in plate			188	large	2.0 hr
0.5xZB[b]		0.5	8.62	144	variable	3.0 hr
ZB		1.1	8.22	150	variable	3.0 hr
2xZB[b]		2.4	8.53	189	variable	2.5 hr
4xZB[b]		5.4	8.32	183	variable	2.0 hr
8xZB[b]		6.9	7.92	222	large	1.5 hr
ZB	0.1% glucose	1.3	7.58	150	variable	2.5 hr
ZB	1% glucose	1.2	5.12	38	tiny	4.5 hr
4xZB[b]		6.0	8.05	231	large	1.5 hr
4xZB[b]	1% glucose	4.3	5.27	65	tiny, turbid	3.5 hr
8xZB[b]		5.5	7.37	220	large	1.5 hr
8xZB[b]	1% glucose	5.8	5.28	45	tiny, turbid	3.5 hr
ZYB		2.5	8.55	175	small	3 hr
ZYB	1% glucose	3.2	5.30	22	small	overnight
M9		2.5	6.10			
M9ZB		7.5	7.02			
M9ZB	2% glucose (total)	5.8	4.57			
ZB	M9 PO_4+NH_4Cl	1.2	7.36			
ZB	1 mM $MgSO_4$	2.1	8.35			
ZB	M9 salts	2.6	7.40			
ZB	1% glucose	1.2	5.12			
ZY[c]		1.7				
ZY[c]	1 mM $MgSO_4$	4.3				

[a] Equivalent numbers of T7 4107 deletion phage particles were plated on 0.25 ml of each culture in 2.5 ml of ZB top agar on ZB plates, both made with N-Z-amine lacking inducing activity (old ZB)

[b] Contained 0.5% NaCl

[c] Grown 14 hr, 37°C, 0.5 ml in 13×100 mm tubes

Table 3

Growth requirements for BL21(DE3) in modified LG media. Cultures were grown 14-15 hr, 37°C from 10^3 dilution, 0.5 ml in 13×100 mm tubes. Cultures were titered after 3-4 weeks in the refrigerator.

	LG medium, no succinate			LG medium + 25 mM succinate		
Glucose	**A_{600}**	**pH**	**Titer (× 10^9)**	**A_{600}**	**pH**	**Titer (× 10^9)**
0	0			0		
0.05%	0.24	6.89		0.66	7.03	1.8
0.10%	0.7	6.76		1.4	7.20	3.6
0.15%	1.2	6.62		2.2	7.33	7.2
0.20%	1.6	6.51		3.1	7.47	11.8
0.25%	1.8	6.42	5.0	3.2	7.47	15.5
0.30%	2.6	6.19	7.1	4.0	7.64	14.5
0.35%	3.0	5.91	8.1	4.2	7.76	12.0
0.40%	3.0	4.85		4.6	7.69	8.8
0.45%	3.8	4.57		5.1	7.87	7.8
0.50%	3.3	4.41	<0.02	5.3	7.84	9.5
Na_2SO_4						
0	0.7	6.67	0.11	0.6	6.67	0.46
0.1 mM	2.0	6.06	~0.14	1.8	6.89	2.1
0.2 mM	2.6	5.50	<0.02	3.2	6.49	3.9
0.5 mM	3.8	4.91	<0.02	6.1	6.75	13.4
1 mM	3.8	4.86		5.9	6.74	12.7
2 mM	3.6	4.84		5.8	6.75	14.2
NH_4Cl						
0	0	6.98	<0.02	0	7.02	
5 mM	1.5	6.51	<0.04	1.5	6.75	~0.08
10 mM	3.0	5.50	<0.04	2.8	6.50	4.7
15 mM	3.4	4.90		4.0	5.95	9.1
20 mM	3.4	4.87		4.9	6.52	16.4
25 mM	3.4	4.87		5.3	6.92	12.5
50 mM	3.5	4.90		5.5	7.10	13.4
Phosphate						
0				0.78	6.75	
1 mM				3.8	6.39	
2 mM				3.9	6.09	
5 mM				5.1	7.75	
10 mM				5.7	7.96	
15 mM				5.8	8.15	
20 mM				5.9	8.19	
25 mM				5.8	8.11	
35 mM				5.5	7.83	
50 mM				5.6	7.15	
$MgSO_4$						
0	0	6.90		0	6.79	
0.1 mM	0.41	6.81	0.12	3.9	6.73	5.0
0.2 mM	1.1	6.61	0.42	5.7	6.28	5.1
0.5 mM	3.7	5.64	<0.02	6.4	6.20	8.8
1 mM	3.7	5.08	<0.02	6.1	6.51	13.2
2 mM	3.7	4.81	<0.02	5.8	6.80	12.3

Table 4

Effectiveness of individual amino acids in balancing pH from 0.5% glucose or in serving as a carbon and energy source. BL21(DE3) was grown from 10^3 dilution, 0.5 ml in 13×100 mm tubes.

	Grown in LG medium				Grown in L medium + 0.18% aa [a]				
Addition	**Conc.**	**A_{600}**		**pH**	**Concentration**		**A_{600}**		**pH**
		8 hr	**22 hr**				**5.7 hr**	**46 hr**	
Glucose	0.5%	0.58	4.0	4.06	0.5%	28 mM	4.6	8.2	4.28
Glycerol					0.5%	54 mM	2.4	9.0	5.72
Succinate	20 mM	0.74	7.6	6.80	0.5%	42 mM	0.80	4.5	8.80
18 aa	0.27%	6.2	6.9	5.66	0.18%	24 mM	0.56	1.4	6.78
D	0.25%	4.8	8.1	7.55	0.5%	38 mM	0.84	3.9	8.28
S	0.25%	0.12	6.9	6.95	0.5%	48 mM	1.3	5.9	7.43
S + 100 ILV [b]	0.25%	0.56	8.7	6.93					
N	0.25%	2.6	7.0	6.86	0.5%	33 mM	0.72	2.0	7.02
G	0.25%	1.1	6.4	6.58	0.5%	67 mM	0.32	1.0	7.43
E	0.25%	3.4	7.5	6.28	0.5%	30 mM	0.72	5.1	7.28
A	0.25%	0.16	4.2	4.53	0.5%	56 mM	0.60	5.1	7.21
A	0.50%		6.2	7.32					
P	0.25%	2.0	5.3	3.63	0.5%	43 mM	0.92	9.6	7.00
T	0.25%	0.52	4.9	3.64	0.5%	42 mM	0.76	3.0	7.09
Q	0.25%	1.3	6.1	3.94	0.5%	34 mM	0.78	1.9	6.71
I	0.25%	1.2	4.3	3.86	0.5%	38 mM	0.44	1.1	6.67
L	0.25%	0.22	3.7	3.65	0.5%	38 mM	0.74	1.5	6.70
V	0.25%	0.26	3.5	3.70	0.5%	43 mM	0.64	1.3	6.73
M	0.25%	0.66	4.3	3.82	0.5%	34 mM	0.72	1.4	6.74
R	0.25%	0.88	4.9	3.63	0.5%	24 mM	0.70	1.5	6.81
K	0.25%	0.90	4.3	3.60	0.5%	27 mM	0.70	1.3	6.75
F	0.25%	0.64	2.3	3.89	0.5%	30 mM	0.68	1.2	6.81
W	0.25%	1.3	4.4	3.68	0.5%	24 mM			
H [c]	0.25%	0.16	4.5	3.69	0.5%	24 mM	0.06	1.0	5.19

[a] 100 μg/ml of each of 18 amino acids (no C or Y)

[b] 0.25% serine plus 100 μg/ml each of isoleucine, leucine and valine

[c] Reconstitution indicated an initial pH ~5.4 for 0.25% histidine in LG and an initial pH ~6.0 for 0.5% histidine in L + 0.18% amino acids

Table 5

Effects of metal ions on saturation density and auto-induction. B832(DE3)RIL/P21 was grown 20 hr, 37°C from 10^3 dilution, 0.5 ml in 13×100 mm tubes.

Medium[a]	Addition	A_{600} at different metal concentrations				Target protein
		Concentration of trace metal mix[b]				
		0	0.1x	1x	10x	
ZYP-5052		18.0				+++
PA-5052		4.4				(+)
PA-5052	metal mix[b]		11.3	12.9	13.1	+++
			Metal ion concentration			
			1 uM	10 uM	100 uM	
PA-5052	$FeCl_3$		7.8	12.7	13.7	+++
PA-5052	$MnCl_2$		11.8	13.2	12.9	+++
PA-5052	$CoCl_2$		11.1	13.6[c]	~0.1[d]	+++
PA-5052	$ZnSO_4$		6.9	8.1	8.7	++
PA-5052	$NiCl_2$		4.8	7.7	5.2	+
PA-5052	Na_2MoO_4		7.6	5.5	6.6	+
PA-5052	$CaCl_2$		6.0	4.7	5.3	(+)
PA-5052	$CuCl_2$		5.6	5.2	4.5	(+)
PA-5052	Na_2SeO_3		5.8	6.3	~0.7[d]	(+)
PA-5052	H_3BO_3		6.1	4.6	5.3	(+)

[a] The media contained 0.625% glycerol rather than the usual 0.5% in 5052. The PA medium contained 200 μg/ml of methionine and 100 μg/m of the other 17 amino acids (no C or Y). Both ZYP and PA media also contained 1 μM each of nicotinic acid, pyridoxine, thiamine, vitamin B_{12}, biotin, rboflavin and folic acid.

[b] The trace metal mix differed from the final formulation. This trace metal mix contained 20 μM $CaCl_2$, 10 μM each of $FeCl_3$, $MnCl_2$, and $ZnSO_4$, 0.1 μM $CoCl_2$, and 0.05 μM each of $CuCl_2$ and $NiCl_2$.

[c] Growth rate was normal after a lag of about an hour.

[d] These cultures had very slow growth.

Table 6

Induction as a function of lactose concentration in ZYP made with N-Z-amine that has no inducing activity. B834(DE3)P35 was grown 13 hr, 37°C from 10^4 dilution, 0.5 ml in 13×100 mm tubes.

Medium	Lactose concentration		A_{600}	Target protein	Titer (× 10^9) [a] plasmid[b]	total[c]
ZYP	0	0	5.8	0	12.6	14.0
ZYP	0.005%	0.14 mM	5.8	+++	10.6	10.7
ZYP	0.01 %	0.28 mM	5.5	+++	6.9	6.7
ZYP	0.02 %	0.56 mM	4.9	+++	4.7	4.6
ZYP	0.05 %	1.4 mM	4.2	+++	~0.12	0.48
ZYP	0.1 %	2.8 mM	4.4	+++	~0.04	~0.16
ZYP	1 %	28 mM	4.1	+++	<0.04	0.9

[a] "~" indicates that titer was based on fewer than 10 colonies

[b] Titer of cells that are resistant to kanamycin and therefore retain plasmid

[c] Titer in the absence of antibiotic, which includes cells with or without plasmid

Table 7

Ability of amino acids to suppress lactose induction and allow growth of B834(DE3)RIL/P21 in P + 1.25% glycerol + 0.1% lactose + 100 μg/ml of methionine. Cultures were grown from 10^3 dilution, 0.5 ml in 13×100 mm tubes.

Addition (100 μg/ml each)	A_{600}	pH	Target protein
19 hr, 37°C			
0	0		
20 aa	12.4	6.28	+++
19 aa (no C)	12.7	5.18	+++
18 aa (no C,Y)	13.2	5.19	+++
GACPTKR	0		
ILVSHNQ	11.3	6.35	+++
FYWDE	0		
ILV	0		
S	4.7	6.69	+++
H	0		
N	0		
Q	0		
14.5 hr, 37°C			
18 aa (no C,Y)	10.6	6.20	+++
17 aa (no S, C, Y)	8.6	6.33	+++

Table 8

Effect of aeration on saturation density and protein production. B834(DE3)RIL/P21 was grown 23 hr, 37°C from 10^3 dilution in ZYP + 0.625% glycerol without added lactose.

Vessel	Culture volume (ml)	Saturation A_{600}	pH	Target protein	Titer (× 10^9) [a] plasmid[b]	total[c]
13×100 mm tube	0.25	15.0		+	21	29
13×100 mm tube	0.5	15.4	7.15	++	11	12
13×100 mm tube	1	15.0	7.19	+++	2.6	6.5
13×100 mm tube	2	8.5	6.34	+++	2.2	2.5
125 ml flask	2.5	13.0	7.04	0	25	25
125 ml flask	5	14.8	6.99	0	20	22
125 ml flask	10	14.0	7.08	++	15	17
125 ml flask	20	14.3	7.09	+++	2.6	6.4
125 ml flask	~39	10.2	6.60	+++	~0.1	3.4

[a] "~" indicates that titer was based on fewer than 10 colonies

[b] Titer of cells that are resistant to kanamycin and therefore retain plasmid

[c] Titer in the absence of antibiotic, which includes cells with or without plasmid

Table 9

Lactose concentration needed for induction as a function of rate of aeration. B834(DE3)RIL/P21 was grown 17 hr, 37°C from 10^4 dilution in ZYP + 0.625% glycerol.

Lactose concentration		Moderate aeration 0.5 ml in 13×100 mm tube			High aeration 1.5 ml in 125-ml Erlenmeyer flask				
		A_{600}	pH	Target protein	A_{600}	pH	Target protein	Titer (× 10^9) plasmid[a]	Titer (× 10^9) total[b]
0	0	15.3	7.19	+++	15.3	6.94	?	12	14
0.0001%	2.8 µM				13.8	6.96	?		
0.0002%	5.6 µM				13.9	6.97	?		
0.0005%	14 µM				14.0	6.98	?		
0.001 %	28 µM				14.2	6.98	?	20	21
0.002 %	56 µM				13.5	7.00	(+)	21	18
0.005 %	0.14 mM				13.9	7.00	+	21	
0.01 %	0.28 mM	16.2	7.19	+++	15.1	6.98	+	17	16
0.02 %	0.56 mM	16.4	7.20	+++	16.9	6.99	++	10	10
0.05 %	1.4 mM	16.4	7.20	+++	18.9	7.01	+++	3.3	6.2
0.1 %	2.8 mM	16.6	7.19	+++	17.9	7.02	+++	2.2	5.0
0.2 %	5.6 mM	16.6	6.93	+++	19.2	7.00	+++	1.5	4.1
0.5 %	14 mM	17.1	6.93	+++	20.4	6.94	+++	0.5	5.4
1 %	28 mM	18.0	6.33	+++	27.4	6.82	+++	<0.1	7.7

[a] Titer of cells that are resistant to kanamycin and therefore retain plasmid

[b] Titer in the absence of antibiotic, which includes cells with or without plasmid

Table 10

Effect of magnesium on saturation density in 2xYT and terrific broth (TRB). BL21(DE3) was grown 15 hr, 37°C from 10^3 dilution, 0.5 ml in 13×100 mm tubes.

Growth medium	Source	A_{600}	pH
ZYM-505	local	12.0	7.05
2xYT	local	5.7	8.37
2xYT + 2 mM $MgSO_4$	local	8.3	8.44
TRB	Gibco/BRL	3.6	7.73
TRB + 2 mM $MgSO_4$	Gibco/BRL	18.6	8.21
TRB	local	12.5	8.06
TRB + 2 mM $MgSO_4$	local	18.1	8.18