Available online at www.sciencedirect.com
SCIENCE DIRECT®
Protein Expression and Purification 40 (2005) 256–267
Protein Expression & Purification
www.elsevier.com/locate/yprep

# Protocols for production of selenomethionine-labeled proteins in 2-L polyethylene terephthalate bottles using auto-induction medium☆

Hassan K. Sreenath*, Craig A. Bingman, Blake W. Buchan, Kory D. Seder, Brendan T. Burns, Holalkere V. Geetha, Won Bae Jeon, Frank C. Vojtik, David J. Aceti, Ronnie O. Frederick, George N. Phillips Jr., Brian G. Fox*

*Department of Biochemistry, Center for Eukaryotic Structural Genomics, University of Wisconsin-Madison, 433 Babcock Drive, Madison, WI 53706-1549, USA*

Received 27 August 2004, and in revised form 22 December 2004
Available online 13 January 2005

**Abstract**

Protocols have been developed and applied in the high-throughput production of selenomethionine labeled fusion proteins using the conditional Met auxotroph *Escherichia coli* B834. The large-scale growth and expression uses a chemically defined auto-induction medium containing 125 mg $L^{-1}$ selenomethionine, salts and trace metals, other amino acids including 10 mg $L^{-1}$ of methionine, vitamins except vitamin $B_{12}$, and glucose, glycerol, and α-lactose. A schematic for a shaker rack that can hold up to twenty-four 2-L polyethylene terephthalate beverage bottles in a standard laboratory refrigerated floor shaker is provided. The growth cycle from inoculation of the culture bottle through the growth, induction, and expression was timed to take ~24 h. Culture growth in the auto-induction medium gave an average final optical density at 600 nm of ~6 and an average wet cell mass yield of ~14 g from 2 L of culture in greater than 150 expression trials. A simple method for visual scoring of denaturing electrophoresis gels for total protein expression, solubility, and effectiveness of fusion protein proteolysis was developed and applied. For the favorably scored expression trials, the average yield of purified, selenomethionine-labeled target protein obtained after proteolysis of the fusion protein was ~30 mg. Analysis by mass spectrometry showed greater than 90% incorporation of selenomethionine over a ~8-fold range of selenomethionine concentrations in the growth medium, with higher growth rates observed at the lower selenomethionine concentrations. These protein preparations have been utilized to solve X-ray crystal structures by multiwavelength anomalous diffraction phasing.


*Keywords:* Selenomethionine; Auto-induction; Polyethylene terephthalate; Cell growth

The critical step in solving a protein structure using X-ray crystallography is the determination of phases [1–3]. Among the methods suitable for addressing this critical problem, the substitution of selenium for sulfur and the application of multiwavelength anomalous diffraction methods have emerged as the standard approach for structural biologists [3], particularly in high-throughput environments. Successful implementation of this approach typically requires high percentage incorporation of selenomethionine (SeMet)[1] in place of methionine in the protein of interest [2,3].

The incorporation of SeMet in recombinant proteins presents a number of obstacles. First, most strains of *Escherichia coli* can synthesize methionine de novo, hence, either the use of methionine auxotrophs or the

☆ This work was supported by the National Institutes of Health, Protein Structure Initiative Grant P50 GM-64598.

* Corresponding authors. Fax: +1 608 262 3453.
*E-mail addresses:* sreenath@biochem.wisc.edu (H.K. Sreenath), bgfox@biochem.wisc.edu (B.G. Fox).

[1] *Abbreviations used:* $OD_{600}$, optical density at 600 nm; PET, polyethylene terephthalate; PCR, polymerase chain reaction; SeMet, selenomethionine; TB, Terrific broth.

1046-5928/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2004.12.022

feedback inhibition of methionine biosynthesis during cell growth and protein expression is required to achieve high-level incorporation [2,3]. Second, due to the substitution of SeMet into all cellular proteins and *S*-adenosylmethionine (presumably leading to toxic side effects), most cells grow slowly in the presence of SeMet and do not easily resume log phase growth after entering stationary phase [2–4]. Moreover, auxotrophic strains undergo lysis upon depletion of either Met or SeMet [5]. Consequently, cell mass yields and levels of total protein expression are often decreased in a chemically defined medium containing SeMet [3,6]. Third, SeMet proteins can undergo post-translational oxidations [7], demanding appropriate protective measures during cell growth, harvest, and protein purification. These problems are only exacerbated in the production environments envisaged to support high-throughput structural biology, where reproducibility of the timing and yield of process are important constraints on all operational protocols.

Polyethylene terephthalate (PET) 2-L beverage bottles were introduced as a convenient bacterial culture vessel [6,8]. As a consequence of their construction, these bottles provide sterility, efficient aeration, and achievement of sufficient cell densities required for the expression of recombinant proteins. The bottle growth approach has been previously investigated in the high-throughput production of SeMet-labeled proteins for X-ray studies [6,9,10] and of $^{13}C$–$^{15}N$ labeled proteins for NMR studies [11] using IPTG as a gratuitous chemical inducer of protein expression. In both cases, high percentages of isotopic incorporation were obtained. However, as these protocols required various additions and manipulations throughout the growth cycle, they were labor intensive.

Recently, Studier has introduced the auto-induction approach for production of labeled proteins for structural studies [12–14]; [14] is available at www.novagen.com. As alternatives to the batch induction reported in two previous efforts using PET bottles [6,11], this work and the following [15] provide results from application of the auto-induction strategy to the production of labeled proteins for structural studies. Our use of conditional methionine auxotropy in *E. coli* B834 and the adoption of the auto-induction process gave reproducible and rapid growth during scale-up, and corresponded to the predicted elimination of time-consuming and imprecise determinations of the appropriate time for culture induction. Results are presented from application of this protocol to greater than 150 target proteins.

## Materials and methods

### *Chemicals*

Unless otherwise stated, bacterial growth reagents, antibiotics, routine laboratory chemicals, and disposable labware were from Sigma–Aldrich (St. Louis, MO), Fisher (Pittsburgh, PA), or other major distributors. L-SeMet was from Acros (Morris Plains, NJ). Preparations of standard laboratory reagents were as previously described [16]. The 2-L PET beverage bottles used for bacterial cell growth were from Ball Corporation (Chicago, IL).

### *Plasmids*

The pVP13-GW expression vector used for these studies was derived from pQE80 (Qiagen, Valencia, CA) to produce an *N*-terminal fusion protein consisting of *S*-tag [17], maltose binding protein [18], and $His_6$ followed by a linker region containing the TEV protease site contiguous with the second residue of the target protein [19]. The Gateway *attB* recombination sites (Invitrogen, Carlsbad, CA), which reside in the linker region before the TEV recognition site, were also incorporated into the plasmid sequence. The codon adaptation plasmid pLacI RARE is from Novagen (Madison, WI).

### *Bacterial strain*

*Escherichia coli* B834 (Novagen) was used as the Met auxotroph for labeling. This strain likely has a mutation in *metE*, and thus requires either methionine or vitamin $B_{12}$ for growth [13]. The cells were made competent by the Z-Competent (Geno Technology, St. Louis, MO) method and then transformed with pLacI RARE (Novagen). The pLacI RARE transformants were also made competent by the same method.

### *Stock solutions*

The recipes and rationale for preparation of the auto-induction medium are the work of Studier [12–14]. All medium components were prepared using distilled and deionized water, and were either filter-sterilized (0.2, 0.5, and 0.75 μm filters, Nalgene, Rochester, NY) or heat-sterilized as indicated. This work describes how we have prepared and used the auto-induction method.

The 20× NPS solution contained 66 g $(NH_4)_2SO_4$, 136 g $KH_2PO_4$, and 142 g $Na_2HPO_4$ per L. This preparation was prepared fresh and heat-sterilized. As a precautionary measure, reagents with precipitation were discarded.

The 50 × 5052 solution was prepared by the combination of 250 g glycerol (0.5% w/v), 730 mL water, 25 g glucose (0.05% w/v), and 100 g α-lactose (0.2% w/v). The constituents were added in the specified order, stirred until dissolved, and the final volume was adjusted to 1 L. Since α-lactose dissolved slowly at room temperature, gentle heating in a microwave oven for 2–3 min was used to speed up the preparation. This solution was prepared fresh, heat-sterilized, used for 1 week, and then discarded as a precautionary measure.

The 50× amino acid mixture was prepared fresh before each use from 10 g each of sodium glutamate, lysine–HCl, arginine–HCl, histidine–HCl, free acid of aspartic acid, and zwitterionic forms of alanine, proline, glycine, threonine, serine, glutamine, asparagine, valine, leucine, isoleucine, phenylalanine, and tryptophan in a total volume of 1 L. Note that aspartic acid and tryptophan dissolved slowly, and that cysteine, tyrosine, and methionine were not included in the mixture. After the amino acids were completely dissolved, the solution was filter-sterilized, wrapped in aluminum foil, and stored at 25 °C until use.

The 10,000× trace metals solution was prepared as a 100 mL aliquot containing 50 mL of 0.1 M $FeCl_3 \cdot 6H_2O$ dissolved in ~0.1 M HCl, 2 mL of 1 M $CaCl_2$, 1 mL of 1 M $MnCl_2 \cdot 4H_2O$, 1 mL of 1 M $ZnSO_4 \cdot 7H_2O$, 1 mL of 0.2 M $CoCl_2 \cdot 6H_2O$, 2 mL of 0.1 M $CuCl_2 \cdot 2H_2O$, 1 mL of 0.2 M $NiCl_2 \cdot 6H_2O$, 2 mL of 0.1 M $Na_2MoO_4 \cdot 5H_2O$, 2 mL of 0.1 M $Na_2SeO_3 \cdot 5H_2O$, and 2 mL of 0.1 M $H_3BO_3$. All stock solutions of individual metals except acidified $FeCl_3 \cdot 6H_2O$ were heat-sterilized and stored at 25 °C until use. The trace metals solution was wrapped in aluminum foil and stored at 25 °C.

The 1000× vitamins solution was prepared as a 100 mL aliquot containing 2 mL of 10 mM nicotinic acid, 2 mL of 10 mM pyridoxine–HCl, 2 mL of 10 mM thiamine–HCl, 2 mL of 10 mM *p*-aminobenzoic acid, 2 ml of 10 mM pantothenate, 5 mL of 100 μM folic acid, 5 mL of 100 μM riboflavin, and 80 mL sterile water. For the vitamins solution used for preparation of the scale-up inoculum, 4 mL of 5 mM vitamin $B_{12}$ solution was added. Note that a different vitamins solution lacking vitamin $B_{12}$ must be used to prepare the medium used for large-scale growth and expression. The vitamins solution was prepared fresh each week, wrapped in aluminum foil, and stored at 25 °C until use.

The 1000× ampicillin solution was made by dissolving 10 g ampicillin in 50 mL water, and then combined with 50 mL of absolute ethanol. The solution was filter sterilized and stored at 4 °C until use. The 1000× chloramphenicol solution was made by dissolving 3.4 g chloramphenicol in 100 mL of absolute ethanol and stored at 4 °C. The 1 M IPTG solution was made by dissolving 23.8 g IPTG into 100 mL water, filter-sterilized, and stored at 4 °C until use. A 40% glucose solution was prepared by adding 40 g glucose to water to give a final volume of 100 mL, thoroughly mixed, and heat-sterilized. This solution was prepared each week. An 80% (w/v) glycerol solution was prepared by dilution of 65.5 mL (80 g) of neat glycerol to a final volume of 100 mL, thoroughly mixed, and heat-sterilized. This solution was prepared each week. The methionine solution (25 mg $mL^{-1}$) was made by dissolving 2.5 g methionine in 100 mL water, heat-sterilized, wrapped in aluminum foil, and stored at 4 °C. The SeMet solution (25 mg $mL^{-1}$) was made by dissolving 2.5 g of SeMet in 100 mL water, filter-sterilized, wrapped in aluminum foil, and stored at 4 °C.

*Growth and expression in SeMet-labeled medium*

The PA-0.5G medium (100 mL) was assembled from the following sterile components: 92 mL water, 100 μL of 1 M $MgSO_4$, 10 μL of the 10,000× trace metals solution, 1.25 mL of 40% glucose, 5.0 mL of the 20× NPS solution, 1.0 mL of 50× amino acids mixture, 0.4 mL of the L-methionine solution, 100 μL of the 1000× vitamins solution including vitamin $B_{12}$, 100 μL of the 1000× ampicillin solution, and 100 μL of the 1000× chloramphenicol solution. This liquid medium was used for the scale-up inoculum. For PA-0.5G plates, 10 g × $L^{-1}$ of agar was added to above components.

Note that the medium compositions we have used for the scale-up inoculum and the large-scale growth and expression are different.

The PASM-5052 medium (1 L) was assembled from the following sterile components: 900 mL water, 1 mL of 1 M $MgSO_4$ solution, 100 μL of the 10,000× trace metals solution, 20 mL of the 50 × 5052 solution, 50 mL of the 20× NPS solution, 20 mL of the 50× amino acids mixture, 0.4 mL of the L-Met solution, 5 mL of the L-SeMet solution, 1 mL of the ampicillin solution, 1 mL of the chloramphenicol solution, and 1 mL of the 1000× vitamins solution lacking vitamin $B_{12}$. The $MgSO_4$ and trace metals solutions were added and well-mixed with water before the 20× NPS solution was added to avoid precipitate formation in both PA-0.5G and PASM-5052 media. This liquid medium was used for large-scale growth and auto-induction.

*Day 1*. A competent glycerol stock of *E. coli* B834 pLacI RARE (100 μL, with transformation efficiency of ~$5 \times 10^5$ cells $\mu g^{-1}$ plasmid DNA) was thawed on ice, 2 μL pVP13-GW expression plasmid DNA was added, and the cells were left on ice for 1 h. The cells were heat shocked at 42 °C for 1 min (to increase the efficiency of transformation) and put back on ice for 5 min. About 200 μL of 37 °C SOC medium was added, and the cells were grown at 37 °C for 3 h on a microplate shaker. The cell mixture was plated onto a PA-0.5G agar plate containing ampicillin (100 μg $mL^{-1}$) and chloramphenicol (35 μg $mL^{-1}$) using sterile glass beads and grown overnight at 37 °C.

*Day 2*. One to three colonies from an overnight transformation were picked from a PA-0.5G plate after 24 h using a sterile inoculation loop, transferred to a 10 mL test tube containing 3 mL PA-0.5G medium, and incubated at 37 °C with shaking at 300 rpm for 7–8 h. This 3 mL culture, pre-incubated at 25 °C for 30 min, was used to inoculate a flask containing 100 mL PA-0.5G medium at 25 °C with shaking at 300 rpm and incubated for 18 h.

*Day 3*. After 18 h, 20 mL aliquots of the 100 mL culture were used to inoculate four 2-L PET beverage

bottles containing 480 mL PASM-5052 medium. The bottles were loosely sealed with aluminum foil and placed into a New Brunswick C25KC refrigerated shaker (New Brunswick Scientific, New Brunswick, NJ) equipped with a custom 24-hole aluminum rack (Fig. 1). The tight fit of the thin-walled PET bottles in this rack helped to prevent mechanical abrasion that could lead to bottle failure at elevated shaking rates over the time

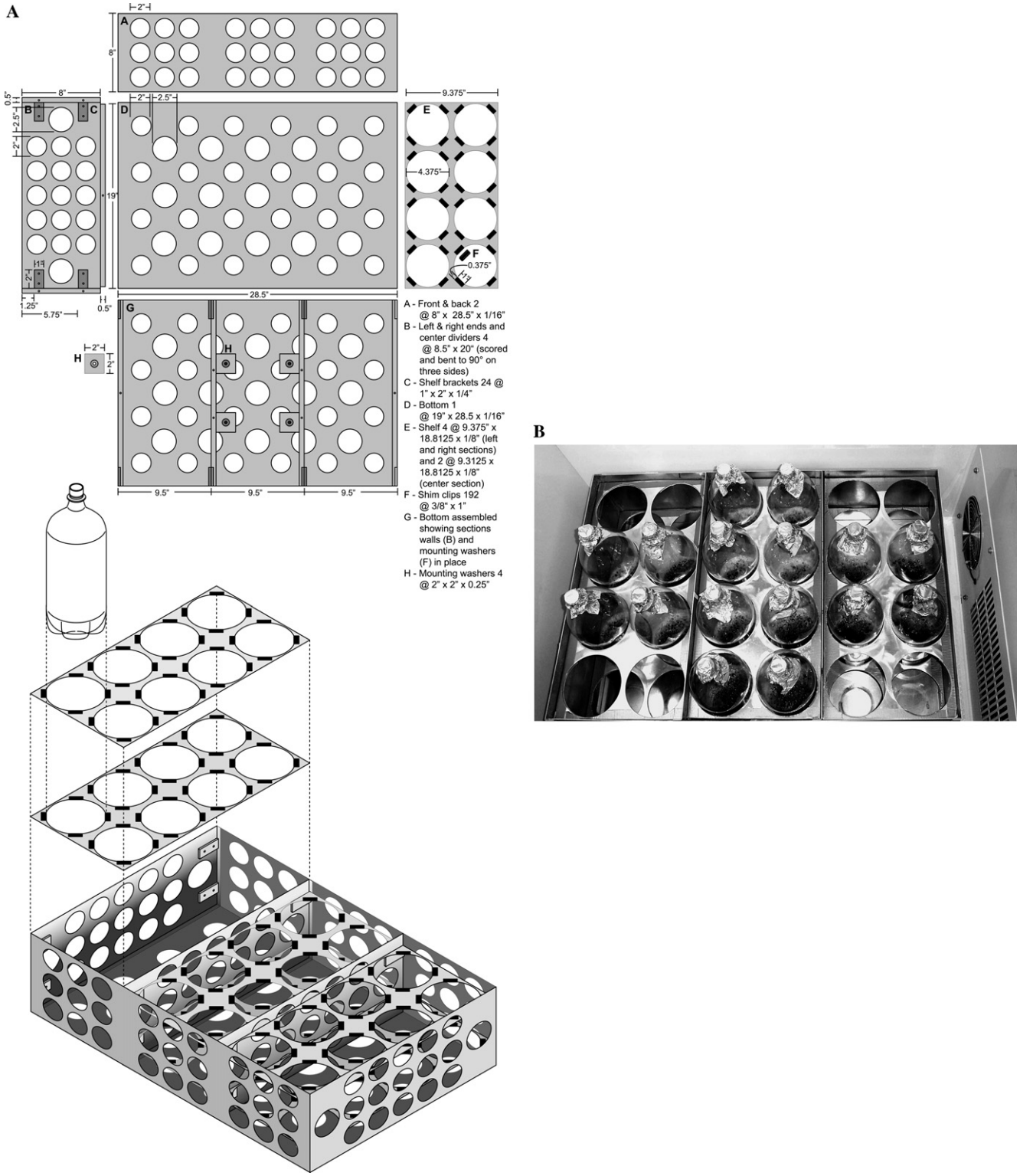


Fig. 1. (A) Schematic drawing of a modified 24-hole aluminum shaker platform. (B) A set of sixteen 2-L PET bottles secured in the modified platform in a New Brunswick C25KC series refrigerated floor shaker.

period required for the culture growth. Up to 24 culture bottles could be incubated at 25 °C with shaking at 250 rpm for the ~24 h required for growth.

*Day 4*. The cultures were centrifuged at 5000*g* for 20 min in a JS 4.2 rotor and an Avanti J-HC centrifuge (Beckman Coulter, Fullerton, CA). The supernatant was treated for 30 min with 20% (v/v) bleach solution and disposed into the sanitary sewer. The cell pellet was resuspended in 30 mL of 50 mM $NaH_2PO_4$, pH 7.4, containing 0.5 M NaCl and 20% (w/v) ethylene glycol, and centrifuged at 5000*g* for 20 min. The washed cell pellet was weighed and stored in ~15 g samples at −84 °C. The empty PET bottles were crushed, heat-sterilized, and disposed.

*Growth and expression in unlabeled medium*

Terrific broth (TB) medium was prepared by mixing 48 g dry TB powder (Sigma–Aldrich, St. Louis, MO) with 1 L water and sterilized by heat. For plates, 10 g $L^{-1}$ of agar was added to the PA 0.5G medium before sterilization. Glycerol (80% w/w) was autoclaved separately. After cooling, 10 mL of the 80% glycerol solution, 1.0 mL ampicillin solution, and 1.0 mL chloramphenicol solution were added and thoroughly mixed into the medium.

Cells from a fresh transformation were transferred from a 24-h-old PA-0.5G plate into 50 mL TB medium in a 250 mL Erlenmeyer flask. The culture was grown at 37 °C with shaking at 300 rpm for 3 h. A 50 mL aliquot of the culture was inoculated into 450 mL TB medium in one 2-L PET beverage bottle loosely sealed with aluminum foil. The culture was incubated at 37 °C with shaking at 300 rpm in the refrigerated shaker and custom rack described above. The growth was continued for ~2 h until the $OD_{600}$ reached 1.0–1.5. The culture was induced by addition of 500 μL IPTG solution and the incubation was continued at 20 °C with shaking at 250 rpm for 20 h.

*Analysis of expression, solubility, and proteolysis*

Cell growth was monitored by $OD_{600}$ measurements using the culture medium as the spectrophotometer blank. Samples of the cell pellet (~0.1 g) were suspended in 0.5 mL of 50 mM $NaH_2PO_4$, pH 7.4, containing 0.5 M NaCl and 20% (w/v) ethylene glycol, and saved for examination of total expression, solubility, and proteolysis. A 150 μL aliquot of the cell suspension was mixed with 50 μL of a 1 mg $mL^{-1}$ lysozyme solution (prepared in 50 mM Tris–HCl, pH 7.5, containing 50% (v/v) glycerol, 0.1 M NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.1% (v/v) Triton X-100) and incubated for 1 h at room temperature. A 15 μL aliquot of the cell lysate was suspended in 85 μL of 15 mM $NaH_2PO_4$, pH 7.5, containing 0.1 M NaCl in a strip tube and sonicated in a Misonix 3000 plate sonicator (Farmingdale, NY) at 160 W and 4 °C for 10 min with a duty cycle of 1 min on, 15 s off. A 40-μL aliquot of the sonicated sample was retained for "total protein (T)" analysis and 60 μL of the sample was centrifuged at 13,200*g* for 10 min. Twenty microliters of supernatant was saved as the "soluble protein (S)" sample, and the pellet was saved as the "insoluble pellet (P)" sample. The total protein sample was mixed with 160 μL of SDS buffer (0.49 g Tris–HCl, pH 6.8, containing 1.0 g SDS, 12.5 mL glycerol, 5 mg bromophenol blue, 2.5 mL β-mercaptoethanol, and water to 50 mL) and heated at 95 °C for 12 min. The pellet sample was suspended in 60 μL water and 240 μL SDS buffer prior to heating at 95 °C for 12 min. For the "protease cleavage (C)" sample, 20 μL of the soluble protein sample was incubated with 1 μL TEV protease (1 mg $mL^{-1}$) at 30 °C for 3 h. Both soluble protein (S, 20 μL) and protease cleavage (C, 20 μL) samples were mixed with 80 μL of the SDS buffer and heated at 95 °C for 12 min.

The low-molecular-weight range protein markers (Bio-Rad, Hercules, CA) were prepared in SDS buffer to give each marker band a concentration of ~1 μg $μL^{-1}$. Samples of the protein markers (8 μL in lane 1 and 2 μL in lane 2) were loaded into precast 4–20% Criterion Tris–HCl gradient gel (Bio-Rad) followed by other protein samples and electrophoresed for 50 min at 200 V. The gel was stained in 0.015% Coomassie blue-250 solution for 45 min and destained until protein bands were distinguished. The image was captured using a Fotodyne imaging system (Hartland, WI) and saved as a high resolution JPEG file.

Fig. 2 shows a representative gel used to score total protein expression, solubility, and proteolysis of the expressed fusion protein. This scoring is based on visual comparison with the stained intensity of bovine serum albumin (66.2 kDa), which is present at ~8 μg in stan-

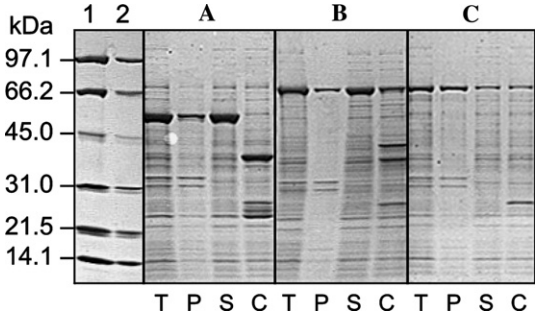


Fig. 2. Analysis of total protein expression, solubility, and proteolysis of fusion protein targets using denaturing gel electrophoresis: A, AK099872; B, AK072216; and C, AK065519. The marked lanes were used to evaluate total expression (*T*), soluble expression (*S*), insoluble or pellet expression (*P*), and TEV proteolysis (*C*). Protein molecular weight markers were loaded in the first two lanes (8 μL in lane 1 and 2 μL in lane 2). In this figure, (A) is an example of a scoring of high total protein expression, high solubility, and high percentage of proteolysis (H, H, and H scoring), (B) is an example of H, H, and M scoring, and C is an example of M, M, and W scoring.

dard lane 1 and ~2 μg in standard lane 2. If the target protein band (~45–80 kDa) was more intense than bovine serum albumin band in lane 1, the total expression was scored high, "H." If the intensity of the target protein was between that of the marker bands in lanes 1 and 2, it was scored medium, "M," and if the intensity of the target protein was less than that of the marker band in lane 2, it was scored weak, "W." Similar assessments were made for the lanes containing the soluble fraction, the pellet fraction, and the TEV protease-treated soluble fraction.

*Purification and determination of SeMet incorporation*

Cells (1 g) were thawed, suspended in 5 mL of 50 mM $NaH_2PO_4$, pH 7.4, containing 0.5 M NaCl, 20% (w/v) ethylene glycol and 35 mM imidazole, and lysed by sonication. The sonicated cells were clarified by centrifugation and the recombinant fusion protein was purified by IMAC in a linear 0–500 mM imidazole gradient. The purified fusion protein was desalted into 20 mM $NaH_2PO_4$, pH 7.5, containing 100 mM NaCl, and reacted with tobacco etch virus (TEV) protease (prepared in-house and containing a $His_6$-tag) overnight at 25 °C. The proteolyzed target was subjected to subtractive IMAC chromatography, and the pure target was desalted into 5 mM Hepes, pH 7.0, containing 50 mM NaCl and concentrated. The identity of the protein and the percentage of SeMet incorporation were determined using a Sciex API 365 triple quadrupole mass spectrometer (Perkin–Elmer, Boston, MA) maintained at the University of Wisconsin Biotechnology Center.

## Results

*Bacterial hosts and expression plasmid*

*Escherichia coli* B834 is widely used for SeMet labeling [2]. This strain has a conditional Met auxotrophy likely arising from disruption of *metE*, which encodes a vitamin $B_{12}$-independent methionine synthase (EC 2.1.1.14) [13,20–22]. The *E. coli* B834 also contains a vitamin $B_{12}$-dependent methionine synthase (*metH*, EC 2.1.1.13) but, as with most *E. coli* strains, is unable to synthesize vitamin $B_{12}$ [13]. Upon addition of vitamin $B_{12}$ to the chemically defined growth medium, complementation of the vitamin $B_{12}$-dependent methionine synthase allows growth on a medium lacking methionine. The auto-induction protocols can take advantage of this conditional auxotrophy to promote rapid growth during culture scale-up but also to provide high-level incorporation of SeMet during large-scale growth and protein expression.

The B834 strain was transformed with pLacI RARE to compensate for codon usage [23–26], and also transformed with pVP13, a plasmid created at the University of Wisconsin Center for Eukaryotic Structural Genomics for expression of recombinant fusion proteins [19]. The simultaneous presence of the codon adaptation and protein expression plasmids conferred resistance to chloramphenicol and ampicillin, respectively. In the PASM5052 auto-induction medium, the specific growth rates of *E. coli* B834 and *E. coli* BL21 (a commonly used prototrophic expression host) were comparable (~0.14 $h^{-1}$ at 25 °C).

The pVP13 expression plasmid was derived from pQE80, which contains the viral T5 promoter under control of tandem copies of the *lac* operator. *E. coli* RNA polymerase recognizes the T5 promoter [27], hence control of expression is achieved by repression of transcription. This control is provided during growth in a medium lacking a synthetic inducer such as IPTG, and can be enhanced by catabolite repression from preferred carbon sources such as glucose. Arising from the presence of the *lac* operator, α-lactose can serve as an inducer of expression in the appropriate host strains upon depletion of other carbon sources [5,28–30].

*Comparison of growth and expression in unlabeled and SeMet-labeling media*

Table 1 shows a comparison of the $OD_{600}$ values and protein expression characteristics obtained for 21 target proteins from *Arabidopsis* (protein designators beginning with At) and rice (protein designators beginning with AK). For TB medium, a single 0.5-L culture was grown as a comparator for $OD_{600}$ values and characteristics of protein expression. For PASM-5052 medium, four replicate 0.5-L cultures were grown. The final $OD_{600}$ values obtained in TB medium were ~2-fold higher than the $OD_{600}$ values obtained with the chemically defined PASM-5052 medium. This difference in $OD_{600}$ also generally tracked the difference in the yield of wet cell mass obtained per L of culture medium at the end of the growth.

Table 1 also shows results of the assignment of total protein expression, solubility, and proteolysis of the *S*-Tag-$His_6$-MBP-target fusion proteins using the protocols given under Materials and methods. Overall, the pattern of protein expression, solubility, and proteolysis observed in PASM-5052 medium was comparable to that observed with the TB medium. A more detailed statistical analysis of this comparison will be presented elsewhere.

Fig. 2 shows representative gels demonstrating the scoring assignment. In Fig. 2A, the AK099872 fusion protein had high total expression, high solubility, and high percentage of cleavage by TEV protease. Thus, the intensity of the AK099872 fusion protein band in lane T was greater than that observed from 8 μg of the bovine serum albumin band in lane 1. The AK099872 fusion also had high solubility as determined by comparison of

Table 1
Comparison of results from growth and expression of proteins from TB medium with IPTG induction and PASM-5052 medium with α-lactose auto-induction and SeMet labeling

| Protein designator | TB medium[a] | | | | PASM-5052 Medium[b] | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $OD_{600}$ | Exp[c] | Sol | Clv | $OD_{600}$ | Cells (g)[d] | Exp[c] | Sol | Clv |
| AK065519 | 8.2 | H | — | — | 7.7 | 14.4 | M | W | W |
| AK065635 | 10.9 | — | — | — | 3.7 | 9.4 | W | H | — |
| AK067283 | 12.7 | — | — | — | 4.9 | 16.0 | — | — | — |
| AK067669 | 8.4 | H | — | — | 4.6 | 10.1 | M | M | — |
| AK072216 | 11.3 | H | H | M | 3.3 | 10.7 | H | H | M |
| AK099872 | 7.7 | H | H | H | 6.8 | 12.8 | H | H | H |
| AK104879 | 12.6 | H | H | H | 5.6 | 12.6 | M | H | H |
| AK104912 | 9.1 | H | W | — | 3.5 | 9.2 | M | M | — |
| AK105632 | 8.7 | H | — | — | 5.1 | 9.0 | M | W | W |
| AK111505 | 12.9 | M | H | H | 7.6 | 13.6 | M | W | H |
| AK111570 | 10.2 | H | H | H | 5.8 | 13.2 | M | H | H |
| At1g06680.1 | 11 | H | H | H | 7.6 | 15.5 | H | H | H |
| At3g17820.1 | 13.9 | H | H | W | 4.8 | 13.2 | M | H | W |
| At3g21360.1 | 10.2 | H | H | H | 7.6 | 19.1 | M | M | H |
| At4g22530.1 | 12.3 | H | H | H | 6.9 | 14.3 | H | H | H |
| At4g34360.1 | 22.6 | H | H | H | 9.4 | 22.0 | M | H | H |
| At5g07680.1 | 12.0 | H | H | H | 5.2 | 12.0 | M | H | H |
| At5g09350.1 | 9.2 | H | H | H | 7.6 | 16.3 | H | H | H |
| At5g36840.1 | 9.4 | H | H | H | 9.4 | 17.0 | H | M | W |
| At5g37415.1 | 20 | M | W | H | 6.0 | 13.2 | — | — | — |
| At5g62780.1 | 13.0 | H | H | H | 7.2 | 16.6 | H | H | H |
| Average[e] | $11.7 \pm 3.7$ | | | | $6.2 \pm 1.8$ | $13.8 \pm 3.3$ | | | |

[a] The 500 mL growth in TB medium was conducted for 22 h at 20 °C with shaking at 250 rpm. The amount of cell paste was not determined.
[b] The SeMet growth was conducted in four bottles with each containing 500 mL of PASM-5052 medium and 125 mg $L^{-1}$ SeMet for 24 h at 25 °C with shaking at 250 rpm.
[c] The abbreviations used for the scoring system are H, high; M, medium; W, weak; and —, no expression detected. Definitions and method used for the scoring are given under Materials and methods.
[d] Cell paste obtained from 2-L of culture medium.
[e] An average cell mass of 27 g was obtained from 2-L of culture medium in 148 expression trials using the TB medium, while an average cell mass of 14.9 g was obtained from 2-L of culture medium in 173 trials using the auto-induction method with SeMet-labeling.

lane P, containing the pellet fraction, and lane S, containing the soluble fraction with the marker bands. In this case, the fusion protein was found almost entirely in the soluble fraction after sonication and fractionation by centrifugation. The AK099872 target was also efficiently proteolyzed from the intact fusion protein as comparison of lane S and lane C showed the loss of the fusion protein after treatment with the protease and the appearance of MBP ($M_r$ ~45 kDa) and the target protein ($M_r$ ~26 kDa). Fig. 2B shows that another example AK072216 had high total expression and high solubility, but medium cleavage by TEV protease. Fig. 2C shows that AK065519 had medium level of total expression, weak solubility, and weak cleavage by TEV protease.

*Routine use of auto-induction medium*

The scale-up inoculum was grown in PA-0.5G medium, which contained low amounts of Met, vitamin $B_{12}$, and other amino acids, but no α-lactose. This medium allowed for rapid, reproducible growth of the host cells to low density in the scale-up phase of culturing without induction of the target protein. The two-step scale-up (single colony into 3 mL and 3 mL into 100 mL) required ~24 h to provide a final $OD_{600}$ of 5–6 in the final scale-up inoculum. The starting OD in the large-scale culture was ~0.2.

For the large-scale growth and expression, the medium was changed to PASM-5052. The PASM-5052 medium contained a 9.4-fold molar excess of SeMet relative to Met and no additional vitamin $B_{12}$ other than carry-over from the scale-up inoculum. The PASM-5052 medium also contained glucose and glycerol as carbon sources, and α-lactose as both a carbon source and an inducer. Fig. 3 shows a time course of the growth and auto-induction in PASM-5052 as detected by denaturing gel electrophoresis. The cell density of the culture increased for ~15 h as glucose was preferentially consumed in the early phase of the culture growth. Expression could be clearly detected after ~15 h, corresponding to the depletion of glucose. The culture growth was continued for another ~9 h under inducing conditions, while both glycerol and α-lactose were utilized as a carbon source. The PASM-5052 cultures reached an $OD_{600}$ of ~6 and yielded ~14 g of cell paste (Table 1) from the 2-L culture volume after 24 h. For comparison, growths in TB medium gave an average $OD_{600}$ of ~12 (Table 1) with an average of ~27 g of wet cells obtained from greater than 150 trials.

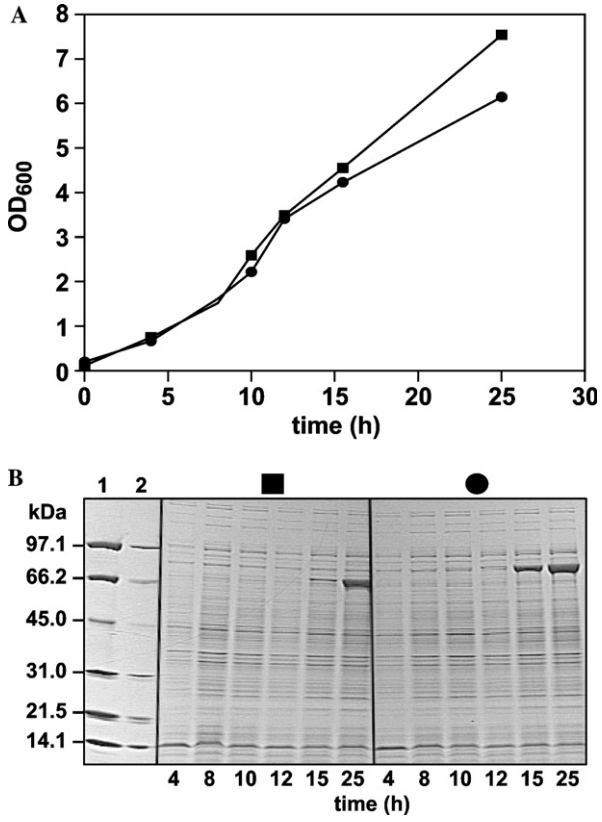


Fig. 3. A time course of growth and expression in PASM-5052 medium at 25 °C for 25 h. (A) Increase in $OD_{600}$ for At4g34215 (●) and At4g09670 (■). (B) SDS–PAGE analysis of protein expression of At4g34215 (●) and At4g09670 (■). The amount of total cell protein analyzed was normalized by $OD_{600}$ measurements. Both gels show visible accumulation of the target protein by ~15 h and received an expression scoring of high at 25 h.

### *Yield of protein from growths in PASM-5052 medium*

The scoring system elaborated in Table 1 and Fig. 2 was used to sort cell pastes for potential outcome upon protein purification. Table 2 shows representative results for purification of SeMet-labeled proteins from cells grown in PASM-5052 auto-induction medium. As a process standardization, all protein purifications were initiated on ~15 g wet of cell mass. The auto-induction method gave total yields of purified SeMet-labeled protein after proteolysis in the range of 2–70 mg. For cells scored to have high total expression, solubility, and cleavage by TEV protease, the most likely outcome from the purification was recovery of sufficient protein for advancement to crystallization trials (73 successful purifications out of 85 attempts, 86%). Table 2 shows representative results for this scoring class. In contrast, for cells given a weak score in any of the total expression, solubility, or TEV protease cleavage assessments, the most likely outcome from the purification was a failure to recover sufficient protein for advancement to crystallization trials. For example, from 155 cell pastes scored to have weak solubility, a protein was successfully purified from only 11 attempts (7%). Moreover, from 55 purifications with a weak cleavage score, only one purification was successful, albeit with a meager total yield of ~2 mg of protein. Further analysis of the application of this scoring scheme to protein purification efforts will be presented elsewhere.

### *SeMet labeling efficiency*

Table 3 and Fig. 4 show the effect of different concentrations of SeMet on the wet cell weight obtained and the percentage of SeMet incorporated into the purified protein. Although there was no apparent change in the level of protein expression, solubility, or protease cleavage as the SeMet concentration was increased (Fig. 4B), the growth rate and the cell mass yield decreased on average as the SeMet concentration was increased (Table 3 and Fig. 4A). Also on average, the highest final concentration of SeMet that did not significantly inhibit cell growth was 125 mg $L^{-1}$. Table 2 shows that the percentage incorporation of SeMet in a set of purified proteins grown in medium containing 125 mg $L^{-1}$ SeMet was 90 ± 6% as determined by ESI- and MALDI-MS. Moreover, Table 3 shows that for the target protein At5g11950, a ~8-fold variation in the SeMet concentration gave only a minor difference in the percentage incorporation of SeMet (95.8 ± 2.2%).

The PASM-5052 medium also contained vitamins other than $B_{12}$ and amino acids other than Met and SeMet. Replicate expression trials of a single protein were studied to determine the effect of changing the concentration of these medium additives on the cell yield and on the percentage incorporation of SeMet. A 4-fold variation in the level of vitamins (other than vitamin $B_{12}$) had only minor effects on the cell yield and expression (data not given). Furthermore, a 4-fold increase in the level of the other amino acids gave a ~2-fold increase in the cell yield at 24 h. However, the increased amount of amino acids was associated with a decrease in the percentage incorporation of SeMet. The origin of this decrease was not identified.

### *Effect of temperature on SeMet labeling*

Fig. 5 shows the average results from growth and expression of three different *Arabidopsis* targets at four different temperatures. For each target, the highest $OD_{600}$ was obtained after 24 h at 25 °C. Also for each target, the growths at 20 °C and 37 °C growths gave noticeably lower $OD_{600}$ values, but for apparently different reasons. In the 20 °C growth, the cultures exhibited slow exponential growth, while in the 37 °C growths, exponential growth was more rapid and led to a stationary culture after ~10 h. The growth at 30 °C was intermediate and apparently dependent on the target. After the 24 h culture period with cells grown at

Table 2
Cell mass, protein yield, and incorporation of SeMet from auto-induction medium

| Protein designator[a] | Cells used (g) | Exp | Sol | Clv | Purified protein (mg) | Protein yield (mg/g) | SeMet incorporation (%) |
|---|---|---|---|---|---|---|---|
| At1g01470 | 16.5 | H | H | H | 43 | 2.6 | 85 |
| At1g06000 | 18 | H | H | M | 1.5 | 0.08 | 95 |
| At1g27020 | 13.5 | H | H | H | 18.7 | 1.4 | 90 |
| At1g49820 | 21.0 | H | H | H | 68.6 | 3.3 | 92 |
| At1g70830.1 | 15.8 | M | H | H | 43.8 | 2.8 | 92 |
| At2g25570 | 16.2 | M | M | nd[b] | 3.1 | 0.19 | 80 |
| At2g34160 | 19.5 | H | H | H | 39.1 | 2.0 | 90 |
| At2g34160 | 21.3 | H | H | H | 22.8 | 1.1 | 85 |
| At2g35830 | 15.7 | H | H | H | 4.7 | 0.3 | 95 |
| At2g44060 | 20.8 | H | M | M | 18.6 | 0.9 | 85 |
| At2g45260 | 21.5 | H | M | nd | 15.4 | 0.71 | 95 |
| At2g45790 | 20.5 | H | H | H | 49.4 | 2.4 | 95 |
| At3g17820.1 | 16.6 | M | H | M | 19.3 | 1.2 | 92 |
| At3g22680 | 14.0 | H | nd | H | 36.0 | 2.6 | 90 |
| At5g01610 | 16.0 | H | M | H | 25.3 | 1.6 | 85 |
| At5g05060 | 18.5 | H | H | H | 32.2 | 1.7 | 90 |
| At5g16510 | 24.0 | H | H | H | 52.9 | 2.2 | 75 |
| At5g16510 | 18.5 | H | M | M | 49.2 | 2.7 | 90 |
| At5g16570 | 22.0 | M | W | H | 17.6 | 0.8 | 100 |
| At5g18200 | 18.2 | H | W | H | 6.4 | 0.4 | 96 |
| At5g44450.1 | 10.6 | H | M | H | 42.3 | 4.0 | 93 |
| Average[c] | $18.0 \pm 3$ | | | | $29 \pm 19$ | $1.7 \pm 1.1$ | $90 \pm 6$ |

[a] Large-scale cultures were grown in PASM-5052 medium containing 125 mg $L^{-1}$ SeMet for as described under Materials and methods.
[b] nd, not determined.
[c] The average purified protein yield of 39.5 mg was obtained from 173 protein purification trials using cells grown with the auto-induction method and SeMet-labeling. The range of protein yield was 0.2–184 mg.

Table 3
Effect of SeMet concentration on the percentage incorporation of SeMet

| SeMet (mg $L^{-1}$)[a] | Cell yield (g $L^{-1}$) | SeMet labeling (%) |
|---|---|---|
| 62.5 | 9.9 | 92.8 |
| 125 | 9.6 | 95.6 |
| 250 | 6.6 | 97.1 |
| 500 | 8.2 | 97.7 |

[a] Cultures expressing At5g11950 in PASM-5052 medium with the indicated amount of SeMet were grown for 24 h at 25 °C with shaking at 250 rpm.

25 °C and 30 °C, the expression, solubility, and protease cleavage were scored as follows for At1g62620 (high, medium, and high), At4g15900 (weak, high, and high), and At4g38540 (high, high, and high). For comparison, these three targets were scored to have weak to negative expression, solubility, and protease cleavage when prepared from cells grown at either 20 or 37 °C after the 24 h culture period.

*Effect of agitation on growth in PASM-5052 medium*

A 500 mL volume of PASM-5052 medium was found to give the best match between required cell mass yield, protein expression, and available laboratory equipment for SeMet labeling. However, higher cell densities could be achieved when smaller volumes of the auto-induction medium were used in the 2-L PET bottles, suggesting that aeration might have an influence on cell growth and expression. Fig. 6 shows that increased agitation of the 500 mL expression cultures for three different expression targets also provided better cell growth. Thus, cell cultures grown with shaking at 250 rpm reached $OD_{600}$ of ~6 and yielded about ~14 g of cell paste (Table 1), while cell growth with shaking at 350 rpm gave a ~1.6-fold increase in $OD_{600}$ and corresponding increase in the mass of cell paste. Overall, the total expression, solubility, and proteolysis characteristics were similar with the higher agitation, which indicates a potential pathway to improvements in process yield.

## Discussion

*Host strain*

The ability of *E. coli* B834 in PASM-5052 to grow with added Met and/or vitamin $B_{12}$ was used to advantage to promote rapid and reproducible growth in the scale-up inocula developed from single colonies. Upon transfer to the large-scale growth medium, the contribution of Met and vitamin $B_{12}$ was minimized by dilution and by the presence of excess SeMet. The growth rate observed in the large-scale culture was dependent on SeMet concentrations, with 62–125 mg $L^{-1}$ of SeMet identified to give faster cell growth without compromising the percentage incorporation.

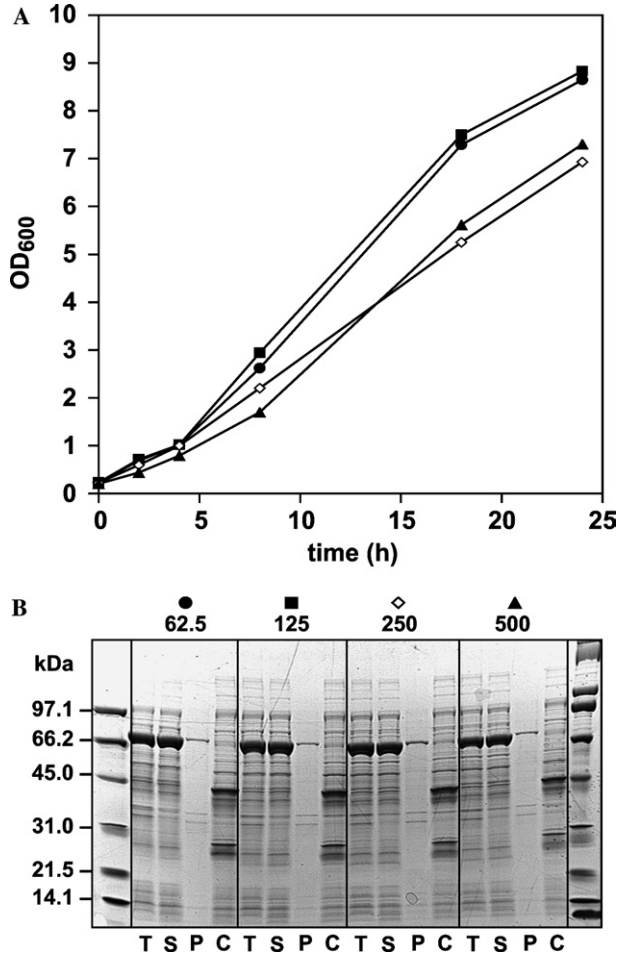


Fig. 4. Effects of increasing SeMet concentration on the growth of *E. coli* B834 expressing pVP13-At5g11950. The cultures were grown at 25 °C with shaking at 250 rpm for 24 h. (A) Growth curves. The SeMet concentrations were (mg $L^{-1}$): 62.5 (●); 125 (■); 250 (◇); and 500 (▲). (B) Denaturing gel analysis: lane 1, 62.5; lane 2, 125; lane 3, 250; and lane 4, 500.

*Routine use*

The auto-induction medium developed by Studier served as the basis for these growths [12–14]. For this work, vitamin $B_{12}$ (other than carry-over from scale-up) was excluded from the PASM-5052 medium used for our large-scale production without apparent adverse effects on cell growth or protein expression. The protocols reported here balance the performance of bacterial host strains and plasmid expression vectors with a time-scheduled process for labeling with SeMet. The results represent greater than 150 applications of this labeling protocol. The process goal was to provide between 10 and 20 g of wet cell weight from a 2-L growth, and to obtain 10 mg or greater of purified protein from 15 g of cell paste. Table 1 compares results of 0.5-L scale growths for expression testing in TB medium and application of this method at the 2-L scale for production of SeMet-labeled proteins. The $OD_{600}$-values obtained with the TB medium were 1.5- to 5-fold higher than with the SeMet-labeling medium, and this result generally corresponded to the amount of wet cell mass obtained. Nevertheless, although the cell mass obtained per unit volume was lower in the SeMet-labeling

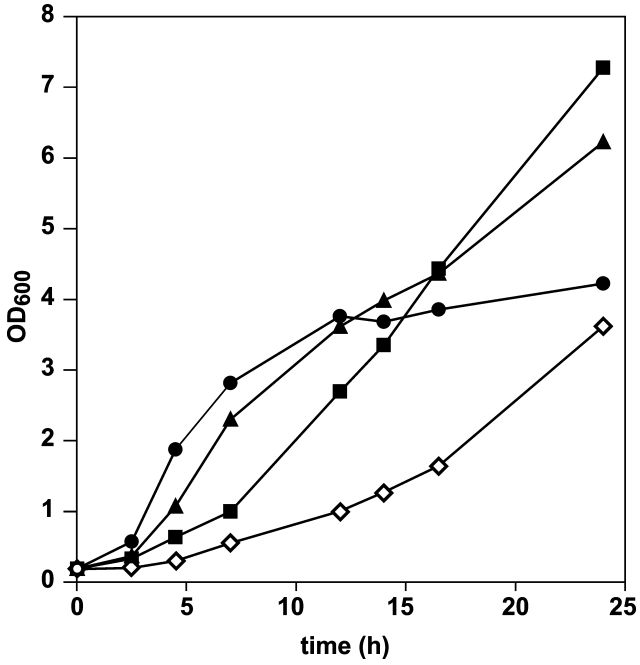


Fig. 5. Effect of temperature on the growth of *E. coli* B834 expressing At4g19003 from pVP13. The growth temperatures were 20 °C (◇), 25 °C (■), 30 °C (▲), and 37 °C (●).

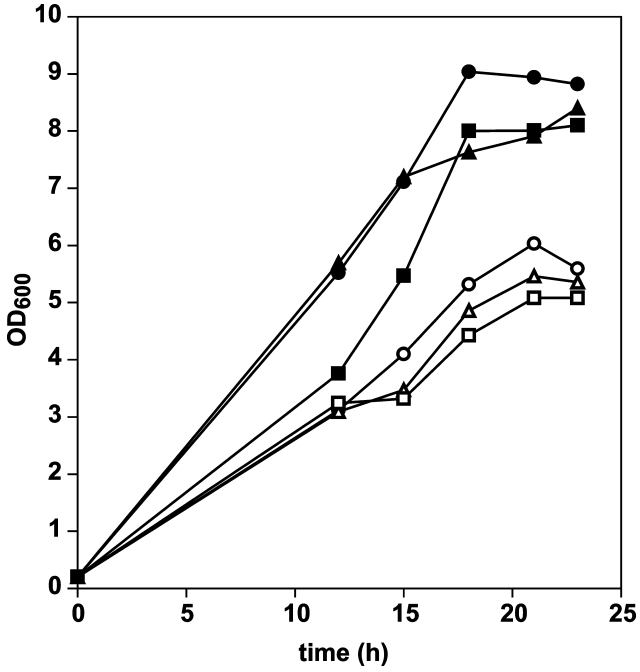


Fig. 6. Effect of agitation on the growth of *Arabidopsis* targets in PASM-5052 medium. At3g28950 (○ 250 rpm; ●] 350 rpm), At4g19003 (△ 250 rpm; ▲ 350 rpm), and At1g22270 (□ 250 rpm; ■ 350 rpm), and were grown at 25 °C for 23 h.

medium as compared to TB medium, the target cell mass (~15 g) was routinely obtained with the SeMet-labeling medium. Other scales of operation would be possible by suitable modification of this protocol.

Table 1 also indicates that protein expression, solubility, and proteolysis were roughly correlated for cells grown in either the TB medium or the chemically defined SeMet-labeling medium. This correlation would allow pre-screening of target genes before attempting growth in SeMet labeling medium. In practice, pre-screening may not present significant advantage, however, as the cost and labor of evaluating target properties using TB medium is only incrementally lower than with the SeMet-labeling medium. Moreover, as structure determinations require the preparation of SeMet-labeled protein, and as SeMet-labeled proteins may crystallize in slightly different conditions than the unlabeled protein, there may be significant logistic advantages to production of SeMet-labeled proteins as the first-pass effort in structural genomics. This work clearly supports the feasibility of this type of first-pass approach. Moreover, for expression trials given the most favorable scoring, the average yield of purified, SeMet-labeled protein was ~30 mg (Table 2). This result also met the originally established goal.

Increased agitation gave a substantial increase in cell mass. However, to achieve the higher agitation rate, it was not possible to fully load the NBS shaker with 24 bottles. Instead, only 16 bottles could be shaken (see Fig. 1) without instrument shut-off due to overheating before the 24 h growth cycle could be completed. This compromise in throughput performance can presumably be overcome by improved engineering of the shaker to accommodate to the loading demands of the PET bottles and custom shaker rack.

*Scoring of expression, solubility, and proteolysis*

The visual scoring of protein expression, solubility, and protease reactivity using SDS–PAGE was found to be a simple and useful predictor for the likelihood of obtaining a purified protein from the expression trials performed in the auto-induction medium. Further refinement of this scoring approach and miniaturization of the culture volume required to perform the predictive analysis would help to focus the large-scale expression efforts on proteins with higher likelihood of success. Moreover, miniaturization of the culture volume would permit more effective iteration of the expression host, vector construction, and culturing conditions in search of the best conditions for expression [31].

*Performance*

The protocols described here are timed for a 4-day production cycle from initial transformation to harvest of the cells. The auto-induction period occurred over a 24 h period in a refrigerated shaker with no labor intervention required after inoculation and placement of the culture bottles into the shaker. The wet cell mass was routinely ~15 g in a standard 2-L growth by the use of trace amounts of vitamin $B_{12}$ and Met in the auto-induction medium containing 125 mg $L^{-1}$ of SeMet. These additions also helped to assure that greater than 10 mg purified protein could be obtained from a standard purification with SeMet incorporation efficiency averaging 90%. The suitability of proteins prepared from these cells for X-ray structure determination has also been established [9,10].

SeMet labeling was originally demonstrated in 2-L PET bottles using M9 medium and induction by IPTG [6]. This original approach gave ~95% incorporation of SeMet into the overexpressed proteins, which were also successfully purified in amounts sufficient to allow X-ray structure determinations. The present work makes use of the auto-induction approach [12–14] and incorporates trace amounts of vitamin $B_{12}$ and Met into the scale-up medium. The first difference minimizes the handling required after the large-scale growth and expression vessel has been assembled, while the second difference makes the scale-up more robust and rapid without compromising the ultimate level of SeMet incorporation. The use of auto-induction may also give rise to higher cell mass recovery as lactose-induced cultures have been observed to continue growth into the expression phase of the culture without the strong inhibitory perturbation typically observed by batch addition of IPTG [5,29,30].

## Acknowledgments

We thank Dr. F. William Studier (Department of Biology, Brookhaven National Laboratory) for generous personal communications on the composition of the auto-induction medium prior to his publication. We acknowledge the help of J. Myron Crawford and Fernando M. Pineda of the Keck Amino Acid Analysis and Protein Sequencing Facility, W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University, for analysis of SeMet incorporation by amino acid analysis. We thank Amy C. Harms, Grzegorz Sabat, and James Brown of the Mass Spectrometry/Proteomics Facility, Biotechnology Center, University of Wisconsin-Madison, for mass spectrometry. We also thank other members of the staff at the University of Wisconsin Center for Eukaryotic Structural Genomics for support of these efforts.

## References

[1] H. Ke, Overview of isomorphous replacement phasing, Methods Enzymol. 276 (1997) 448–461.

[2] S. Doublie, Preparation of selenomethionyl proteins for phase determination, Methods Enzymol. 276 (1997) 523–530.
[3] W.A. Hendrickson, J.R. Horton, D.M. LeMaster, Selenomethionyl proteins produced for analysis by multiwavelength anomalous diffraction (MAD): a vehicle for direct determination of three-dimensional structure, EMBO J. 9 (1990) 1665–1672.
[4] T.H. Grossman, E.S. Kawasaki, S.R. Punreddy, M.S. Osburne, Spontaneous cAMP-dependent derepression of gene expression in stationary phase plays a role in recombinant expression instability, Gene 209 (1998) 95–103.
[5] J.M. Studts, B.G. Fox, Application of fed-batch fermentation to the preparation of isotopically labeled- or selenomethionine-labeled proteins, Protein Expr. Purif. 16 (1999) 109–119.
[6] L. Stols, C. Sanville Millard, I. Dementieva, M.I. Donnelly, Production of selenomethionine-labeled proteins in two-liter plastic bottles for structure determination, J. Struct. Funct. Genom. 5 (2003) 95–102.
[7] L. Shepherd, R.E. Huber, Some chemical and biochemical properties of selenomethionine, Can. J. Biochem. 47 (1969) 877–881.
[8] C. Sanville Millard, L. Stols, P. Quartey, Y. Kim, I. Dementieva, M.I. Donnelly, A less laborious approach to the high-throughput production of recombinant proteins in *Escherichia coli* using 2-liter plastic bottles, Protein Expr. Purif. 29 (2003) 311–320.
[9] D.W. Smith, K.A. Johnson, C.A. Bingman, D.J. Aceti, P.G. Blommel, R.L. Wrobel, R.O. Frederick, Q. Zhao, H. Sreenath, B.G. Fox, B.F. Volkman, W.B. Jeon, C.S. Newman, E.L. Ulrich, A.D. Hegeman, T. Kimball, S. Thao, M.R. Sussman, J.L. Markley, G.N. Phillips Jr., Crystal structure of At2g03760, a putative steroid sulfotransferase from *Arabidopsis thaliana*, Proteins: Struct. Funct. Genet. Bioinf. 57 (2004) 854–857.
[10] C.A. Bingman, K.A. Johnson, F.C. Peterson, R.O. Frederick, Q. Zhao, S. Thao, B.G. Fox, B.F. Volkman, W.B. Jeon, D.W. Smith, C.S. Newman, E.L. Ulrich, A. Hegeman, M.R. Sussman, J.L. Markley, G.N. Phillips Jr., Crystal structure of the protein from gene At3g17210 of *Arabidopsis thaliana*, Proteins Struct. Funct. Genet. 57 (2004) 218–220.
[11] Q. Zhao, R. Frederick, K. Seder, S. Thao, H. Sreenath, F. Peterson, B.F. Volkman, J.L. Markley, B.G. Fox, Production in two-liter beverage bottles of proteins for NMR structure determination labeled with either $^{15}$N- or $^{13}$C–$^{15}$N, J. Struct. Funct. Genom. 5 (2004) 87–93.
[12] F.W. Studier, Auto-Induction for Protein Production in Inducible T7 Expression Systems, Annual Meeting of the American Crystallographic Association, Chicago, IL, 2004 July 17–22.
[13] F.W. Studier, Protein production by auto-induction in high-density shaking cultures, Protein Expr. Purif. (2005) (in press).
[14] A. Grabski, M. Mehler, D. Drott, Unattended high-density cell growth and induction of protein expression with the overnight express TM autoinduction system, Innovations 17 (2003) 3–6.
[15] R.C. Tyler, H.K. Sreenath, S. Singh, D.J. Aceti, C.A. Bingman, J.L. Markley, B.G. Fox, Auto-induction medium for the production of [*U*-$^{15}$N]- and [*U*-$^{13}$C, *U*-$^{15}$N]- labelled proteins for NMR screening and structure determination, Protein Expr. Purif., in press, doi:10.1016/j.pep.2004.12.024.
[16] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, third ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001.
[17] B.R. Keleman, T.A. Klink, M.A. Behlke, S.R. Eubanks, P.A. Leland, R.T. Raines, Hypersensitive substrates for ribonucleases, Nucleic Acids Res. 27 (1999) 3696–3701.
[18] R.B. Kapust, D.S. Waugh, *Escherichia coli* maltose binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused, Protein Sci. 8 (1999) 1668–1674.
[19] D.J. Aceti, P.G. Blommel, Y. Endo, B.G. Fox, R.O. Frederick, A.D. Hegeman, W.B. Jeon, T.L. Kimball, J.M. Lee, C.S. Newman, F.C. Peterson, T. Sawasaki, K.D. Seder, M.R. Sussman, E.L. Ulrich, R.L. Wrobel, S. Thao, D.A. Vinarov, B.F. Volkman, Q. Zhao, Role of nucleic acid and protein manipulation technologies in high-throughput structural biology efforts, in: A. Steinbüchel (Ed.), Biopolymers, vol. 8, Wiley-VCH, Weinheim, 2002, pp. 469–496.
[20] I.G. Old, I.S. Girons, C. Richaud, Physical mapping of scattered methionine genes on the *Escherichia coli* chromosome, J. Bacteriol. 175 (1993) 3689–3691.
[21] J.T. Mulligan, W. Margolin, J.H. Krueger, G.C. Walker, Mutations affecting regulation of methionine biosynthetic genes isolated by use of *met-lac* fusions, J. Bacteriol. 151 (1982) 609–619.
[22] M.E. Maxon, B. Redfield, X.-Y. Cai, R. Shoeman, K. Fujita, W. Fisher, G. Stauffer, H. Weissbach, N. Brot, Regulation of methionine expression in *Escherichia coli*: effect of MetR protein on the expression of the metE and metR genes, Proc. Natl. Acad. Sci. USA 86 (1989) 85–89.
[23] A.H. Rosenberg, E. Goldman, J.J. Dunn, F.W. Studier, G. Zubay, Effects of consecutive AGG codons on translation in *Escherichia coli*, demonstrated with a versatile codon test system, J. Bacteriol. 175 (1993) 716–722.
[24] G.T. Chen, M. Inouye, Role of the AGA/AGG codons, the rarest codons in global gene expression in *Escherichia coli*, Genes Dev 8 (1994) 2641–2652.
[25] J.F. Kane, Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*, Curr. Opin. Biotechnol. 6 (1995) 494–500.
[26] C. Gustafsson, S. Govindarajan, J. Minshull, Codon bias and heterologous protein expression, Trends Biotechnol. 22 (2004) 346–353.
[27] L.M. Hsu, N.V. Vo, C.M. Kane, M.J. Chamberlin, In vitro studies of transcript initiation by *Escherichia coli* RNA polymerase. 1. RNA chain initiation, abortive initiation, and promoter escape at three bacteriophage promoters, Biochemistry 42 (2003) 3777–3786.
[28] P. Neubauer, C. Wolff, M. Hecker, K. Hoffmann, L. Meyer, P. Kruschke, H.W. Heinrich, Introduction of the *tac*-promoter by lactose under fermentation conditions, Acta Biotechnol. 11 (1991) 23–29.
[29] P. Neubauer, K. Hofmann, O. Holst, B. Mattiasson, P. Kruschke, Maximizing the expression of a recombinant gene in *Escherichia coli* by manipulation of induction time using lactose as an inducer, Appl. Microbiol. Biotechnol. 36 (1992) 739–744.
[30] B.J. Hoffman, J.A. Broadwater, P. Johnson, J. Harper, B.G. Fox, W.R. Kenealy, Lactose fed-batch overexpression of recombinant metalloproteins in *Escherichia coli* BL21(DE3): Process control yielding high levels of metal incorporated, soluble protein, Protein Expr. Purif. 6 (1995) 646–654.
[31] Y.-P. Shih, W.-M. Kung, J.-C. Chen, C.-H. Yeh, A.H.-J. Wang, T.-F. Wang, High-throughput screening of soluble recombinant proteins, Protein Sci. 11 (2002) 1714–1719.