

Bacterial Bioreactors for High Yield Production of Recombinant Protein*

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We developed a new bacterial expression system that utilizes a combination of attributes (low temperature, induction of an mRNA-specific endoribonuclease causing host cell growth arrest, and culture condensation) to facilitate stable, high level protein expression, almost 30% of total cellular protein, without background protein synthesis. With the use of an optimized vector, exponentially growing cultures could be condensed 40-fold without affecting protein yields, which lowered sample labeling costs to a few percent of the cost of a typical labeling experiment. Because the host cells were completely growth-arrested, toxic amino acids such as selenomethionine and fluorophenylalanine were efficiently incorporated into recombinant proteins in the absence of cytotoxicity. Therefore, this expression system using *Escherichia coli* as a bioreactor is especially well suited to structural genomics, large-scale protein expressions, and the production of cytotoxic proteins.

MazF is an *Escherichia coli* toxin possessing single-stranded RNA- and ACA-specific endoribonuclease activity (1) that selectively degrades almost all cellular mRNAs *in vivo*, resulting in a precipitous drop in total protein synthesis and leading to complete cell growth arrest. However, when a gene encoding a target protein is engineered to transcribe an ACA-less mRNA, its transcript is stably co-expressed and translated in MazF-induced cells (2). Codon degeneracy enables alteration of an ACA triplet to a cleavage-resistant sequence without changing the protein amino acid sequence, regardless of its position in the reading frame.

MazF growth-arrested cells are “quasi-dormant” because they can be coaxed out of metabolic quiescence by exposure to an appropriate trigger (in this case through exposure to an ACA-less mRNA). Remarkably, the host cell translation machinery remains functional, enabling the expression of recombinant proteins at high levels without significant background cellular protein synthesis. In fact, despite being growth-arrested, MazF-induced cells

retain the full spectrum of biosynthetic functions necessary to support mRNA transcription and translation (2).

Here we exploit the properties of MazF to develop an *E. coli* expression system that can sustain high level, virtually pure target protein production under condensed culture conditions, creating an *E. coli* bioreactor for production of a target protein without any cellular protein synthesis. In fact, culture condensation imparts dramatic cost savings without sacrificing protein yield. In addition, unlike any existing expression technology in bacteria or eukaryotes, this expression system supports high level incorporation of toxic amino acid analogs (e.g. selenomethionine). The applications for this bioreactor are multifold and of particular utility for large scale structural genomics projects that employ both NMR and x-ray crystallography and for production of cytotoxic proteins.

EXPERIMENTAL PROCEDURES

Culture Condensation—*E. coli* BL21(DE3) transformed with pACYCmazF and pColdI(SP-4)envZB(-ACA) was grown in 1000 ml of M9-glucose medium at 37 °C. When the A_{600} reached 0.5, the culture was chilled in an ice-water bath to quickly reach 15 °C and incubated at 15 °C for 45 min to acclimate the cells to cold shock conditions. Cells were harvested and resuspended in 10 ml of chilled M9-glucose medium containing 1 mM IPTG.² Three ml of this 100-fold condensed culture was transferred into a 25-ml culture flask. The remaining 7 ml was appropriately diluted with chilled M9 medium containing 1 mM IPTG to make 3 ml of 50-, 40-, 30-, 20-, 10-, and 1-fold condensed cultures. Each culture was transferred into a 25-ml culture flask and incubated in a water bath shaker at 15 °C for 21 additional hours to induce both MazF and EnvZB. Cells were collected by centrifugation and resuspended such that each sample comprised an extract from an equal number of cells/ml, and equal volumes were loaded and subjected to SDS-PAGE followed by Coomassie Blue staining.

Incorporation of Amino Acid Analogs—EnvZB and CspA were expressed in order to demonstrate the efficiency of incorporation of selenomethionine and *p*-fluoro-*L*-phenylalanine (F-Phe), respectively. For selenomethionine incorporation, *E. coli* BL21(DE3) transformed with both pACYCmazF and pColdI(SP-4)envZB(-ACA) was grown in 200 ml of M9-glucose medium at 37 °C. When the A_{600} reached 0.5, the culture was shifted to 15 °C for 45 min to acclimate the cells to cold shock

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² The abbreviations used are: IPTG, isopropyl-1-thio- β -D-galactopyranoside; UTR, untranslated region; F-Phe, *p*-fluoro-*L*-phenylalanine.

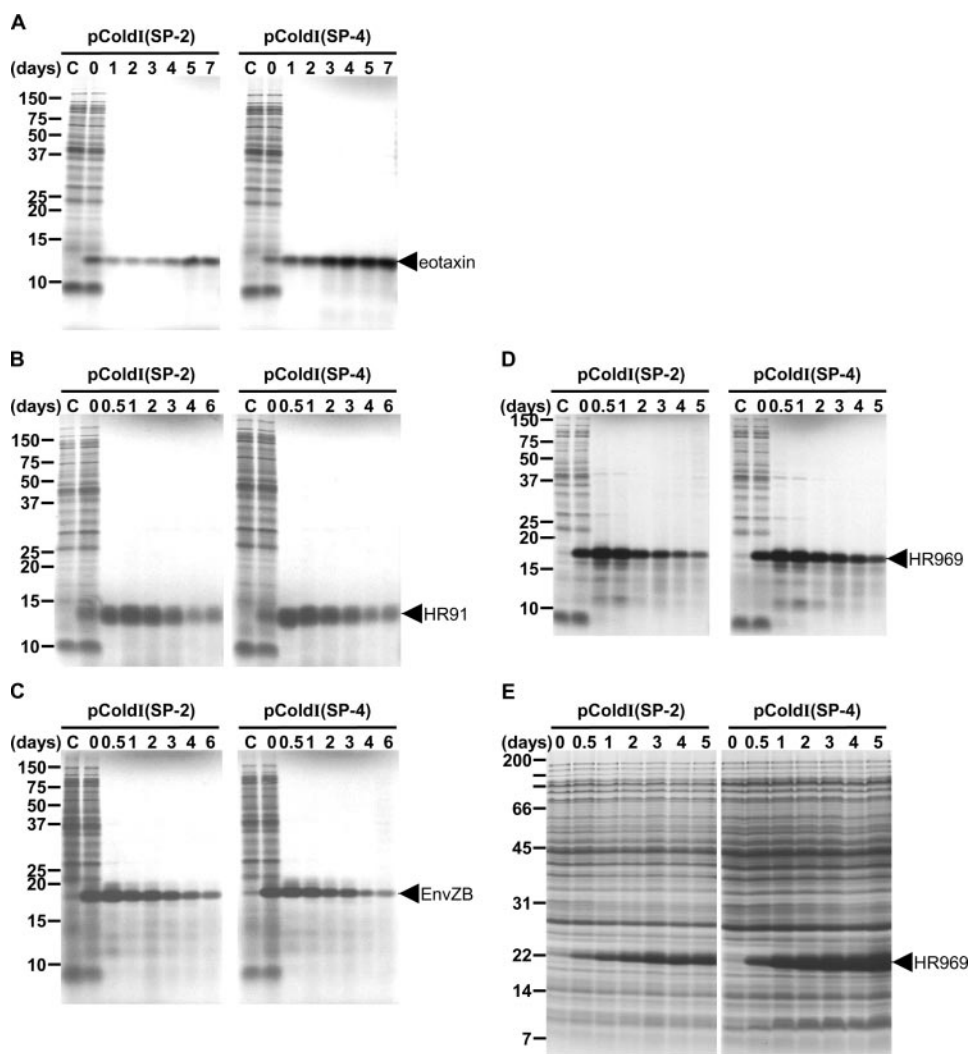


FIGURE 1. A–D, pColdI(SP-2) and pColdI(SP-4) vectors support sustained, high level target protein expression. Mature human eotaxin (A), HR91 (B), EnvZB (C), or HR969 (D) was expressed from pColdI(SP-2) (one ACA in 3'-UTR) or pColdI(SP-4) (no ACA in 3'-UTR) along with MazF from pACYCmazF. Upon reaching an A_{600} of 0.5, cultures were shifted from 37 to 15 °C for 45 min. New protein synthesis was then monitored by isotopic labeling with [35 S]methionine for 15 min before (control lane C) or at intervals after IPTG induction for up to 7 days for eotaxin, 6 days for HR91 and EnvZB, and 5 days for HR969. Equivalent amounts of cell lysate derived from equal culture volumes were subjected to SDS-PAGE followed by autoradiography. E, total cellular protein derived from the unlabeled samples otherwise subjected to the same condition as in D was analyzed by SDS-PAGE and assessed by Coomassie Blue staining. Molecular mass markers are on the left; the position of the protein of interest is designated by an arrowhead 32 on the right.

conditions. Cells were harvested and suspended with 5 ml of M9-glucose medium containing Lys (100 μ g/ml), Phe (100 μ g/ml), Thr (100 μ g/ml), Ile (50 μ g/ml), Leu (50 μ g/ml), and Val (50 μ g/ml) (40 \times concentrated culture). Cultures were incubated at 15 °C for 15 min to inhibit endogenous methionine biosynthesis, and then 25 μ l of 12 mg/ml seleno-L-methionine (final concentration of 60 μ g/ml) and 5 μ l of 1 M IPTG (final concentration of 1 mM) were added to the culture. Both CspA and EnvZB were expressed at 15 °C for 21 h. For the incorporation of F-Phe, 50 μ l of 6 mg/ml F-Phe was added immediately after concentrating. In contrast to selenomethionine labeling conditions, endogenous phenylalanine biosynthesis was not prevented during F-Phe incorporation.

Incorporation of 15 N—*E. coli* BL21(DE3) transformed with pACYCmazF and pColdI(SP-4)envZB(-ACA) was grown in

1000 ml of M9-glucose medium at 37 °C. When the A_{600} reached 0.5, the culture was shifted to 15 °C for 45 min to acclimate the cells to cold shock conditions. IPTG (1 mM) was added to induce expression of both MazF and EnvZB followed by a 3-h incubation at 15 °C to eliminate isotope incorporation into background cellular proteins. Cells were harvested and resuspended with 50 ml of [15 N]M9-glucose medium (1 g of [15 N]NH $_4$ Cl/liter) containing 1 mM IPTG (20 \times concentrated culture). EnvZB was expressed at 15 °C for 15 h.

RESULTS

pColdI(SP-2) and *pColdI(SP-4)* Vectors Support Sustained, High Level Target Protein Expression—

We originally developed pCold vector backbone to facilitate high yield protein expression under low temperature conditions to enhance the stability of expressed proteins (3). pColdI features include a *cspA* promoter and translation-enhancing element to drive high levels of target protein transcription and translation upon cold shock; a His $_6$ tag sequence; a factor Xa cleavage site; and an amino-terminal His-Met-encoding sequence comprising an NdeI site that enables the in-frame insertion of any target gene. With the goal of enhancing expression levels when a target protein is coincuded along with a second plasmid expressing MazF, the plasmid pColdI(SP-4)³ was created from the plasmid designated pColdI(SP-2). Both pColdI(SP-2) and pCold-

I(SP-4) were engineered to resist cleavage by MazF. The pColdI(SP-2) vector was modified such that all of the ACAs were removed from the relevant 5'-sequences (2). Subsequent removal of a single ACA from the 3'-untranslated region (UTR) of pColdI(SP-2) resulted in the pColdI(SP-4) vector.

The efficacy of pColdI(SP-4) relative to its pColdI(SP-2) progenitor was first tested by assessing both the levels and duration of expression of the test protein eotaxin, a 74-amino acid human chemokine (Fig. 1A). New synthesis of eotaxin from either pColdI(SP-2) or pColdI(SP-4) was assessed for up to 7 days after its coincubation with MazF by monitoring [35 S]methionine incorporation by SDS-PAGE and autoradiography.

³ pColdI(SP-4), as well as pColdII(SP-4), pColdIII(SP-4), and pColdIV(SP-4), is now available from TaKaRa-Bio, Inc.

Eotaxin synthesis rates from pColdI(SP-4) gradually increased from day 1 to 3, peaked between days 3 and 4, and were sustained at that level through the final 7-day time point. In contrast, eotaxin expression levels from pColdI(SP-2) were relatively modest initially, increased only marginally, peaked later (day 5), and sustained peak expression through the 7-day time point. Therefore, pColdI(SP-4) supports higher levels of eotaxin expression, suggesting that removal of ACAs at the 3'-UTR of the vector would generally enhance expression levels. In addition, these results demonstrated that quasi-dormant *E. coli* cells are able to translate proteins for at least 7 days despite being completely growth-arrested.

The efficacy of both plasmids was then tested with three additional proteins: EnvZB, the 161-residue ATP-binding domain of the *E. coli* EnvZ histidine kinase; a 72-residue human protein of unknown function designated HR91 by the Northeast Structural Genomics Consortium (NESG); and another 139-residue NESG human target protein of unknown function called HR969. In contrast to eotaxin, HR91 (Fig. 1B), EnvZB (Fig. 1C), and HR969 (Fig. 1D) expression efficiency was somewhat better but almost identical in pColdI(SP-4) versus pColdI(SP-2). The expression patterns also differed from eotaxin; they peaked earlier (0.5–1 day) and then gradually decreased through day 7. Therefore, the eotaxin results did not reflect a general trend, but instead, the target protein appeared to dictate the expression characteristics and protein synthesis rates from either the original pColdI(SP-2) or the modified pColdI(SP-4) vector. However, because synthesis rates of new target proteins are never worse, and sometimes even better, when using pColdI(SP-4), we selected this modified vector for the remainder of the expression studies described in this study.

Next, we used HR969 to examine the cumulative levels of target protein present in the cell during the 5-day incubation by analyzing total cellular proteins by Coomassie staining (Fig. 1E). The levels of HR969 protein accumulated steadily to the 5-day time point, representing ~30% of the total cellular protein, notably without any discernible changes in the density of any other cellular proteins; this is consistent with Fig. 1D, where no background cellular protein synthesis was observed during the 5-day incubation. We obtained comparable results for both EnvZB and HR91 (data not shown), indicating that the pColdI(SP-4) expression vector supported enhanced, sustained, and stable recombinant protein production during the 5-day reaction.

Expression Can Occur under Highly Condensed Culture Conditions without Significant Reduction in Target Protein Yields—Because cell growth is completely inhibited upon MazF induction, we examined whether cell cultures could tolerate substantial condensation for protein production without affecting the protein yield. *E. coli* BL21 cultures harboring pACYCmazF and pColdI(SP-4)envZB were subjected to normal induction conditions. However, the cells were first pelleted and resuspended in medium to achieve cell concentrations 10-, 20-, 30-, 40-, 50-, and 100-fold higher than normal inducing conditions (A_{600} of 0.5, corresponding to $\sim 3 \times 10^8$ cells/ml). The total cellular protein profiles of samples containing equivalent cell numbers were then analyzed by SDS-PAGE followed by Coomassie Blue staining (Fig. 2). Overall, the cells tolerated concentration remarkably well and continued to express and translate high

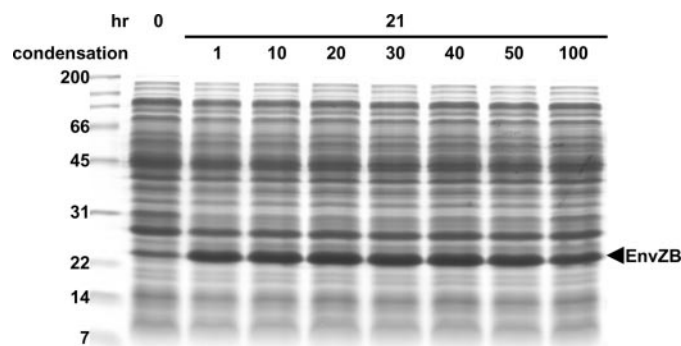


FIGURE 2. Expression cultures can be highly condensed without sacrificing yield. ACA-less EnvZB was expressed from pColdI(SP-4) along with MazF from pACYCmazF. Cultures were grown to an A_{600} of 0.5, shifted to 15 °C for 45 min, concentrated to the levels shown, and then induced with IPTG for 0 or 21 h in M9 medium. Samples were subjected to SDS-PAGE followed by Coomassie Blue staining. Molecular mass markers are on the left; the position of EnvZB is designated by an arrowhead on the right.

levels of target protein. In fact, after 21 h of induction, EnvZB represented the most abundant stainable protein in the full range of concentrated cultures that we analyzed. Concentration did not reduce protein yield; the amounts of EnvZB produced for the uncondensed sample ($\sim 3 \times 10^8$ cells/ml) compared with the 40-fold ($\sim 1 \times 10^{10}$ cells/ml) sample were almost identical. Only upon culture condensation of 50-fold or greater was a reduction in EnvZB protein levels observed. Therefore, MazF-induced cells in a quasi-dormant state can withstand substantial (40-fold) condensation without a reduction in the yield of target protein.

Condensed Culture Conditions Support High Efficiency Selenomethionine Incorporation without Toxicity—The ability to produce high levels of a single protein in condensed cultures may have applications for structural analysis of proteins by x-ray crystallography or NMR. Heavy atoms are used as phasing centers for multiwavelength anomalous diffraction (MAD) in synchrotron crystallography (4). Selenium addition to proteins through *in vivo* (5, 6) or *in vitro* (7) incorporation of precursor selenomethionine is the most common approach to enable MAD phasing. However, *in vivo* selenomethionine incorporation into *E. coli* proteins is cytotoxic and leads to growth inhibition. Alternatively, the *E. coli in vitro* translation system is technically difficult to construct in the laboratory and expensive if purchased commercially.

We projected that selenomethionine-induced cytotoxicity and growth inhibition should not be an issue for the condensed expression approach because these cells are already growth-arrested. More importantly, upon MazF and target protein coinfection in condensed cultures, we have demonstrated that the target protein alone accounts for virtually all of the new protein synthesis. Therefore, there should be negligible background synthesis of other cellular proteins that typically contribute to cytotoxicity when they are also selenomethionine-labeled along with the target protein.

Using 40-fold condensed culture conditions (the maximal condensation for the highest yield), we assessed whether selenomethionine substitution affected the yield of target protein and also measured the efficacy of selenomethionine replacement into EnvZB (Fig. 3A). The amount of selenomethionine-

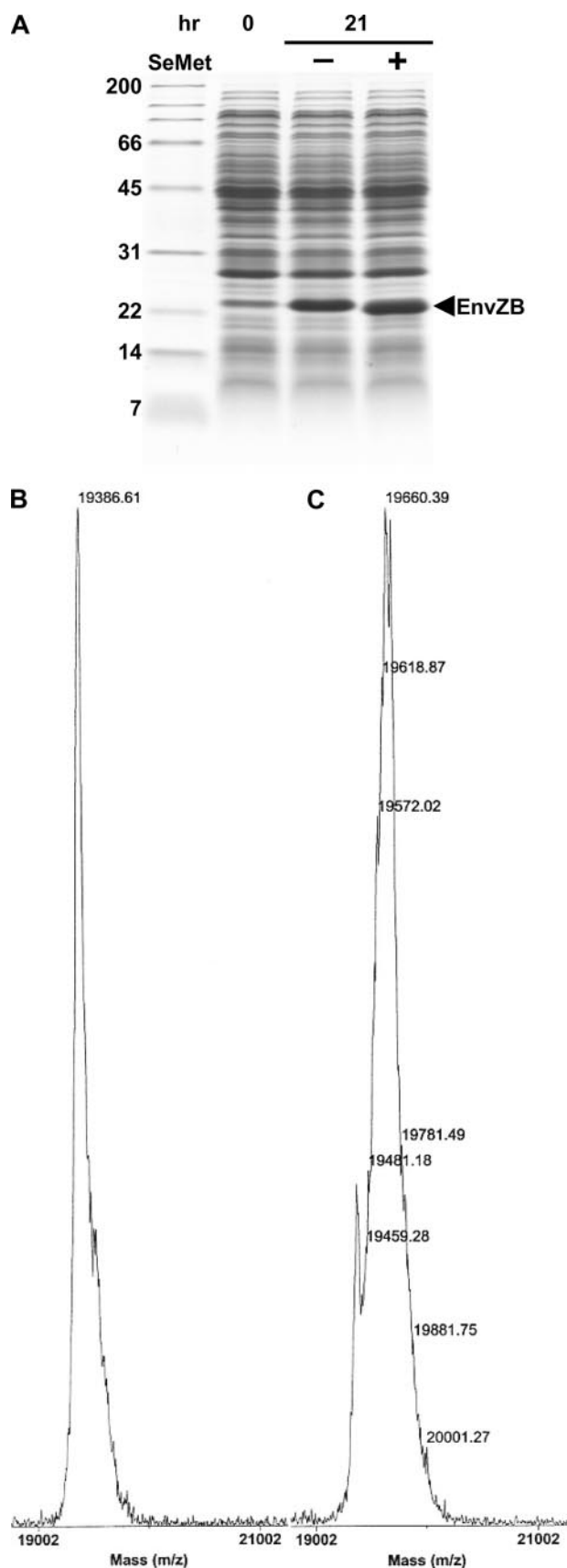


FIGURE 3. High efficiency selenomethionine substitution without toxicity. A, bacterial cultures expressing ACA-less EnvZB from pColdI(SP-4) and MazF from pACYCmazF were grown to an A_{600} of 0.5, shifted to 15 °C for 45 min, concentrated 40-fold, and then induced with IPTG for 0 h (left lane) or for

21 h in M9 medium with (right lane) or without (middle lane) selenomethionine. Samples were subjected to SDS-PAGE followed by Coomassie Blue staining. Molecular mass markers are on the left; the position of EnvZB is designated by an arrowhead on the right. B and C, EnvZB expressed with (C) or without selenomethionine (B) was affinity-purified through a nickel-nitrilotriacetic acid column, and its molecular mass was analyzed by mass spectrometry.

containing EnvZB (lane 4) was almost identical to that in the control experiment done under the same conditions but without selenomethionine (lane 3), indicating that selenomethionine incorporation did not adversely affect EnvZB protein production in our expression system. Histidine-tagged EnvZB was then purified, and the molecular mass of wild type versus selenomethionine-substituted EnvZB was analyzed using mass spectrometry (Fig. 3, B and C). Calculation of mass increase indicated that ~90% of the methionines (on average 6.3 of the 7 methionine residues, excluding the initiation methionine) were substituted with selenomethionine.

Effective Fluorophenylalanine Substitution without Toxicity— Replacement of phenylalanine residues in a protein with F-Phe can be implemented for the structural analysis of a protein by ^{19}F NMR (8, 9). However, as with selenomethionine substitution, F-Phe substitution is cytotoxic. We followed the expression of the CspA in a manner analogous to that demonstrated for selenomethionine EnvZB. CspA was expressed with pColdI(SP-4)cspA for 21 h with and without the addition of F-Phe (Fig. 4A). The addition of F-Phe did not adversely affect CspA production in our expression system. We then affinity-purified the 86-residue CspA product and used mass spectrometry to determine the extent of F-Phe substitution (Fig. 4, B and C). Although endogenous Phe biosynthesis was not inhibited, substantial incorporation of F-Phe (~60–70% of total Phe residues) was observed, indicating that toxic F-Phe can be efficiently incorporated into a protein without affecting its yield.

Condensed Expression Cultures Support Very High Efficiency ^{15}N Incorporation— Because concomitant expression of MazF with a target protein results in an extremely high signal-to-noise ratio upon [^{35}S]methionine labeling, our condensed expression system may be ideal for ^{15}N and ^{13}C isotope labeling of proteins for NMR structural analysis. To test this possibility, EnvZB was expressed in a 20-fold condensed culture containing [^{15}N]NH $_4$ Cl. A control culture was also prepared in the identical manner, except that the medium instead contained [^{14}N]NH $_4$ Cl. We observed no difference in protein yields between [^{15}N]NH $_4$ Cl and [^{14}N]NH $_4$ Cl incorporation experiments (data not shown). Both proteins were then affinity-purified, and their tryptic peptides were analyzed by mass spectrometry.

We chose the tryptic peptide YGNGWIK from EnvZB (which contains 10 nitrogen atoms and 40 carbon s) for comparative analysis using mass spectrometry. The control peptide has three distinct masses due to the presence of naturally abundant ^{13}C (~1%, Fig. 5A). Of the three peaks, the major peak I contains only ^{14}N and ^{12}C , and peaks II and III contain one and two ^{13}C atoms, respectively, in the peptide. Assuming that the peak heights are proportional to the amount of peptide in the peaks, the level of the naturally abundant ^{13}C is estimated to be 1.0%. The analysis of the peptide from ^{15}N -labeled EnvZB

21 h in M9 medium with (right lane) or without (middle lane) selenomethionine. Samples were subjected to SDS-PAGE followed by Coomassie Blue staining. Molecular mass markers are on the left; the position of EnvZB is designated by an arrowhead on the right. B and C, EnvZB expressed with (C) or without selenomethionine (B) was affinity-purified through a nickel-nitrilotriacetic acid column, and its molecular mass was analyzed by mass spectrometry.

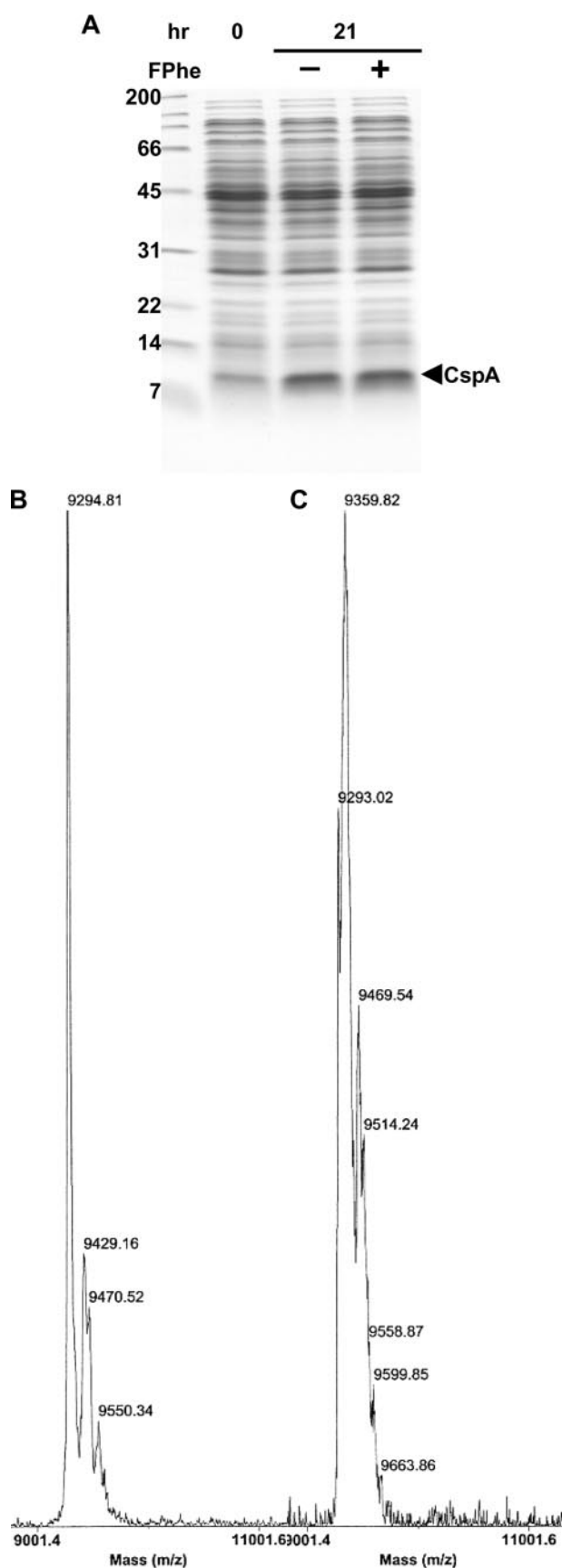


FIGURE 4. Efficient fluorophenylalanine substitution without toxicity. A, *E. coli* cultures expressing ACA-less CspA from pColdI(SP-4) and MazF from pACYCmazF were grown to an A_{600} of 0.5, shifted to 15 °C for 45 min,

shows two distinct peak profiles (Fig. 5B); the peak profile at the left is identical to the pattern in Fig. 5A, indicating that these peaks were derived from EnvZB produced before isotope labeling. This background ^{14}N -labeled EnvZB is estimated to comprise ~20% of the total EnvZB produced under these conditions. Seven major peaks constitute the ^{15}N -labeled peptides. Major peak 5 represents a mixture of the peptide consisting of 9 ^{15}N , 1 ^{14}N , 0 ^{13}C , and 40 ^{12}C atoms and the peptide containing 8 ^{15}N , 2 ^{14}N , 1 ^{13}C , and 39 ^{12}C atoms. Peak 4 comprises a mixture of two peptides: one contains 8 ^{15}N , 2 ^{14}N , 0 ^{13}C , and 40 ^{12}C atoms, and the other contains 7 ^{15}N , 3 ^{14}N , 1 ^{13}C , and 39 ^{12}C atoms. On the other hand, peak 6 consists of two peptides, 10 ^{15}N , 0 ^{14}N , 0 ^{13}C , and 40 ^{12}C atoms and 9 ^{15}N , 1 ^{14}N , 1 ^{13}C , and 39 ^{12}C atoms. On the basis of the peak distribution due to the naturally abundant ^{13}C (Fig. 5A), peptide one containing 10 ^{15}N atoms represents 6% of the total peptides analyzed; peptide two (9 ^{15}N), 38%; peptide three (8 ^{15}N), 31%; and peptide four (7 ^{15}N), 15%. These results demonstrate that ~90% of the peptides produced after the addition of ^{15}N -labeled NH_4Cl contain at least 7 ^{15}N atom substitutions out of the 10 total nitrogen atoms.

DISCUSSION

There are several major attributes of the new expression system described in this study. First, we have demonstrated that target protein synthesis can occur for at least 7 days, indicating that recycling of the components required for translation (tRNA and ribosomes) undoubtedly occurs. Therefore, there appears to be no theoretical limit to the size of the protein that can be synthesized by our expression system. Second, with the goal of reducing the cost of the amino acid substitution and isotope labeling steps for x-ray crystallography and NMR structure applications, we first demonstrated that a 40-fold culture condensation step (to $>10^{10}$ cells/ml) does not significantly reduce protein yields (the expressed protein comprises 20–30% of total cellular proteins or the yield of as high as 1–1.5 mg/ml for target protein). Third, the cost efficiencies afforded by this condensed expression method result in a decrease (to only 2.5% the expense of typical incorporation experiments) in the cost of medium containing highly expensive isotope-labeled amino acids, amino acid analogs, or carbon sources (such as deuterated ^{13}C glucose).

Finally, in contrast to any existing *in vivo* protein expression technology, incorporation of cytotoxic amino acid analogs using our condensed expression system does not result in cell toxicity. The toxicity of selenomethionine, fluorophenylalanine, or any other amino acid analogs limits the level of incorporation into proteins, whereas without toxicity there should be no limit to incorporation. The ability of the cells to tolerate culture condensation and avoid amino acid analog cytotoxicity stems from the unique physiological state of the *E. coli* cells upon MazF expression. MazF-induced quasi-dormant cells are not actively growing, because MazF acts to selectively degrade

concentrated 40-fold, and then induced with IPTG for 0 h (left lane) or for 21 h in M9 medium with (right lane) or without F-Phe (middle lane). Samples were subjected to SDS-PAGE followed by Coomassie Blue staining. Molecular mass markers are on the left; the position of CspA is designated by an arrowhead on the right. B and C, CspA expressed with (C) or without (B) F-Phe was affinity-purified, and its molecular mass was analyzed by mass spectrometry.

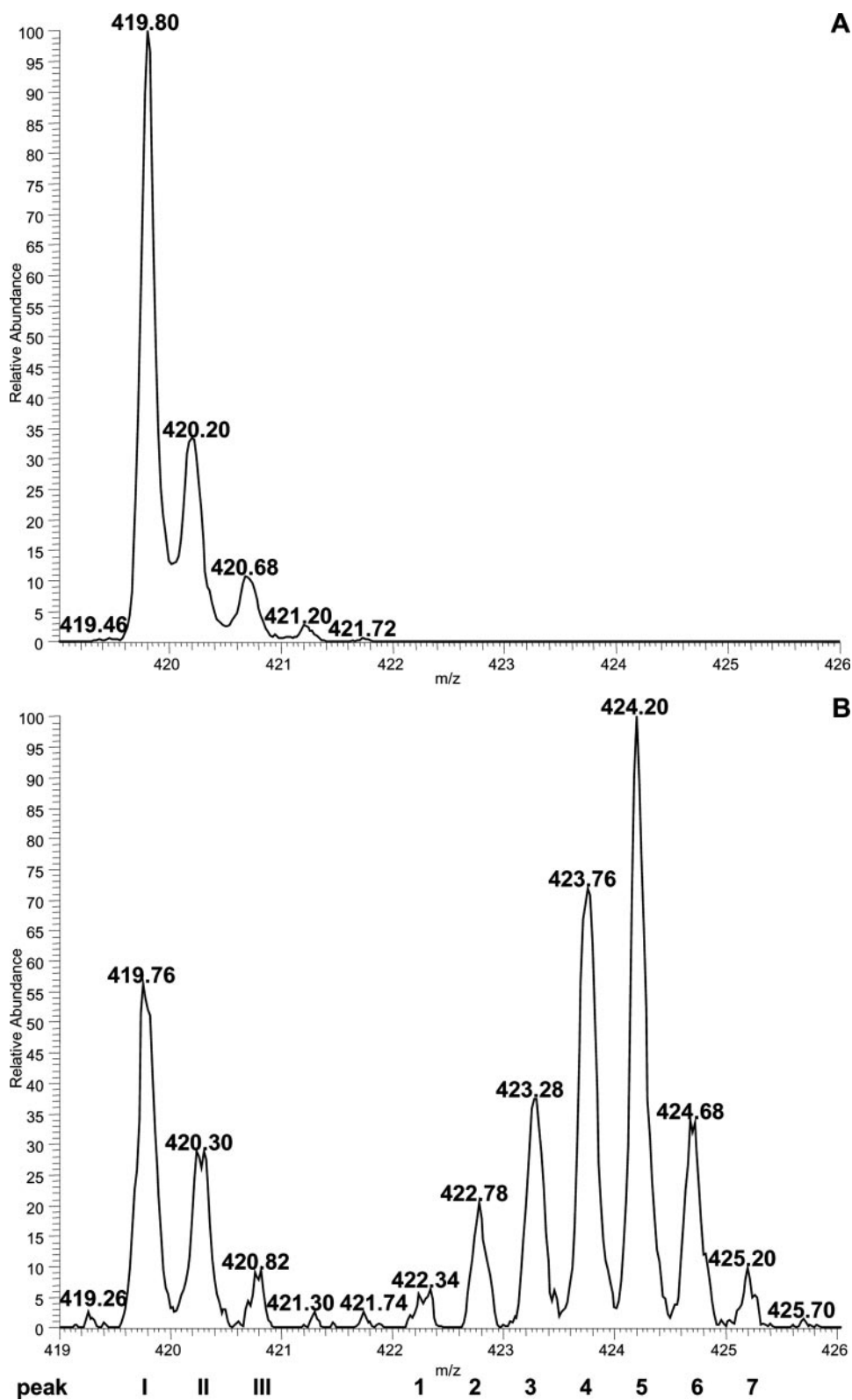


FIGURE 5. High efficiency ^{15}N incorporation. Both panels represent mass spectroscopy of a seven-residue YNGGWIK tryptic fragment derived from 20-fold concentrated cultures of ACA-less EnvZB expressed from pColdI(SP-2) along with MazF from pACYCmazF grown in either M9 medium (A) or ^{15}N M9 medium (B).

virtually all cellular mRNAs. Therefore, almost no background protein synthesis occurs, so that selenomethionine or other cytotoxic amino acid analogs are not incorporated into any cellular proteins. This enables the production of pure target pro-

A tein with high levels of selenomethionine or other amino acid analogs without the secondary cytotoxic effects.

Although this expression system requires the construction of an ACA-less target gene (thus encoding an ACA-less mRNA), there are two approaches that would quickly and economically address this requirement. If the target gene contains only a few ACA sequences, these sequences can be altered by oligonucleotide-directed, site-specific mutagenesis so that they are no longer cleavable by MazF. However, if a gene is large and/or has many ACA sequences, the best approach is to chemically synthesize the entire gene using one of the commercially available gene synthesis technologies (e.g. Codon Devices, Cambridge, MA (10)). This general approach has several advantages. First, the gene sequences are guaranteed for accuracy so that the subsequent sequence verification step that typically follows PCR mutagenesis is no longer necessary. Second, the synthetic gene can be directly inserted into a vector of choice, eliminating the need for a cloning step. Third, in an effort to improve translation efficiency, the gene sequence can be optimized to the codon usage preferences of the particular organism used for expression. Finally, the time and manpower savings more than offset the modest cost of whole gene synthesis. As with new technologies in general, the cost should continue to fall as the methods used for gene synthesis are further improved. In fact, we expect that this technology will completely replace PCR-mediated site-directed mutagenesis for creation of ACA-less genes in the next few years, when the cost of synthesizing a gene for a 200-residue protein may drop to less than \$200.

Our expression system generates high signal-to-noise ratios without a purification step because essentially only the target protein is labeled with isotopes, as background cellular protein synthesis is virtually absent. Therefore, NMR structural studies of the protein may be carried out without purification. This attribute

is especially attractive for membrane proteins. Furthermore, this system enables us to explore the potential for stabilizing the folded state of a protein by compartmentalization inside the living cell using in-cell NMR spectroscopy (11–14). In-cell NMR is the only way to learn about the actual structures and dynamics of proteins inside of living cells under truly physiological conditions.

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