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Effects of a Minor Isoleucyl tRNA on Heterologous Protein Translation in *Escherichia coli*

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In *Escherichia coli*, the isoleucine codon AUA occurs at a frequency of about 0.4% and is the fifth rarest codon in *E. coli* mRNA. Since there is a correlation between the frequency of codon usage and the level of its cognate tRNA, translational problems might be expected when the mRNA contains high levels of AUA codons. When a hemagglutinin from the influenza virus, a 304-amino-acid protein with 12 (3.9%) AUA codons and 1 tandem codon, and a mupirocin-resistant isoleucyl tRNA synthetase, a 1,024-amino-acid protein, with 33 (3.2%) AUA codons and 2 tandem codons, were expressed in *E. coli*, product accumulation was highly variable and dependent to some degree on the growth medium. In rich medium, the flu antigen represented about 16% of total cell protein, whereas in minimal medium, it was only 2 to 3% of total cell protein. In the presence of the cloned *ileX*, which encodes the cognate tRNA for AUA, however, the antigen was 25 to 30% of total cell protein in cells grown in minimal medium. Alternatively, the isoleucyl tRNA synthetase did not accumulate to detectable levels in cells grown in Luria broth unless the *ileX* tRNA was coexpressed when it accounted for 7 to 9% of total cell protein. These results indicate that the rare isoleucine AUA codon, like the rare arginine codons AGG and AGA, can interfere with the efficient expression of cloned proteins.

In most organisms, there is a noticeable preference for certain codons in highly expressed genes with a subset of so-called rare codons that are generally found in poorly expressed genes (20, 22). While this has some interesting evolutionary ramifications for codon selection, there is a more pragmatic problem that these rare codons present with respect to the overexpression of heterologous proteins in a host such as *Escherichia coli*. There appears to be a correlation between the frequency of codon usage and the level of its cognate tRNA (10). Thus, the expression of mRNA molecules containing a large number of these codons, or tandem rare codons, can result in translational problems. If the level of certain tRNAs was low, then one would predict translational difficulties in decoding mRNA species containing large numbers of these rare codons. This situation would be exacerbated when that particular heterologous mRNA was suddenly induced and represented the major species inside the cell. Since the ribosomes would be more likely to encounter that heterologous mRNA, there would be a need for increased levels of the appropriate acylated rare tRNA species. In the absence of these acylated molecules, the ribosomes would stall while awaiting the appropriate tRNA species (17, 18, 21, 22, 31, 32), and a stalled ribosome is more likely to produce translational errors such as frameshifts or hops. Such effects have been found when the heterologous mRNA contained the rare codons AGG and AGA (11, 18, 23, 24). This paper addresses the effect of another rare codon, namely, AUA, on the quality and quantity of heterologous proteins expressed in *E. coli.*

In *E. coli*, the AUA codon is used at a frequency of less than 0.4% (26, 33) and, in an analysis by Zhang et al. (33), is the least frequently utilized codon after AGG and AGA. The gene encoding the tRNA that decodes AUA is called *ileX* (16) and has the interesting property of containing a CAU anticodon. Although this is the anticodon for methionine (AUG), the C residue becomes lysinylated to allow it to base pair with the AUA rather than the AUG codon. This posttranscriptional modification is essential for both acylation by isoleucyl tRNA synthetase (IRS) with isoleucine and for recognizing the AUA codon (16).

The impact of the AUA codon on expression was measured by using two proteins in two different *E. coli* hosts. The first protein was a hybrid polypeptide containing the first 81 amino acids of the viral NS1 nonstructural protein and the first 223 amino acids of the HA2 subunit of A/Puerto Rico/8/34 subtype H1N1 hemagglutinin (B/LeeHA) (30). (The hemagglutinin is a major surface glycoprotein of the influenza virus, consisting of two polypeptide chains, HA1 and HA2 [13].) The coding sequence for B/LeeHA contains 12 (3.9% of the total codons) rare isoleucine codons (AUA), including one tandem. The distribution of these codons is illustrated schematically in Fig. 1. The second protein was a mupirocin-resistant (Mup^r) IRS (6). Mupirocin (pseudomonic acid) is used as an antimicrobial agent which is effective against staphylococcal infections; its target is the bacterial IRS (9). The coding sequence for Mup^r IRS contains 33 (3.2% of the total codons) AUA codons, including two tandems (Fig. 2). In both cases, accumulation of these two proteins was low and variable. It was postulated that the poor expression of these proteins was related to the high number of AUA codons and that coexpression of the minor isoleucyl tRNA would relieve the translational stress present during heterologous gene expression in *E. coli*. Toward this end, the minor isoleucyl $tRNA_{LAU}$ (L = lysinylated C) DNA sequence (*ileX*) was synthesized with its own promoter and

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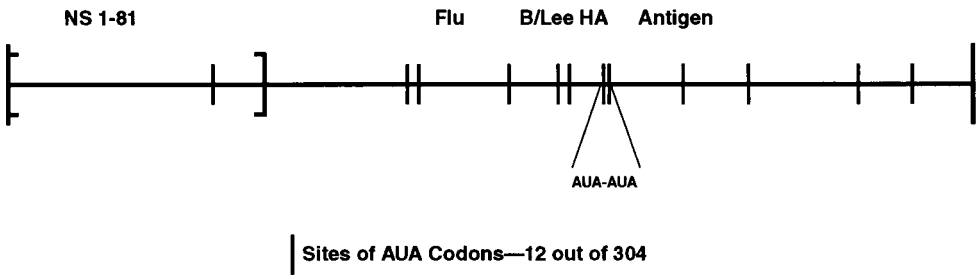

FIG. 1. Schematic diagram of the coding sequence for the influenza B/LeeHA antigen. This sequence codes for a total of 304 amino acids, of which 6.6% (i.e., 20) are isoleucine and 3.9% (i.e., 12) are rare isoleucine (AUA) codons. The bracketed portion corresponds to the first 243 nucleotides (81 amino acids) and reflects the junction between the two sequences in the hybrid message-protein.

termination sequences and inserted into three pBR322-compatible plasmids. These *ileX*-containing plasmids were introduced into *E. coli* expressing either the B/LeeHA or Mup^r IRS. Expression of *ileX* tRNA was qualitatively confirmed by reverse transcriptase PCR (RT-PCR) in the three plasmid vectors, while B/LeeHA expression was monitored by reverse-phase high-performance liquid chromatography (RP-HPLC), and Mup^r IRS expression was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and enzyme activity.

The levels of expression of these proteins were markedly different, but each was enhanced by the presence of the cloned *ileX* gene. The B/LeeHA was expressed fairly well in rich broth (about 16% of total cell protein) but was poorly expressed in defined medium (<3% total cell protein) unless coexpressed with the *ileX* gene (25 to 30% of total cell protein). The difference in expression in rich versus minimal medium was not due to the higher level of isoleucine available in the rich medium since supplementation of minimal medium with isoleucine did not increase expression. The Mup^r IRS, on the other hand, was undetectable in rich medium but accumulated to 7 to 9% of total cell protein when coexpressed with the *ileX* gene. These results clearly demonstrate the negative impact that the AUA codon can have on expression and suggest that problems in expression can be avoided by a careful inspection of the coding sequence and inclusion of appropriate tRNA genes or necessary site-specific mutations.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* K-12 strain LW14 was the host used to express B/LeeHA and was constructed as follows. A P1 phage stock grown on strain AR58 (*thr galE*::Tn*10* *c*I857 *bio uvrB rpsL*) was used to transduce *E. coli* K-12 strain W3110 [F$^-$ IN(*rrnD-rrnE*)1] to tetracycline resistance. Strain LW14 was one such transductant and had the genotype F$^-$ IN(*rrnD-rrnE*)1 *galE*::Tn*10* *c*I857 *bio uvrB*. The gene encoding B/LeeHA was cloned into a pBR322-based vector with a p_L promoter-operator which contained the λ temperature-sensitive *c*I repressor, the *nutL* and the *nutR* transcription antitermination sequences, the *c*II ribosome binding site, the NS181-B/LeeHA fusion gene, the *oop* RNA termination region (28), and the kanamycin resistance gene, NPTII, encoding a kanamycin phosphotransferase (pOTS208B/LeeHA). *E. coli* K-12 strain DH1 (*supE44 recA1 endA1 thi-1 hsdR17 gyrA96 rfbD1 relA1 spoT1*) expressed the Mup^r IRS from the *tac* promoter in plasmid pBROC466. The plasmid pBROC466 was constructed by a combination of PCR and subcloning to contain the Mup^r gene as an *Eco*RI-*Msc*I insert in the *Eco*RI-*Sma*I sites of pDB575, an expression vector containing the *tac* promoter, the *lacI*^{q} gene, and an ampicillin resistance gene (3).

The *ileX* DNA insert (Fig. 3) with the indigenous isoleucyl tRNA promoter and terminator sequences (12) was assembled with synthesized 5′ and 3′ tRNA-specific primers for fill-in by PCR and contained *Hin*dIII ends. The *ileX* gene was inserted into three pBR322-compatible vectors, namely, pDPT489 and pDPT289, differing in copy number only (2 to 4 and 20 to 40 copies per cell, respectively), and pDC952, a pACYC184 derivative containing the *argU* gene (encoding an arginyl tRNA which reads AGG and AGA codons), generously supplied by James Walker at the University of Texas. The *ileX* gene-containing plasmids were termed pI289, pI489, and pRI952.

Culture media and fermentation conditions. The seed medium for shake flasks was Luria-Bertani broth. The seed medium for fermentations involving B/LeeHA was 1× SB which contained the following (in grams per liter of deionized water): tryptone, 12; yeast extract, 24; glycerol, 13; K_2HPO_4, 15.3; KH_2PO_4, 1.7. The pH of the medium was adjusted to 7.0 before sterilization. All shake flask studies involving B/LeeHA were performed with defined medium, which consisted of the following (in grams per liter of deionized water): K_2HPO_4, 6; KH_2PO_4, 3; NH_4SO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 0.48; biotin, 0.001; and glucose, 2. The pH of the medium was adjusted to 7.0 before sterilization. The minimal medium used in the fermentation studies involving B/LeeHA has been detailed previously (1). Where indicated, isoleucine supplements were added to the culture medium to a final concentration of 0.6 g/liter. All fermentation studies involving Mup^r IRS were performed in Luria-Bertani broth.

Studies involving shake flasks were performed in 250-ml Erlenmeyer flasks with a working volume of 50 ml. Cultures were grown at 32°C and 220 rpm for approximately 16 h. At an optical density at 650 nm (OD_{650}) of 0.6, the flasks were transferred to a shaking water bath set at 39.5°C and 220 rpm to inactivate the temperature-sensitive *c*I repressor and thereby induce the production of the B/LeeHA. The induction was carried out for 3 h, and cell pellets were collected at induction start and 3 h postinduction (T_3). Samples were stored at −70°C until analyzed. All fermentations involving B/LeeHA were performed with 15-liter stirred-tank LSL Biolafitte (Princeton, N.J.) fermentors with a working volume of 10 liters. These fermentors are under direct digital control capable of controlling temperature, pH, aeration rate, and agitation rate. Both aeration and agitation rates were increased as needed to maintain the dissolved oxygen level at 25%. The 10-liter fermentors were inoculated with 1 liter of an overnight 1× SB culture containing the appropriate antibiotic(s). The cells were grown in batch culture at 32°C until an OD_{650} of 5.0 was reached. The temperature was raised to 39.5°C to induce the production of B/LeeHA. The fermentations were continued for up to 6 h (T_6). Cell pellets were collected every hour (T_1, T_2, and T_3, etc.) and stored at −70°C until analyzed.

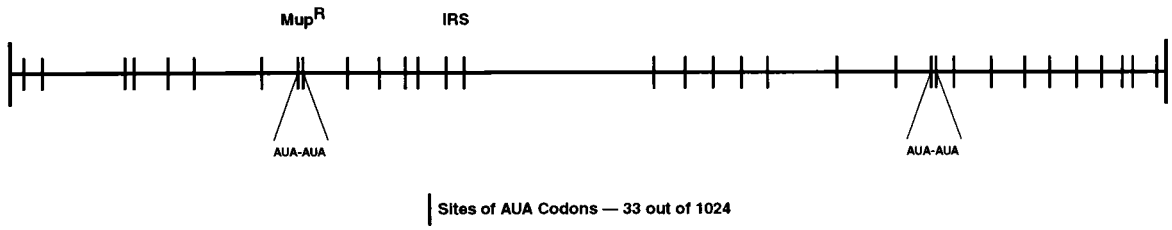

FIG. 2. Schematic diagram of the coding sequence for the Mup^r IRS protein. This sequence codes for a total of 1,024 amino acids, of which 9.0% (i.e., 92) are isoleucine and 3.2% (i.e., 33) are rare isoleucine (AUA) codons.

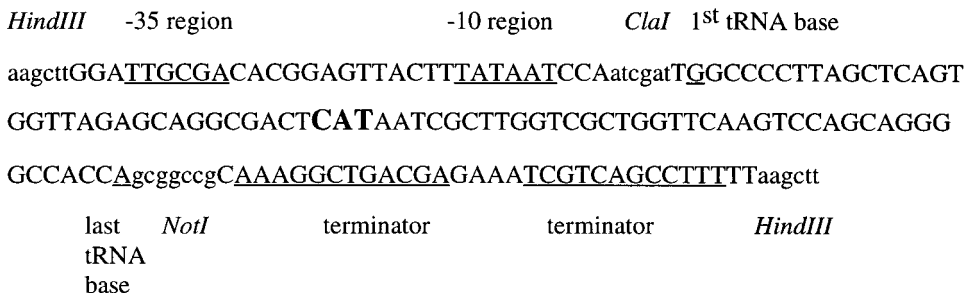

FIG. 3. Gene sequence for the *ileX* tRNA insert. The sense DNA strand only is indicated. Restriction sites, including the 5′ and 3′ *Hin*dIII cloning sites, promoter sites (−10 and −35 regions), anticodon site (**CAT**), and transcription termination sites, are illustrated.

All fermentations involving Mup^r IRS were performed in 2-liter B. Braun Biolab (Allentown, Pa.) fermentors with a working volume of 1.2 liters. These fermentors were operated under the following conditions: temperature at 37°C, agitation at 1.4-m/s tip speed, and aeration of 0.5 vol/vol/min. The primary seed stage was inoculated with an 0.5% (vol/vol) concentration of a liquid-nitrogen-preserved stock culture; the shake flasks were incubated at 37°C and 240 rpm for 4 h. The secondary seed fermentation mixture was inoculated with a 0.1% (vol/vol) concentration of primary seed culture and incubated under the same conditions for 16 h. The final-stage fermentation mixtures were inoculated with a 2% (vol/vol) concentration of secondary shake flask seed. The culture was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1.0 mM when the OD_{550} was approximately 1.0. The fermentation mixtures were incubated for up to 4 h following induction. Expression of Mup^r IRS was evaluated with whole-cell samples taken at regular intervals following induction.

Analytical methods. Total cell protein was measured by the method of Lowry et al. (15) with an automated robotics system (5). Samples for Western blot (immunoblot) analysis were prepared by the method described by Rosenberg and coworkers (19). Western blot analysis was performed by the method of Towbin et al. (25), with the following modifications. A 10% polyacrylamide gel was prepared and loaded to reflect a constant protein concentration. Gels were transblotted onto nitrocellulose, and B/LeeHA was detected with an enhanced chemiluminescence system (Amersham Corp., Arlington Heights, Ill.). A primary rabbit polyclonal antibody specific to the NS181 fusion protein, followed by a secondary goat anti-rabbit horseradish peroxidase-conjugated polyclonal antibody (Cappel Laboratories, Malvern, Pa.), was used. Chemiluminescence was detected by exposure of the blot to Hyperfilm-ECL. The film was developed with an Alphatek AX 700 LE X-ray processor.

RP-HPLC chromatographic separation was performed with a Polymer Laboratories PLRP-S 4,000-Å (400-nm) polymeric reverse-phase column (4.6 by 50 mm). The procedure was developed with a Hewlett-Packard 1090 HPLC System II for automated use, from sample injection to data analysis. The 5-min chromatographic separation provided resolution of the B/LeeHA antigen from host cell proteins, lower-molecular-weight species, and product aggregates. Whole broth samples (1 ml) were subjected to centrifugation, and the cell pellet was suspended in 1 ml of Tris-EDTA (10 mM Tris [pH 7.4] containing 1 mM EDTA) buffer. Lysozyme (1 mg) was added, and the suspension was incubated at 37°C for 15 min. Nuclease treatment (500 U of benzonase [Nycomed Pharma A/S, Copenhagen, Denmark] per ml of culture broth) followed, to eliminate excess nucleic acids, and the suspension was left standing for 5 min at room temperature. Samples (100 μl) were centrifuged in a microcentrifuge and used for RP-HPLC analysis.

An RP-HPLC procedure was used to quantitate isoleucine in *E. coli* supernatant samples. The Hewlett-Packard 1090 AminoQuant HPLC system utilizes precolumn derivatization with *ortho*-phthaldialdehyde and fluorescence detection by diode array for the identification and quantitation of primary and secondary amino acids. The chromatographic separation was performed with a Hewlett-Packard AminoQuant column. Supernatant samples (1 ml) were centrifuged through a 0.45-μm-pore-size cellulose acetate filter unit for 15 min and then through a Millipore Ultrafree-MC low-binding cellulose filter unit for 30 min.

Mup^r IRS SDS-PAGE analysis. Soluble expression of the Mup^r IRS was examined in samples of sonicated cell-free lysates prepared from harvested broth. SDS-PAGE analysis was carried out using a Pharmacia (Piscataway, N.J.) Phast system with 10 to 15% gradient polyacrylamide gels. The percentage expression was determined by scanning stained gels with a Pharmacia (LKB) Phast Image analyzer.

Mup^r IRS activity. The specific activity of the soluble enzyme was determined in cell-free lysates serially diluted twofold to 1:128, from an initial protein concentration of 1 mg/ml, essentially as described previously (7). Mupirocin (5 μM) was added to the diluted samples to inhibit the host's isoleucyl tRNA synthetase. Enzyme activity was determined by measuring the incorporation of ^{14}C-isoleucine into the aminoacyl-tRNA complex over the linear response range of the dilution series.

PCR analysis. Expression of isoleucyl tRNA was confirmed by an RT-PCR method with isoleucyl tRNA-specific 5′ and 3′ primers. Total RNA was isolated from cell pellet samples with the TRIzol reagent system (4). The RNA isolation kit is a single-step extraction with a DNase incubation step (1 h at 37°C) added to ensure elimination of DNA contamination. RT-PCR was performed on a Perkin-Elmer GeneAmp 9600 system. RT-PCR products were visualized on 2% agarose gels stained with ethidium bromide. Each total RNA sample (excluding DNase treatment) was also subjected to PCR amplification with *Taq* DNA polymerase by use of primers made for the original DNA insert which flanked both *ileX* cloning sites. Following *Taq* PCR, samples were visualized on 1% agarose gels stained with ethidium bromide.

RESULTS

Expression of isoleucyl tRNA. RT-PCR was used to confirm, in a qualitative manner, the expression of the synthesized *ileX* tRNA gene. A 74-bp fragment was found in RNA samples isolated from T_6 samples of cells containing pI289, pI489, and pRI952. No such fragment was seen in cultures of cells lacking the cloned *ileX* gene (Fig. 4). Similarly, no fragment was amplified when primers outside of the tRNA coding region were used (data not shown). Since the amplified fragment did not originate from contaminating plasmid DNA, we concluded that there was an increased concentration of the rare isoleucyl tRNA in strains bearing these plasmids.

Expression of B/LeeHA. There did not appear to be any detrimental effect of increased levels of the minor isoleucyl tRNA on preinduction growth rates (32°C) in either shake flasks or fermentors. There were, however, major effects of

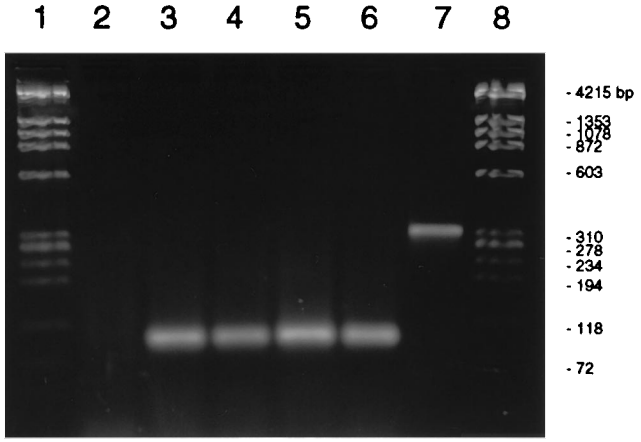

FIG. 4. Confirmation of *ileX* expression by RT-PCR. Total RNA was isolated from T_6 fermentation samples and incubated with primers specific to the tRNA sequence within the *ileX* insert. Lanes: 1 and 8, DNA fragment markers; 2, the negative control sample (no *ileX*-containing plasmid); 3 to 5, RNA from strains carrying the three *ileX*-containing plasmids (pI289, pI489, and pRI952, respectively); 6, pI489-purified plasmid DNA (positive control); 7, RT-PCR kit control.

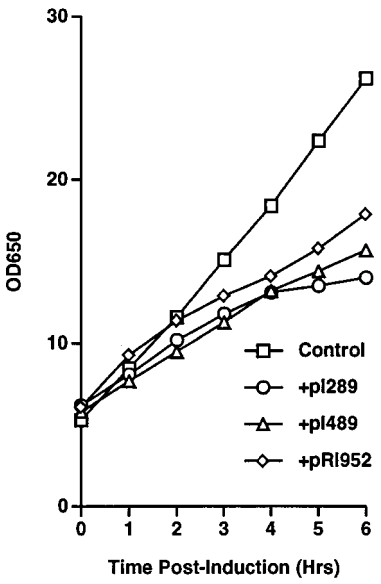

FIG. 5. Growth rates of cells from 10-liter fermentation cultures expressing the influenza B/LeeHA antigen with and without the *ileX* tRNA gene. Cell growth was measured by the OD_{650} at times postantigen induction for 6 h.

these plasmids on the postinduction (39.5°C) physiology. The optical density in the control (no cloned *ileX* gene) increased in a linear fashion at 39.5°C, whereas that of the cultures containing the cloned *ileX* gene did not increase as rapidly and appeared to be leveling off (Fig. 5). While this is clearly an effect of expression of the rare tRNA molecules, that is, dependent upon the level of tRNA, it is manifested as a result of increased expression of the B/LeeHA antigen. Microscopic examination of the cultures indicated that the control cells were small and motile, whereas the cells containing the tRNA vectors were elongated and contained inclusion bodies at their poles (data not shown). These inclusion bodies were typical of those seen in cells expressing various flu hemagglutinins. These qualitative conclusions were supported by quantitative RP-HPLC analysis (Table 1). Expression of B/LeeHA in shake flask cultures was increased two- to threefold when the isoleucyl tRNA was coexpressed (data not shown). This enhancing effect of the isoleucyl tRNA was even more evident in the fermentation cultures. In this case, the level of accumulation increased on the order of 8- to 10-fold. Not surprisingly, the

TABLE 1. Coexpression of *ileX* and flu B/LeeHA in 10-liter fermentation cultures[a]

Sample[b]	OD_{650}	TCP (mg/ml)	B/LeeHA concn (mg/mg of TCP)
Control	29.1	5.01	<0.125
pI289	15.9	2.57	0.31
pI489	15.6	2.74	0.28
pRI952	18.8	2.94	0.22

[a] All values except OD_{650} values are the means of two separate studies. TCP, total cellular protein.
[b] Sample analysis for control cells and for pI289-, pI489-, and pRI952-containing cells was done at 6 h postinduction.

total cell protein for the cultures containing the tRNA vectors was less than that seen in the control cultures (Table 1).

The difference in copy number between the pI289 and pI489 plasmids did not appear to affect B/LeeHA expression levels, suggesting that sufficient isoleucyl tRNA was available even from a plasmid with a low copy number. Alternatively, the *ileX* promoter sequence may be more active on the plasmid than in its native configuration in the chromosome, thereby resulting in higher expression than might be expected from mere copy number considerations. Unexpectedly, the pRI952 plasmid containing both the *argU* and *ileX* genes showed a slightly diminished level of expression compared with that of the other two plasmids (i.e., *ileX* gene alone). While this was not a dramatic effect in shake flasks, the same pattern was observed with the cultures grown in a fermentor and was a significant effect (Table 1). While the implications of this are not clear at this point, the pRI952 vector nevertheless supported accumulation of B/LeeHA to 22% of total cell protein.

Western blot analysis of the B/LeeHA antigen suggested that the presence of the tandem AUA codons did not have a pronounced, if any, effect on the quality of the protein made, as evidenced by the lack of lower-molecular-weight species (Fig. 6). The Western blot results, however, do confirm the increased levels of B/LeeHA in hosts coexpressing the *ileX* gene.

When the host vector was grown and induced in a rich broth, the B/LeeHA antigen represented about 16% of the total cell protein, and coexpression of *ileX* had no effect. To determine whether this increased B/LeeHA expression resulted from an increased availability of isoleucine, the host vector with and without plasmid pI289 was grown in minimal medium supplemented with three different concentrations of isoleucine (150,

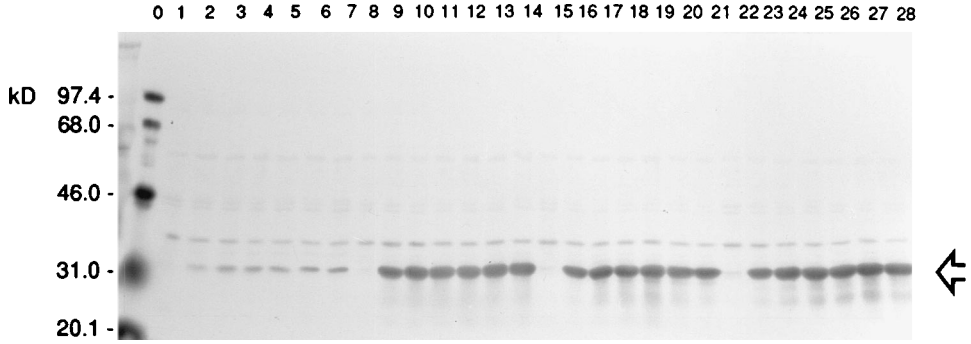

FIG. 6. Western blot analysis of influenza B/LeeHA product quality in 10-liter fermentations. Lanes: 0, molecular mass markers; 1 to 7, control (no cloned *ileX* gene) at T_0, T_1, T_2, T_3, T_4, T_5, and T_6, respectively; 8 to 14, pI289 at T_0, T_1, T_2, T_3, T_4, T_5, and T_6, respectively; 15 to 21, pI489 at T_0, T_1, T_2, T_3, T_4, T_5, and T_6, respectively; 22 to 28, pRI952 at T_0, T_1, T_2, T_3, T_4, T_5, and T_6, respectively. Total cell lysates of equal total cellular protein (20 μg) were loaded. The arrow indicates the influenza B/LeeHA protein band.

TABLE 2. Coexpression of *ileX* and Mup^r IRS in 1.2-liter fermentation cultures[a]

Sample[b]	Mup^r IRS (mg/mg of TCP)	Sp act (U/mg of TCP)
Control	<0.020	0.003
pI489	0.070	0.020
pRI952	0.090	0.090

[a] All values are the means of two separate studies. TCP, total cellular protein.
[b] Sample analysis for control cells and for pI489- and pRI-952-containing cells was done at 6 h postinduction.

300, and 600 mg/liter). There were no significant differences in the level of B/LeeHA in control cultures with or without isoleucine supplementation to the medium (data not shown), even though we confirmed the presence of high levels of isoleucine in the growth medium from T_1 through T_6.

Expression of Mup^r IRS. The Mup^r IRS behind the *tac* promoter was expressed very poorly in *E. coli* K-12 strain DH1, while mupirocin-sensitive (Mup^s) IRS was overexpressed in both *Staphylococcus aureus* and *E. coli*. An examination of the coding sequence for Mup^r IRS indicated that there were a large number of AUA codons with tandem AUAs about 25 and 80% into the coding sequence (Fig. 2), while the Mup^s IRS sequence contained no AUA codons (sequence not shown). When this host vector was grown in Luria-Bertani medium, the Mup^r IRS was <2% of the total cell protein. In the presence of pI489 or pRI952, levels of expression were increased to 7 and 9% of total cell protein, respectively (Table 2). The *argU* gene, which encodes an arginyl tRNA that reads AGG and AGA codons, increased specific activity more than twofold over that of pI489 (Table 2); this was not unexpected since there are 22 AGG and AGA residues in the mRNA.

Fermentation development studies were carried out with various combinations of nitrogen-enriched media. The specific activity of the Mup^r IRS from fermentation samples increased more than twofold under nitrogen source enrichment (data not shown); total Mup^r IRS activity increased nearly sixfold under the same conditions (Fig. 7). The increase in biomass was more dependent on the yeast extract concentration than on the tryptone concentration; however, tryptone influenced the productivity per unit biomass more than yeast extract. As we observed with the B/LeeHA antigen, additional arginine and/or isoleucine in the medium (1 mM) did not improve expression or enzyme activity (data not shown).

DISCUSSION

As the number of genes that are being expressed in *E. coli* continues to increase, the likelihood of encountering mRNA sequences with high numbers of poorly utilized codons will increase. The AUA codon is one such example. It is rarely found in *E. coli* genes, and its cognate tRNA has an unusual modification, lysinylation of the Wobble base in the anticodon. This modification is essential for its biological activity with its cognate amino acid isoleucine. The results of this study unequivocally demonstrate that the AUA codon caused translational problems manifested mainly by reduced levels of expression and that these problems could be ameliorated by coexpressing the cognate tRNA.

It was clear from the RT-PCR results that the tRNA vectors in LW14pOTS208B/LeeHA produced increased levels of isoleucyl tRNA. What was not clear, however, was whether the increased levels of isoleucyl tRNA would be modified properly at the anticodon. While we did not isolate the isoleucyl tRNA and determine that the C residue was lysinylated, the fact that the coexpression of the cloned *ileX* gene positively affected expression of two different proteins inside the two different hosts, LW14 and DH1, indicated that the isoleucyl tRNA was indeed functional and limiting the expression of both B/LeeHA and Mup^r IRS. The absence of an amplified tRNA fragment from the control culture was most probably the result of the low number of PCR cycles used and the presence of only a single copy of the *ileX* gene on the chromosome.

An additional question was examined with the B/LeeHA antigen for which we had an antibody; namely, did the presence of the AUA codons have any effects on the quality of the proteins made. This question was raised because of past experience with AGG and AGA codons which cause in-frame hops (11) and frameshifts (21, 24, 29). If ribosomes were to stall at the tandem AUA codons, then one might expect the ribosome to frameshift either +1 or −1 (14). A +1 frameshift would encounter a UAA stop codon immediately, and a −1 frameshift would reach a UGA stop codon after the addition of two amino acids. In either case, a prematurely terminated polypeptide about 188 amino acids in length would be generated. These polypeptides would be expected to migrate at about 15 to 20 kDa. This observation assumes that the polyclonal antibody used for Western blot detection included specificity for epitopes in the N-terminal 188 amino acids. No such bands were observed on a Western blot (Fig. 6), suggesting that the ribosome did not frameshift at these tandem rare codons. This result is consistent with the recent studies of Goldman et al. (8), who also found no signs of frameshifting when ribosomes stalled at CUA (rare leucine) codons.

Overexpression of tRNA genes has been shown to be deleterious to the cell (17, 27). It was for this reason that the indigenous *ileX* promoter was used to control expression of the tRNA from low-copy-number plasmids. We were, therefore, somewhat concerned when there appeared to be a negative effect of the tRNA plasmids on postinduction cell growth of LW14pOTS208B/LeeHA and DH1pBROC466Mup^r IRS constructs. The control cells reached cell densities about 45% higher than that of any of the tRNA-containing cultures. Upon further examination, however, it was noted that the control cells did not accumulate much of either heterologous protein.

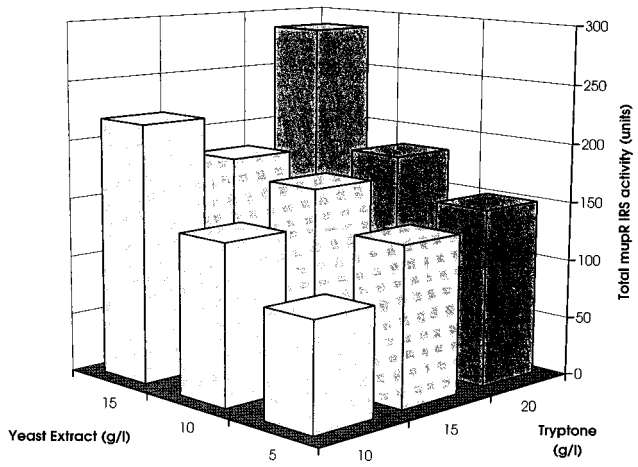

FIG. 7. Total Mup^r IRS activity in 1.2-liter fermentation cultures. Total enzyme was measured in activity units (*z* axis) at 4 h post-Mup^r IRS induction as a function of increasing concentrations of yeast extract (*y* axis) and tryptone (*x* axis).

In the past, we had seen that accumulation of some flu hemagglutinins inhibited cell growth, although the molecular mechanism for this remains unclear. We propose that the continued cell growth seen in this study merely reflects the poor expression and accumulation of B/LeeHA and Mup^r IRS proteins. When the *ileX* tRNA was coexpressed, expression of the proteins significantly increased (Tables 1 and 2), and as a result, cell growth (increase in OD_{650}) was inhibited. Another possibility is that the *ileX* promoter could be more active at higher temperatures, thereby producing more isoleucyl tRNA, which might account for these deleterious effects.

When a gene product with rare codon preferences such as B/LeeHA or Mup^r IRS is expressed at high efficiency, the demand for the acylated cognate tRNA isoacceptors will exceed the supply. The expected result would be ribosomal stalling at these codons. We observed that the B/LeeHA was made at very reasonable levels (about 16% of total cellular protein) when the culture was grown in rich broth. One possible explanation for the poorer performance in minimal medium may be the low pool levels of the cognate amino acid isoleucine. However, isoleucine supplementation of cultures in shake flasks or fermentors did not increase expression, suggesting that the limiting factor was indeed the *ileX* gene product. It is possible that the *ileX* promoter is expressed at a higher level in cells grown in rich medium compared with those grown in a minimal glucose medium. Similarly, enhanced Mup^r IRS activity in the enriched medium may be due to higher levels of isoleucine and arginine, although supplementation with these amino acids failed to increase activity in the cultures. Alternatively, the differences between the media could simply reflect that in rich medium there is much less demand for the endogenous minor isoleucyl tRNA, thereby sparing this tRNA such that it is more available to translate the AUA codons in the heterologous proteins. In minimal medium, there may be more competition for the limited supply of this minor tRNA because of more messages present with AUA codons; thus, the heterologous proteins might be limited in translation.

This research supports the hypothesis that the rare codon AUA directly affects expression of heterologous proteins in *E. coli*. Furthermore, this work provides evidence that the coexpression of minor tRNAs such as *ileX* or *argU* (formerly called *dnaY*) (2) can be utilized to overcome these translational stresses rather than resorting to the synthesis of the genes by use of the host's codon preferences.

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