Enhanced Production of Recombinant Mycobacterium tuberculosis Antigens in Escherichia coli by Replacement of Low-Usage Codons

DAVID L. LAKEY,1,2,3,4,5* RAMA K. R. VOLADRI,2 KATHRYN M. EDWARDS,1 CYNTHIA HAGER,1 BUKA SAMTEN,4 ROBERT S. WALLIS,6 PETER F. BARNES,4,5 AND DOUGLAS S. KERNODLE1,3

Divisions of Infectious Diseases, Departments of Pediatrics1 and Medicine,2 Vanderbilt University School of Medicine, Nashville, Tennessee 37232; Department of Veterans Affairs Medical Center, Nashville, Tennessee 37212; Center for Pulmonary and Infectious Disease Control4 and Departments of Medicine and Cell Biology,5 The University of Texas Health Center at Tyler, Tyler, Texas 75708; and Department of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

Received 22 February 1999/Returned for modification 14 September 1999/Accepted 4 October 1999

A major obstacle to development of subunit vaccines and diagnostic reagents for tuberculosis is the inability to produce large quantities of these proteins. To test the hypothesis that poor expression of some mycobacterial genes in Escherichia coli is due, in part, to the presence of low-usage E. coli codons, we used site-directed mutagenesis to convert low-usage codons to high-usage codons for the same amino acid in the Mycobacterium tuberculosis genes for antigens 85A and 85B and superoxide dismutase. Replacement of five codons in the wild-type gene for antigen 85B increased recombinant protein production in E. coli 54-fold. The recombinant antigen elicited proliferation and gamma interferon production by lymphocytes from healthy tuberculin reactors and was recognized by monoclonal antibodies to native antigen 85, indicating that the recombinant antigen contained T-cell and B-cell epitopes. Northern blotting demonstrated only a 1.7- to 2.5-fold increase in antigen 85B mRNA, suggesting that the enhanced protein production was due primarily to enhanced efficiency of translation. Codon replacement in the genes encoding antigen 85A and superoxide dismutase yielded four- to sixfold increases in recombinant protein production, suggesting that this strategy may be generally applicable to overexpression of mycobacterial genes in E. coli.

Despite widespread administration of bacillus Calmette-Guérin vaccine throughout the world, tuberculosis remains the leading cause of death from a single pathogen (3). Development of subunit tuberculosis vaccines has been spurred by findings that partial immunity is conferred by vaccination of animals with culture filtrate proteins (1, 18), purified antigen (9, 23), and infectors to produce mycobacterial proteins in baculo virus expression systems (2) or to use bacteria that are phylogenetically closer to mycobacteria, such as Streptomyces lividans, Corynebacterium spp., and Mycobacterium smegmatis (7, 10, 16, 26).

When the gene encoding antigen 85B was first sequenced and cloned behind a tac promoter in E. coli, less than 0.5 mg of recombinant antigen 85B per liter was produced (15). Whereas this yield can be improved to 10 mg/liter by using a stronger promoter such as T7 (9), problems with achieving higher-level expression and solubility of antigen 85B have remained. Therefore, investigators studying antigen 85B and many other selected M. tuberculosis antigens have generally purified them from M. tuberculosis (9, 12). This is extremely inefficient, since growth of M. tuberculosis for 2 to 3 weeks in 150 liters of broth culture was required to produce 100 mg of antigen 85B (9, 12). The yield of recombinant antigen 85B per liter can be improved 5- to 10-fold and the time until cultures are harvested can be shortened from weeks to days by overexpression in rapidly growing, nonpathogenic mycobacterial species such as M. smegmatis and Mycobacterium vaccae (10). However, for mycobacterial proteins to be used for large-scale immunization, more efficient means to produce large amounts of these proteins must be developed.

Although several codons can encode the same amino acid, E. coli contains more tRNA for certain high-usage codons than for other low-usage codons. Observations while working with antigens 85A, 85B, and 85C led us to consider the possibility that part of the problem with overexpressing mycobacterial genes in E. coli might derive from problems with translation rather than transcription. In this study, we tested the hypothesis that selective replacement of low-usage E. coli codons in mycobacterial genes by high-usage E. coli codons might enhance production of recombinant mycobacterial proteins. We find that this strategy has a dramatic effect on the yield of antigen 85B, and our experience with other mycobacterial genes suggests that selective codon replacement can enhance the overexpression of a wide variety of mycobacterial proteins in E. coli.

MATERIALS AND METHODS

Bacterial strains and DNA vectors. M. tuberculosis H37Rv (ATCC 25618) was obtained from the American Type Culture Collection, Rockville, Md. E. coli TOP 10 and plasmids pTrcHisB and pRSETB were purchased from Invitrogen (Carlsbad, Calif.). The plasmids pTrcHisB and pRSETB are E. coli expression vectors containing the ampicillin resistance gene, the tac and T7 promoters, respectively, an

* Corresponding author. Mailing address: Center for Pulmonary and Infectious Disease Control, The University of Texas Health Center at Tyler, 11937 U.S. Highway 271, Tyler, TX 75708-3154. Phone: (903) 877-5957. Fax: (903) 877-7989. E-mail: dlakey@uthct.edu.
ATG start codon, the sequence for an N-terminal fusion tag encoding six histidines and a monoclonal antibody (Anti-Xpress; Invitrogen) epitope, and a multiple-cloning site. E. coli JM109 DE3 was obtained from Promega (Madison, Wis.), and phagemid pBCSK+ was purchased from Stratagene (La Jolla, Calif.).

**Cloning mycobacterial genes.** Mycobacterial chromosomal DNA was isolated from *M. tuberculosis* by the freeze-boil method (20) and used as a template for amplification by PCR in a Perkin-Elmer DNA thermal cycler, using oligonucleotide primers based on the DNA sequences of the antigen 85 genes (6, 15) (Table 1). 10% formamide, and vent DNA polymerase (New England Biolabs, Beverly, Mass.). PCR was performed with the following settings: 94°C for 1.5 min, followed by 40 cycles of 94°C for 1 min plus 55°C for 2 min and 72°C for 3 min, and amplification by PCR in a Perkin-Elmer DNA thermal cycler, using oligonucleotide primers noted above, except that the annealing temperature was 55°C. The PCR products were cloned into the phagemid pBCSK+ and transformed into E. coli DH5α, which served as an intermediate vector and host, respectively.

Separate promoters were constructed to amplify the gene encoding each mature exoprotein, without the promoter or leader sequence, from pBCSK+ was used. The PCR products were cloned into E. coli expression plasmids, and the recombinant proteins were resolubilized. Cell pellets from 50-ml cultures following overexpression, purification, and resolubilization of recombinant proteins were resolubilized. Cell pellets from 50-ml cultures following overexpression, purification, and resolubilization of recombinant proteins were resolubilized. The recombinant protein was eluted by lowering the pH to 3.8. Recombinant protein production was quantified spectrophotometrically with an extinction coefficient of A280 of 1.0 for a 1.0-mg/ml protein solution.

For the Western blotting with antibodies to antigen 85 and for the studies evaluating lymphocyte proliferation and gamma interferon production, the recombinant exoproteins were resolubilized. Cell pellets from 50-ml cultures following overexpression, purification, and resolubilization of recombinant proteins were resolubilized. The recombinant proteins were eluted by lowering the pH to 3.8. Recombinant protein production was quantified spectrophotometrically with an extinction coefficient of A280 of 1.0 for a 1.0-mg/ml protein solution.

For the Western blotting with antibodies to antigen 85 and for the studies evaluating lymphocyte proliferation and gamma interferon production, the recombinant exoproteins were resolubilized. Cell pellets from 50-ml cultures following overexpression, purification, and resolubilization of recombinant proteins were resolubilized. The recombinant proteins were eluted by lowering the pH to 3.8. Recombinant protein production was quantified spectrophotometrically with an extinction coefficient of A280 of 1.0 for a 1.0-mg/ml protein solution.

For the Western blotting with antibodies to antigen 85 and for the studies evaluating lymphocyte proliferation and gamma interferon production, the recombinant exoproteins were resolubilized. Cell pellets from 50-ml cultures following overexpression, purification, and resolubilization of recombinant proteins were resolubilized. The recombinant proteins were eluted by lowering the pH to 3.8. Recombinant protein production was quantified spectrophotometrically with an extinction coefficient of A280 of 1.0 for a 1.0-mg/ml protein solution.

TABLE 1. Primers used for cloning and site-directed mutagenesis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Nucleotide sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>85A</td>
<td>85A left</td>
<td>GAT GAA TTC GCG GAA ATG CCA CTT TCA G</td>
</tr>
<tr>
<td>85A</td>
<td>85A right</td>
<td>GAT GGA TTC AGA TGT GTC TGT TGC GAG</td>
</tr>
<tr>
<td>85A</td>
<td>85A left 2</td>
<td>GATGATCCGA(12)TTT CCG CCG GGG GGT CCG GTG(24)</td>
</tr>
<tr>
<td>85A</td>
<td>85A right 2</td>
<td>GAT GAA TTC GCA CTT CCG CCG GGG GGT CCG GTG(24)</td>
</tr>
<tr>
<td>85A</td>
<td>85A left mut 9</td>
<td>GATGATCCGA(12)TTT CCG CCG GGG GGT CCG GTG(24)</td>
</tr>
<tr>
<td>85A</td>
<td>85A mut 339</td>
<td>A(357)GCC GCG CTT AAC GTG ATG AGC GTT CCG CCA GCC(319)</td>
</tr>
<tr>
<td>85A</td>
<td>85AR mut 9</td>
<td>A(357)GCC GCG CTT AAC GTG ATG AGC GTT CCG CCA GCC(319)</td>
</tr>
<tr>
<td>85B</td>
<td>85B left</td>
<td>GAT GAA TTC GCT CGA GTA ATA TCA CTT GAG</td>
</tr>
<tr>
<td>85B</td>
<td>85B right</td>
<td>GAT GGA TTC GCA CTT CCG CCG GGG GGT CCG GTG(24)</td>
</tr>
<tr>
<td>85B</td>
<td>85B left 9/15</td>
<td>GATGATCCGA(12)TTT CCG CCG GGG GGT CCG GTG(24)</td>
</tr>
<tr>
<td>85B</td>
<td>85B R 627 mut</td>
<td>A(627)ACCA CCA CAG AGC GTT GCT TGC CAG CTT GGG GAG GTT CTT(585)</td>
</tr>
<tr>
<td>85B</td>
<td>85B probe L</td>
<td>A(613)GCG CTG TGG GTT TAT TGG TGC GGC ACC CCC(647)</td>
</tr>
<tr>
<td>85B</td>
<td>85B probe R</td>
<td>A(612)GTC GGG TGG CAG CAC GTA CCG GCC TTC GTA(682)</td>
</tr>
<tr>
<td>85C</td>
<td>85C left</td>
<td>GAT GAA TTC GCT GGG ATT GAT AGT AGC TAT GAC</td>
</tr>
<tr>
<td>85C</td>
<td>85C right</td>
<td>GATGATCCGA(12)TTT CCG CCG GGG GGT CCG GTG(24)</td>
</tr>
<tr>
<td>85C</td>
<td>85C left 2</td>
<td>GATGATCCGA(12)TTT CCG CCG GGG GGT CCG GTG(24)</td>
</tr>
<tr>
<td>85C</td>
<td>85C right 2</td>
<td>GAT GAA TTC GCT GGG ATT GAT AGT AGC TAT GAC</td>
</tr>
<tr>
<td>SOD</td>
<td>SOD left</td>
<td>GATGATCCGA(12)TTT CCG CCG GGG GGT CCG GTG(24)</td>
</tr>
<tr>
<td>SOD</td>
<td>SOD right</td>
<td>GATGATCCGA(12)TTT CCG CCG GGG GGT CCG GTG(24)</td>
</tr>
<tr>
<td>SOD</td>
<td>SOD Lmut 192</td>
<td>G(192)CAC GAG ACC GGC GTC GTT AAC ACT GGC(213)</td>
</tr>
<tr>
<td>SOD</td>
<td>SOD Rmut 192</td>
<td>G(201)GAG GGT GAA ACC CAG GAT TTT TTT CAG CAC(175)</td>
</tr>
<tr>
<td>SOD</td>
<td>SOD Lmut 414</td>
<td>C(406)GCT ATC TCC CAG TAC GGT(429)</td>
</tr>
<tr>
<td>SOD</td>
<td>SOD Rmut 414</td>
<td>G(426)GAT CCG CAA CCG AGA GGC GGC GGC(482)</td>
</tr>
</tbody>
</table>

a Nucleotides are numbered beginning with the first base in the portion of the gene encoding the mature exoprotein. Bases corresponding to nucleotide substitutions introduced by site-directed mutagenesis are underlined.
transformation of the mycobacterial genes into E. coli strain TOP 10 were cloned into an M. tuberculosis gene was confirmed by endonuclease restriction and DNA cloning antibody to the N-terminal polyhistidine tag.

42°C in the same buffer containing 2 ng of the32P-labeled probe. The membrane was prehybridized for 4 h at 42°C in a 0.15-ml/cm² buffer solution containing 1× Denhardt’s solution, 6× SSC, 100 μg of salmon sperm per ml, and 0.5% SDS in 20 mM sodium phosphate (pH 7.4). Hybridization was performed for 18 h at 42°C in the same buffer containing 2 ng of the32P-labeled probe. The membrane was washed, and DNA-RNA hybridization was detected with X-Omat AR film (Eastman Kodak, Rochester, N.Y.) and quantified with a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, Calif.).

Western blotting. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes with a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, Calif.) by standard methods (8). After blocking with a solution containing 10 mM Tris (pH 8.0), 0.5 M sodium chloride, and 0.5% Tween 20, the nitrocellulose was incubated with the monoclonal antibodies to the N-terminal polyhistidine tag. The membrane was washed, and DNA-RNA hybridization was detected with X-Omat AR film (Eastman Kodak, Rochester, N.Y.) and quantified with a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, Calif.).

Lympocyte proliferative responses and gamma interferon production. Peripheral blood mononuclear cells (PBMC) from four healthy subjects with tuberculin reactions were isolated and cultured by standard methods (24) and plated in triplicate 200-μl wells at 2 × 10⁶ cells/well in medium alone, with heat-killed M. tuberculosis Erdman (10 μg/ml), or with increasing concentrations of recombinant antigens 85A, 85B, and 85C. Proliferative responses were determined by measurement of [3H]thymidine incorporation (24). Standard errors of the mean were <20% of mean values in all cases.

Previous studies have shown that gamma interferon concentrations produced in the preceding threonicine codon (ACC to ACG) to add a MluI endonuclease restriction site, which produced a rapid means to screen for desired mutants. Furthermore, we changed nucleotides 7 to 15 from CCG-CGG-GGG, which codes for arginine-proline-glycine, to CGT-CTG, which codes for the same amino acids. By substituting these five high-usaged codons for low-usaged codons in the wild-type 85B gene in a manner that did not alter the amino acid sequence, the expression of recombinant antigen 85B improved more than 50-fold from less than 0.5 to 27 mg/liter (Fig. 2). To confirm the effects of codon replacement on expression of a second mycobacterial gene, we substituted high-usaged codons for low-usaged codons in the antigen 85B gene (Table 2) (25).

Enhanced expression of antigens 85A and 85B by codon replacement. Differential production of antigens 85A, 85B, and 85C occurred despite the high degree of homology of the antigen 85 genes. Because the growth curves of the E. coli transformants producing each antigen were indistinguishable, poor expression of antigen 85B was unlikely to be due to its toxic effects on E. coli. Furthermore, since each gene was cloned downstream of the trc promoter, transcription of each gene should be comparable. Therefore, the most likely reason for the observed differences in production of recombinant protein was differential mRNA stability or differential efficiency of mRNA translation.

Examination of the antigen 85 genes revealed that the gene for 85B contains more low-usaged codons that are inefficiently translated in E. coli than the antigen 85A gene (Table 2) (25). We hypothesized that the differences in recombinant protein yields were due to these differences in the distribution of low-usaged codons. To test our hypothesis, we first identified two adjacent low-usaged codons in the antigen 85B gene, CCG and CTA at nucleotides 616 to 621, coding for arginine and leucine, respectively. Using PCR-based site-directed mutagenesis (11), we changed these low-usaged codons to the high-usaged codons CTT and CTG, which encode the same amino acids. We produced a silent mutation in the preceding threonicine codon (ACC to ACG) to add a MluI endonuclease restriction site, which produced a rapid means to screen for desired mutants. Furthermore, we changed nucleotides 7 to 15 from CCG-CGG-GGG, which codes for arginine-proline-glycine, to CTG-CGG-GGT, which codes for the same amino acids. By substituting these five high-usaged codons for low-usaged codons in the wild-type 85B gene in a manner that did not alter the amino acid sequence, the expression of recombinant antigen 85B improved more than 50-fold from less than 0.5 to 27 mg/liter (Fig. 2). To confirm the effects of codon replacement on expression of a second mycobacterial gene, we substituted high-usaged codons for low-usaged codons in the antigen 85A gene as follows: (i) CTG (arginine) was substituted for CCG (arginine) at nucleotides 7 to 9; (ii) CTG (arginine) was substituted for CCA (serine) at nucleotides 10 to 12; and (iii) the remaining low-usaged codons were substituted for high-usaged codons at nucleotides 13 to 15. These results indicated that the differences in expression of recombinant antigens 85A and 85B were due to the differences in the distribution of low-usaged codons in these genes.
AGG (arginine) at nucleotides 337 to 339; (iii) GTT (valine) was substituted for GTC (valine) at nucleotides 343 to 345; (iv) CCG (proline) was substituted for CCC (proline) at nucleotides 349 to 351; (v) GGT (glycine) was substituted for GGA (glycine) at nucleotides 355 to 358. These substitutions increased the yield of antigen 85A from 20 to 80 mg/liter (Fig. 2).

Codon replacement in the SOD gene. The SOD gene (26) was amplified by PCR from H37Rv genomic DNA, sequenced, and cloned behind the trc promoter. Using the induction methods outlined above, recombinant SOD protein was undetectable. When the SOD gene was cloned behind the T7 promoter, only 8 mg of recombinant protein per liter was produced. The low-usage asparagine and leucine codons AAT and CTG at nucleotides 190 to 195 and the isoleucine codon ATA at positions 412 to 414 were exchanged for the high-usage codons AAC, CTG, and ATC, respectively. When this synthetic gene was cloned behind the T7 promoter and transformed into E. coli JM109 DE3, recombinant protein production increased sixfold to 50 mg/liter (Fig. 2).

Production of recombinant antigen 85B behind the T7 promoter. The experiments outlined above were performed with the synthetic antigen 85 genes ligated behind the trc promoter. To characterize protein production with another promoter, the synthetic genes were ligated behind the T7 promoter in the plasmid pRSETB, which was transformed into E. coli JM109 DE3 and induced to overexpress by adding IPTG. This expression system yielded over 250 mg of antigen 85B per liter, which was approximately 50% of the total protein produced by the transformant. Similar yields were obtained with the synthetic antigen 85A gene behind the T7 promoter (data not shown).

Recognition of recombinant antigens by monoclonal antibodies. To confirm the identity of the proteins produced by the synthetic antigen 85A and 85B genes, Western blotting was performed with the TBC-27 antibody, which was raised against antigen 85B (21) and reacts more strongly with native antigen 85B than with antigen 85A (Robert Wallis, unpublished data). TBC-27 reacted with recombinant antigens 85A and 85B in a manner parallel to that seen with the respective native antigens (Fig. 3). A second anti-antigen 85 monoclonal antibody, HYT27, also reacted with both recombinant antigens 85A and 85B (data not shown).

Effect of codon replacement on mRNA expression. We hypothesized that replacement of low-usage codons enhanced translation of mRNA of the mycobacterial genes. To evaluate the alternative possibility that mRNA production or stability was increased by codon replacement, Northern hybridization was performed. Cultures of the E. coli TOP 10 expression vectors containing either the wild-type or synthetic antigen 85B gene cloned into pTrcHisB were grown to an A600 of 0.6 and induced with IPTG. Total cellular RNA was isolated from 1-ml aliquots from each culture harvested at 0 min, 30 min, 1 h, and 5 h, postinduction. Northern hybridization, using a 594-bp radiolabeled probe amplified from the unmodified region of the wild-type antigen 85B gene, demonstrated a 1.7- to 2.5-fold increase in antigen 85B mRNA concentration at all time points postinduction for the expression system containing the synthetic gene (Table 3). Thus, the 50-fold increase in recombinant protein production seen by replacing low-usage codons is not explained by increased mRNA production or stability.

T-cell responses elicited by recombinant antigens 85A and 85B. To determine if the recombinant antigens 85A and 85B contained epitopes recognized by human T cells, we cultured PBMC from four healthy subjects with tuberculin reactions with 1 to 100 μg of recombinant antigens 85A and 85B per ml from which endotoxin had been removed. Cells were also cultured in medium alone or with 10 μg of heat-killed M. tuberculosis per ml as controls. The concentration of recombinant antigen that yielded optimal proliferation varied widely in different donors, and the maximal responses are shown in Fig. 4A. Significant responses to antigen 85A were found in two subjects (delta cpm of 4,248 and 51,806, with stimulation indices of 21 and 193, respectively). Proliferative responses to antigen 85B were observed in three subjects (delta cpm of 3,457 to 18,782, with stimulation indices of 17 to 70). PBMC from all subjects proliferated in response to M. tuberculosis, with a delta cpm of 20,854 to 135,319 and stimulation indices of 46 to 497. No significant proliferation was observed in response to antigens 85A and 85B in two healthy tuberculin-negative donors (Fig. 4A).

![FIG. 2. Coomassie blue-stained PAGE gels of recombinant antigens recovered from E. coli containing the wild-type and mutant genes for antigen 85A (top), 85B (middle), and SOD genes (bottom). Lane numbers represent the fractions that were eluted from the nickel resin affinity column. The protein on the gel was harvested from 0.5 ml of the original broth culture.](http://iai.asm.org/)
pressed behind protein yield. Our finding that only very small amounts of tetanus toxin C resulted in a threefold increase in recombinant 85B out of 452 codons (62%) in the open reading frame of the tetanus toxin C fragment (14). In this case, substitution has been reported to increase recombinant protein yield of prokaryotic bacteria has not been explored very well. To our knowledge, the only part of the antigen 85B gene corresponding to the mature extracellular protein increased the yield of recombinant antigen 85B 54-fold, compared to that of the wild-type gene. Furthermore, when this synthetic antigen 85B gene was cloned behind the T7 promoter, more than 250 mg of recombinant protein per liter was produced; this comprised about 50% of the total protein produced by the bacterial culture. Codon substitution also increased production of antigen 85A and SOD four- to sixfold, suggesting that this strategy may be generally applicable to overexpression of mycobacterial genes in E. coli.

Although replacement of low-usage codons increased expression of antigen 85B markedly, it was produced primarily as insoluble inclusions. A urea-based resolubilization protocol, similar to methods used in refolding other recombinant proteins, resulted in production of soluble recombinant antigen 85, which elicited proliferation and gamma interferon production by PBMC from healthy subjects with tuberculin reactions. It has been postulated that low-usage codons inhibit protein production through ribosome pausing and resultant exposure of RNA to RNA endonuclease activity or rho-dependent RNA polymerase termination (4, 17, 19, 22). In E. coli, as in all prokaryotic organisms, transcription and translation are coupled. Therefore, when a ribosome pauses to find a rare tRNA, the length of exposed mRNA between it and the preceding ribosome or transcribing RNA polymerase lengths, decreasing stability and production of mRNA. In contrast, our data substituting one of them (CGG-CTA) along with three other low-usage codons in the portion of the antigen 85B gene corresponding to the mature extracellular protein increased the yield of recombinant antigen 85B 54-fold, compared to that of the wild-type gene. Furthermore, when this synthetic antigen 85B gene was cloned behind the T7 promoter, more than 250 mg of recombinant protein per liter was produced; this comprised about 50% of the total protein produced by the bacterial culture. Codon substitution also increased production of antigen 85A and SOD four- to sixfold, suggesting that this strategy may be generally applicable to overexpression of mycobacterial genes in E. coli.

**DISCUSSION**

Striking advances have been made in our understanding of the basic biology and immunology of tuberculosis in the past decade, capped by sequencing of the genome of M. tuberculosis (5). These advances will lead to identification of mycobacterial proteins that may be useful vaccine components or diagnostic reagents. However, a critical obstacle is the inability to produce large quantities of these proteins, using a laboratory organism such as E. coli, that does not require stringent containment precautions. We found that selective replacement of E. coli low-usage codons by high-usage codons in mycobacterial genes markedly enhanced production of recombinant mycobacterial proteins in E. coli. These recombinant proteins stimulated proliferation and gamma interferon production by human mononuclear cells and were recognized by monoclonal antibodies to the native mycobacterial proteins, indicating that the recombinant proteins contained T-cell and B-cell epitopes and are likely to be immunogenic. The strategy of selective codon replacement has the potential to greatly facilitate production of mycobacterial proteins for development of vaccines and diagnostic reagents.

Although codon usage preference is a recognized problem in expressing eukaryotic proteins in prokaryotic cells, the utility of codon exchange in expressing prokaryotic proteins in other prokaryotic bacteria has not been explored very well. To our knowledge, the only part of the antigen 85B gene in which codon replacement has been reported to increase recombinant protein yield is the tetanus toxin C fragment (14). In this case, substitution of 280 out of 452 codons (62%) in the open reading frame of tetanus toxin C resulted in a threefold increase in recombinant protein yield. Our finding that only very small amounts of recombinant antigen 85B are produced when its gene is expressed behind trc in E. coli confirms the earlier report of Matsuo et al., who estimated a yield of 0.2 to 0.4 mg/liter (15).

*pressed behind protein yield. Our finding that only very small amounts of tetanus toxin C resulted in a threefold increase in recombinant 85B out of 452 codons (62%) in the open reading frame of the tetanus toxin C fragment (14). In this case, substitution has been reported to increase recombinant protein yield of prokaryotic bacteria has not been explored very well. To our knowledge, the only part of the antigen 85B gene corresponding to the mature extracellular protein increased the yield of recombinant antigen 85B 54-fold, compared to that of the wild-type gene. Furthermore, when this synthetic antigen 85B gene was cloned behind the T7 promoter, more than 250 mg of recombinant protein per liter was produced; this comprised about 50% of the total protein produced by the bacterial culture. Codon substitution also increased production of antigen 85A and SOD four- to sixfold, suggesting that this strategy may be generally applicable to overexpression of mycobacterial genes in E. coli.

Although replacement of low-usage codons increased expression of antigen 85B markedly, it was produced primarily as insoluble inclusions. A urea-based resolubilization protocol, similar to methods used in refolding other recombinant proteins, resulted in production of soluble recombinant antigen 85, which elicited proliferation and gamma interferon production by PBMC from healthy subjects with tuberculin reactions. It has been postulated that low-usage codons inhibit protein production through ribosome pausing and resultant exposure of RNA to RNA endonuclease activity or rho-dependent RNA polymerase termination (4, 17, 19, 22). In E. coli, as in all prokaryotic organisms, transcription and translation are coupled. Therefore, when a ribosome pauses to find a rare tRNA, the length of exposed mRNA between it and the preceding ribosome or transcribing RNA polymerase lengths, decreasing stability and production of mRNA. In contrast, our data substituting one of them (CGG-CTA) along with three other low-usage codons in the portion of the antigen 85B gene corresponding to the mature extracellular protein increased the yield of recombinant antigen 85B 54-fold, compared to that of the wild-type gene. Furthermore, when this synthetic antigen 85B gene was cloned behind the T7 promoter, more than 250 mg of recombinant protein per liter was produced; this comprised about 50% of the total protein produced by the bacterial culture. Codon substitution also increased production of antigen 85A and SOD four- to sixfold, suggesting that this strategy may be generally applicable to overexpression of mycobacterial genes in E. coli.

Although replacement of low-usage codons increased expression of antigen 85B markedly, it was produced primarily as insoluble inclusions. A urea-based resolubilization protocol, similar to methods used in refolding other recombinant proteins, resulted in production of soluble recombinant antigen 85, which elicited proliferation and gamma interferon production by PBMC from healthy subjects with tuberculin reactions.

It has been postulated that low-usage codons inhibit protein production through ribosome pausing and resultant exposure of RNA to RNA endonuclease activity or rho-dependent RNA polymerase termination (4, 17, 19, 22). In E. coli, as in all prokaryotic organisms, transcription and translation are coupled. Therefore, when a ribosome pauses to find a rare tRNA, the length of exposed mRNA between it and the preceding ribosome or transcribing RNA polymerase lengths, decreasing stability and production of mRNA. In contrast, our data substituting one of them (CGG-CTA) along with three other low-usage codons in the portion of the antigen 85B gene corresponding to the mature extracellular protein increased the yield of recombinant antigen 85B 54-fold, compared to that of the wild-type gene. Furthermore, when this synthetic antigen 85B gene was cloned behind the T7 promoter, more than 250 mg of recombinant protein per liter was produced; this comprised about 50% of the total protein produced by the bacterial culture. Codon substitution also increased production of antigen 85A and SOD four- to sixfold, suggesting that this strategy may be generally applicable to overexpression of mycobacterial genes in E. coli.

Although replacement of low-usage codons increased expression of antigen 85B markedly, it was produced primarily as insoluble inclusions. A urea-based resolubilization protocol, similar to methods used in refolding other recombinant proteins, resulted in production of soluble recombinant antigen 85, which elicited proliferation and gamma interferon production by PBMC from healthy subjects with tuberculin reactions.

It has been postulated that low-usage codons inhibit protein production through ribosome pausing and resultant exposure of RNA to RNA endonuclease activity or rho-dependent RNA polymerase termination (4, 17, 19, 22). In E. coli, as in all prokaryotic organisms, transcription and translation are coupled. Therefore, when a ribosome pauses to find a rare tRNA, the length of exposed mRNA between it and the preceding ribosome or transcribing RNA polymerase lengths, decreasing stability and production of mRNA. In contrast, our data substituting one of them (CGG-CTA) along with three other low-usage codons in the portion of the antigen 85B gene corresponding to the mature extracellular protein increased the yield of recombinant antigen 85B 54-fold, compared to that of the wild-type gene. Furthermore, when this synthetic antigen 85B gene was cloned behind the T7 promoter, more than 250 mg of recombinant protein per liter was produced; this comprised about 50% of the total protein produced by the bacterial culture. Codon substitution also increased production of antigen 85A and SOD four- to sixfold, suggesting that this strategy may be generally applicable to overexpression of mycobacterial genes in E. coli.

Although replacement of low-usage codons increased expression of antigen 85B markedly, it was produced primarily as insoluble inclusions. A urea-based resolubilization protocol, similar to methods used in refolding other recombinant proteins, resulted in production of soluble recombinant antigen 85, which elicited proliferation and gamma interferon production by PBMC from healthy subjects with tuberculin reactions.

It has been postulated that low-usage codons inhibit protein production through ribosome pausing and resultant exposure of RNA to RNA endonuclease activity or rho-dependent RNA polymerase termination (4, 17, 19, 22). In E. coli, as in all prokaryotic organisms, transcription and translation are coupled. Therefore, when a ribosome pauses to find a rare tRNA, the length of exposed mRNA between it and the preceding ribosome or transcribing RNA polymerase lengths, decreasing stability and production of mRNA. In contrast, our data substituting one of them (CGG-CTA) along with three other low-usage codons in the portion of the antigen 85B gene corresponding to the mature extracellular protein increased the yield of recombinant antigen 85B 54-fold, compared to that of the wild-type gene. Furthermore, when this synthetic antigen 85B gene was cloned behind the T7 promoter, more than 250 mg of recombinant protein per liter was produced; this comprised about 50% of the total protein produced by the bacterial culture. Codon substitution also increased production of antigen 85A and SOD four- to sixfold, suggesting that this strategy may be generally applicable to overexpression of mycobacterial genes in E. coli.

Although replacement of low-usage codons increased expression of antigen 85B markedly, it was produced primarily as insoluble inclusions. A urea-based resolubilization protocol, similar to methods used in refolding other recombinant proteins, resulted in production of soluble recombinant antigen 85, which elicited proliferation and gamma interferon production by PBMC from healthy subjects with tuberculin reactions.
indicate that increased production of recombinant protein by codon replacement was not due to increased mRNA production or stability, as mRNA concentrations were increased a maximum of 2.5-fold, whereas protein production increased over 50-fold. This suggests that the major detrimental effect of low-usage codons is decreased translational efficiency. When low-usage codons are encountered, the corresponding tRNA is difficult to locate, and the resultant prolonged ribosomal pause may cause ribosomal instability, disassociation of the ribosome-mRNA complex, and termination of protein production. Codon substitution increased production of recombinant antigen 585B 54-fold but increased production of 85A only 4-fold, even though the same numbers of low-usage codons were replaced. As the antigen 85A and 85B genes contained DNA encoding the same 3-kb fusion protein at the 5′ end just behind the promoter, this marked difference was not due to ribosomal binding or synthesis initiation. The low-usage codons in the antigen 85B gene were adjacent, whereas those in the 85A gene were staggered or isolated, suggesting that adjacent low-usage codons more drastically reduced translational efficiency.

Secreted mycobacterial proteins are believed to be important in engendering protective immune responses, in comparison to cytoplasmic proteins. Harth and colleagues described an overexpression system in which M. smegmatis produced and secreted M. tuberculosis proteins which conferred protective immunity against tuberculosis (10). Our overexpression system yielded proteins that were not exported, and it may be advantageous to develop systems that yield proteins secreted by E. coli, since such proteins may be more likely to mimic the native mycobacterial antigens.

In summary, we demonstrated that selective codon replacement of mycobacterial genes can markedly enhance production of recombinant proteins in E. coli. Widespread application of this strategy has the potential to facilitate production of many mycobacterial proteins for basic research, as well as for development of vaccines and diagnostic reagents.

ACKNOWLEDGMENTS

D.L.L. was supported by the Pediatric Infectious Disease Society through a grant sponsored by Merck, by NIH training grant T 32 AI 07474, and by the Cain Foundation Endowment for Infectious Disease Research. D.S.K. was supported by NIH grant AI35250 and a grant from the Research Service of the Department of Veterans Affairs. K.M.E. was supported by NIH grant AI37671. P.F.B. was supported by NIH grant AI27285 and holds the Margaret E. Byers Cain Chair for Tuberculosis Research.

We thank Thomas Shinnick for providing the HYT27 monoclonal antibody.

REFERENCES


Editor: S. H. E. Kaufmann