

Simple and Efficient Method for Heterologous Expression of Clostridial Proteins

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Many clostridial proteins are poorly produced in *Escherichia coli*. It has been suggested that this phenomena is due to the fact that several types of codons common in clostridial coding sequences are rarely used in *E. coli* and the quantities of the corresponding tRNAs in *E. coli* are not sufficient to ensure efficient translation of the corresponding clostridial sequences. To address this issue, we amplified three *E. coli* genes, *ileX*, *argU*, and *leuW*, in *E. coli*; these genes encode tRNAs that are rarely used in *E. coli* (the tRNAs for the ATA, AGA, and CTA codons, respectively). Our data demonstrate that amplification of *ileX* dramatically increased the level of production of most of the clostridial proteins tested, while amplification of *argU* had a moderate effect and amplification of *leuW* had no effect. Thus, amplification of certain tRNA genes for rare codons in *E. coli* improves the expression of clostridial genes in *E. coli*, while amplification of other tRNAs for rare codons might not be needed for improved expression. We also show that amplification of a particular tRNA gene might have different effects on the level of protein production depending on the prevalence and relative positions of the corresponding codons in the coding sequence. Finally, we describe a novel approach for improving expression of recombinant clostridial proteins that are usually expressed at a very low level in *E. coli*.

Clostridial proteins, such as tetanus toxin and seven serologically distinct botulinum neurotoxins (botulinum neurotoxin serotype A [BoNT/A], BoNT/B, BoNT/C, BoNT/D, BoNT/E, BoNT/F, and BoNT/G) that are produced by *Clostridium tetani*, *Clostridium botulinum*, *Clostridium argentiensis*, and *Clostridium baratti*, are powerful tools for studying the mechanisms of synaptic vesicle exocytosis (3, 20–23). These toxins have been also used for therapeutic purposes, such as the treatment of strabismus, blepharospasms (24, 25), and many other neurological conditions, as well as in clinical dermatology (4).

Currently, BoNT/A and other clostridial neurotoxins and their fragments are purified from native *Clostridium* strains by using traditional purification protocols. Because these microorganisms are anaerobes, they pose technical problems. In addition, gene manipulation methods have not been developed for these microorganisms. Therefore, it has been difficult to construct *Clostridium* strains that produce derivatives of neurotoxins and other proteins. Genes for all eight clostridial neurotoxins have been cloned, and their sequences have been identified (2, 6, 9, 18, 30, 31). Many attempts to express fragments of clostridial neurotoxins in *Escherichia coli* have failed because of the unusually high AT content of clostridial DNA. Makoff et al. successfully expressed a tetanus toxin fragment in *E. coli* (12) by optimizing sequences for codon usage in *E. coli* by complete synthesis of these sequences de novo. This approach, however, is very laborious and expensive.

Recently, several groups of workers have demonstrated that rarely used codons can have a pronounced effect on the translation efficiency of cloned genes in *E. coli* (5, 8, 26). Molecular studies have shown that the ATA, AGA, and CTA codons are rarely used in *E. coli*. At the same time, these codons are abundant in clostridial genes. To investigate the impact of these codons on translation of clostridial genes in *E. coli*, we amplified in *E. coli* the *ileX*, *argU*, and *leuW* genes (7, 11, 14,

15), which encode tRNAs that translate the ATA, AGA, and CTA codons, respectively. We demonstrated that amplification of the *ileX* gene resulted in dramatic increases in production of most of the clostridial proteins tested. Indeed, when we examined fragments of tetanus toxin, BoNT/A, BoNT/B, BoNT/C, BoNT/E, the Ia protein of *Clostridium perfringens* iota toxin, and the C3 protein from *C. botulinum*, we observed significant increases in production in *E. coli* for all of these proteins except C3. Amplification of the *argU* gene also had moderate positive effects on the levels of production of these proteins. Amplification of the *leuW* gene, however, did not have a noticeable effect.

MATERIALS AND METHODS

Cells and plasmids. *E. coli* JM109 cells were used to propagate plasmids. *E. coli* BL21(λDE3) was used for expression of recombinant proteins (29).

The pGEM-T vector (Promega) was used to clone PCR products. The vectors pETA32-22, pET28b(+), pPhe23-1, and pETSynB53Km were used to construct expression plasmids encoding clostridial proteins, and the vector pACYC184 was used to amplify genes for tRNA. Plasmid pET28b(+) is a commercial vector obtained from Novagen. Plasmid pETA32-22 is a derivative of pET3b (23) that encodes mutagenized fragment A of diphtheria toxin (unpublished data). Plasmid pPhe23-1 was constructed previously and contains a sequence encoding a *Pseudomonas* exotoxin A derivative (33). Both pETA32-22 and pPhe23-1 were used in this study because they contain a promoter of bacteriophage T7 and efficient signals for initiation of translation. Plasmids pETSynB53Km and pETPA5 were constructed previously by using plasmid pET28b(+) (unpublished data). Plasmid pETSynB53Km encodes a soluble portion of rat synaptobrevin 2 (SynB), and plasmid pETPA5 encodes a fragment of *Pseudomonas* exotoxin A.

DNA-modifying enzymes. Restriction enzymes *Acc65I*, *Bam*HI, *Bgl*II, *Eco*52I, *Eco*ICRI, *Eco*RI, *Hind*III, *Nco*I, *Nde*I, *Sac*I, *Sal*GI, *Stu*I, and *Xho*I, as well as T4 DNA polymerase, were produced by Promega. A rapid DNA ligation kit and an Expand high-fidelity PCR system were supplied by Boehringer Mannheim.

Oligonucleotides. The oligonucleotides used for PCR, as well as the oligonucleotides used for cloning, are listed in Table 1. All of these oligonucleotides were synthesized by Promega.

Nucleic acids. Total DNAs from *C. botulinum* strains producing serotype A, B, C, and E neurotoxins, as well as DNAs from *C. tetani* and *C. perfringens*, were kindly provided by Uri Vertiev (Moscow, Russia). Total-RNA preparations were purified from exponential cultures of *E. coli* BL21(λDE3) containing either plasmid pACYC184 or plasmid pACYC-IRL10 by an alternative protocol for rapid isolation of RNA from gram-negative bacteria described previously (1). Then RNA preparations were treated with RNase-free DNase I for 60 min at 37°C, and RNAs were purified by phenol-chloroform extraction and precipitation with ethanol.

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TABLE 1. Oligonucleotides used

Primer	Sequence	Amplified sequence ^a
5'-Ile-tRNA	5'-AAGCTTTGGATTGCGACACGGAGTTACTTT	<i>ileX</i>
3'-Ile-tRNA	5'-GCTTTTGTATCTCTCGAGAAAAGAAAAGGCTGACGATTTCTCGTCAGC	<i>ileX</i>
5'-Arg-tRNA	5'-CTTTTTCTTTTCTCGAGAGATCAAAAGCCATTGACTCAGCA	<i>argU</i>
3'-Arg-tRNA	5'-GTCGACTCAGGCGTCCCATTATCAGTG	<i>argU</i>
Leu-5'	5'-AACACAAAGTCGACAATAATGACGAATATAGCGCC	<i>subB-E</i>
Leu-3'	5'-GTCAACATCGCGGCCGACATTGAATGAACGC	<i>subB-E</i>
BoNT/A-N	5'-ATAAGAGGATCCGCGGATGCAATTTGTTAATAACAATTTAATT	BoNT/A-L
BoNT/A-LC	5'-TATCTTCTGAGAATTCCTATGTCGACATCCAATTGTTAACTTTGATACATAAATC	BoNT/A-L
BoNT/B-N	5'-GGATCCGCGGATGCCAGTTACAATAAATAATTTAATT	BoNT/B-L
BoNT/B-LC	5'-GAATTCCTATGTCGACATACATATTCTGGAGCTTTAAC	BoNT/B-L
BoNT/B-HN	5'-CCATGGGACATCATCACCATCACCACGGGGATCCACAAGCTTATGAAGAAATTAGCAA	BoNT/B-H
BoNT/B-HC	5'-GAATTCGGATCCTATTATTCAGTCCACCCCTCAT	BoNT/B-H
BoNT/C-N	5'-GGATCCGCGGATGCCAATAACAATTAACAACCTTAATTTATT	BoNT/C-L
BoNT/C-LC	5'-GAATTCCTATGTCGACCTACAATCAATGTTTATTATA	BoNT/C-L
BoNT/C-HN	5'-GGATCCTGTACAAAATAGGAAAATATATCTTTC	BoNT/C-H
BoNT/C-HC	5'-AGATCTTATTCACTTACAGGTACAAAACC	BoNT/C-H
BoNT/E-N	5'-GGATCCGCGGATGCCAAAATTAATAGTTTAAATTTATA	BoNT/E-L
BoNT/E-LC	5'-GAATTCCTATGTCGACATACATATTGATTCCTTATGCC	BoNT/E-L
BoNT/E-HN	5'-GGATCCAAATTTAAATCCTAGAATTTATACACCAA	BoNT/E-H
BoNT/E-HC	5'-AGATCTTATTTTCTTGCCATCCATGTTCTT	BoNT/E-H
TetLN	5'-GGAGATGATACATATGCCAATAACCATAAATAAAT	TeNT-L
tet-LC	5'-AAGTTAAATCAAGCTTTTATGTCGACATACATAAATTCCTCCTAAATCTGT	TeNT-L
TetCN	5'-TGCTTTTAGACATATGGATGGATCAGGCCTAGTTT	TeNT-H
TetHC	5'-TGAACATATCAAGCTTTTAAATCATTGTCATCC	TeNT-H
iota/IaN	5'-ATTATATTACGGATCCAGCTTTTATGAAAGACCAGAAG	Iota Ia
iota/IaC	5'-ATTTATATTACTCGAGTTAATTTATCAATGTTGCATCCAAAAT	Iota Ia
N-C3	5'-GGATCCAGGAGGGGTTTTATGAAAGGGATAAGAAAGTCAATTTTATGTTTAG	C3
IC3-C	5'-AGATCTGAATTTCTTAAATATCATTGCTGTAATCATAAT	C3
Ile1	5'-GGCCCCCTTAGCTCAGTGTT	Ile-tRNA
Ile2	5'-CCCCTGCTGGACTTGAACCA	Ile-tRNA
Arg1	5'-GCGCCCTTAGCTCAGTTGGA	Arg-tRNA
Arg2	5'-TGGCGCGCCCTGCAGGATTC	Arg-tRNA
Leu1	5'-GCGGGAGTGGCGAAATTTGGT	Leu-tRNA
Leu2	5'-TGGTGCGGGAGCGAGACTT	Leu-tRNA
Ile10	5'-TATGATAATAATAATAATAATAATAATAATAATATCGAGCT	NA
Ile10-comp	5'-CGATATATTATTATTATTATTATTATTATTATCA	NA
Arg10	5'-TAGTAGAAGAAGAAGAAGAAGAAGAAGAAGATCGAGCT	NA
Arg10-comp	5'-CGATCTTCTTCTTCTTCTTCTTCTTCTTCTTCTCTAC	NA
Leu10	5'-TATGCTACTACTACTACTACTACTACTACTACTATCGAGCT	NA
Leu10-comp	5'-CGATAGTAGTAGTAGTAGTAGTAGTAGTAGTAGCA	NA

^a TeNT-L, light chain of tetanus toxin; TeNT-H, heavy chain of tetanus toxin; NA, not applicable.

RT-PCR. Reverse transcription-PCR (RT-PCR) was performed by using *E. coli* total RNA, primers listed in Table 1 (Ile1 and Ile2 for amplification of the *ileX* gene fragment, Arg1 and Arg2 for amplification of the *argU* gene fragment, and Leu1 and Leu2 for amplification of the *leuW* gene), and an Access RT-PCR system (Promega) as recommended by the manufacturer.

Construction of plasmids. pGEM-IleArg7 was constructed by cloning into vector pGEM-T a PCR-amplified fragment containing the *ileX* and *argU* genes. As shown in Fig. 1, fragments containing the *ileX* and *argU* genes were originally amplified from *E. coli* chromosomal DNA as separate DNA fragments by using primers 5'-Ile-tRNA and 3'-Ile-tRNA for *ileX* and primers 5'-Arg-tRNA and 3'-Arg-tRNA for *argU* (Table 1). These fragments were then combined in a separate PCR mixture by using primers 5'-Ile-tRNA and 3'-Arg-tRNA.

pACYC-IleArg10 was constructed by joining the *Hind*III-*Sal*GI fragment of plasmid pGEM-IleArg7 containing the *ileX* and *argU* genes with the large *Hind*III-*Sal*GI fragment of plasmid pACYC184 (Fig. 1).

pACYC-IleArgLeu17 was generated by combining the large *Eco*52I-*Sal*GI fragment of plasmid pACYC-IleArg10 with the fragment of *E. coli* chromosomal DNA encoding the *subB-E* tRNA operon. The fragment was amplified by PCR performed with primers Leu-5' and Leu-3' (Table 1) and was treated with the *Sal*GI and *Eco*52I restriction endonucleases (Fig. 1).

pACYC-IRL10 was generated by treating plasmid pACYC-IleArgLeu17 with *Eco*52I and *Acc*65I and then with T4 DNA polymerase and ligase.

pACYC-Ile7 was constructed by treating plasmid pACYC-IleArg10 with *Xho*I and *Sal*GI endonucleases, T4 DNA polymerase, and ligase.

pACYC-Arg34 was generated by treating pACYC-IleArg10 DNA with *Hind*III and *Xho*I endonucleases, T4 DNA polymerase, and ligase.

pACYC-L10 is a derivative of pACYC-IRL10 and was constructed by treating plasmid pACYC-IRL10 with *Sal*GI, *Hind*III, T4 DNA polymerase, and ligase (Fig. 1).

pACYC-RL5 was constructed by treating plasmid pACYC-IRL10 with *Hind*III and *Xho*I endonucleases, T4 DNA polymerase, and ligase (Fig. 1).

pET₁₀PA10, pET₁₀PA25, and pET₁₀PA32 were constructed by joining the large *Nde*I-*Sac*I fragment of plasmid pETPA5 with synthetic DNA fragments formed by the oligonucleotide pairs Ile10-Ile10-comp, Arg10-Arg10-comp, and Leu10-Leu10-comp (Table 1), respectively.

pGEM-BoNT/B-L5, pGEM-BoNT/C-L2, pGEM-BoNT/E-L13, pGEM-BoNT/B-H13, pGEM-BoNT/C-H6, and pGEM-BoNT/E-H10 encoding the light and heavy chains of BoNT/B, BoNT/C, and BoNT/E were constructed by cloning DNA fragments amplified from corresponding clostridial genome DNAs into the vector pGEM-T.

pETBoNT/B-L10, pETBoNT/C-L20, and pETBoNT/E-L31 encoding the light chains of BoNT/B, BoNT/C, and BoNT/E, respectively, were constructed by replacing the small *Bam*HI-*Eco*RI fragment in plasmid pETA32-22 with the small *Bam*HI-*Eco*RI fragments from plasmids pGEM-BoNT/B-L5, pGEM-BoNT/C-L2, and pGEM-BoNT/E-L13, respectively.

pETBoNT/A-L22Km encoding the light chain of BoNT/A was constructed by replacing the small *Bam*HI-*Eco*RI fragment in plasmid pET28b(+) with the fragment amplified from *C. botulinum* by using primers BoNT/A-N and BoNT/A-LC (Table 1).

pETBoNT/B-H18 was constructed by replacing the small *Nco*I-*Eco*RI fragment of plasmid pET28b(+) with the small *Nco*I-*Eco*RI fragment from plasmid pGEM-BoNT/B-H13.

pETBoNT/C-H14 and pETBoNT/E-H10 were generated by subcloning into the *Bam*HI site of plasmid pET28b(+) light *Bam*HI-*Bgl*II fragments from plasmids pGEM-BoNT/C-H6 and pGEM-BoNT/E-H10, respectively.

pGEM-C3-20 encoding the C3 protein was generated as a result of cloning into the pGEM-T vector the DNA fragment amplified from *C. botulinum* DNA with primers N-C3 and IC3-C (Table 1).

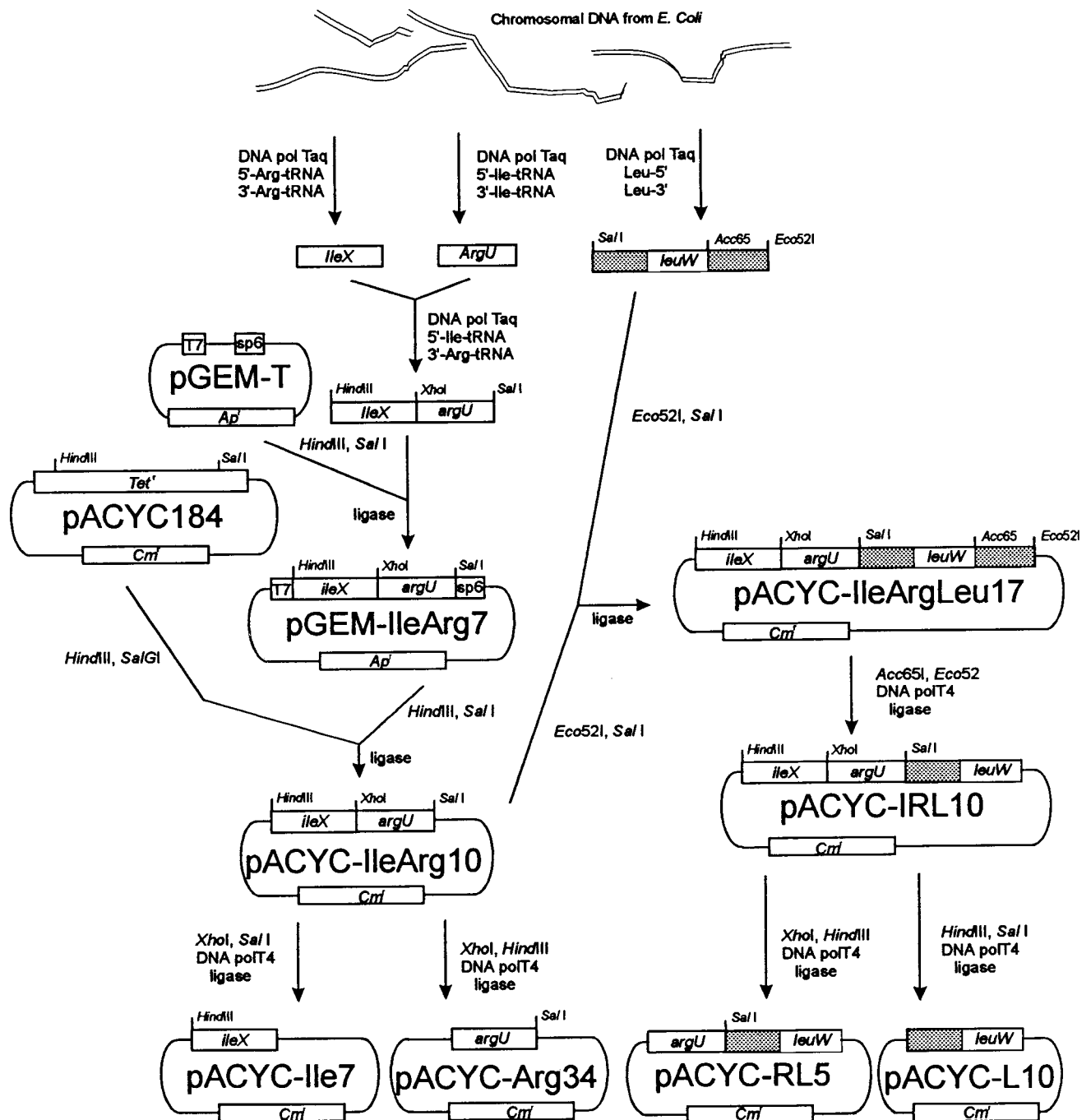


FIG. 1. Construction of plasmids encoding tRNAs. The *ileX*, *argU*, and *leuW* sequences encode tRNAs that recognize the ATA, AGA, and CTA codons, respectively. The *Ap^r*, *Tet^r*, and *Cm^r* sequences encode genes for antibiotic resistance. The T7 and Sp6 sequences encode promoters from bacteriophages T7 and sp6, respectively. The shaded areas represent sequences of *subB-E* tRNA operons other than *leuW*. DNA pol Taq, *Taq* DNA polymerase; DNA polT4, T4 DNA polymerase.

pTSC3-7 encoding the C3 protein was constructed by joining the large fragment of plasmid pPhe23-1 with the small fragment of plasmid pGEM-C3-20. A fragment of plasmid pPhe23-1 was generated by treating pPhe23-1 DNA with *Hind*III, T4 DNA polymerase, and *Bam*HI. A fragment of plasmid pGEM-C3-20 was generated by treating pGEM-C3-20 DNA with *Nde*I, T4 DNA polymerase, and *Bgl*II.

pETiota11Km encoding the iota toxin Ia protein was generated by replacing the small *Bam*HI-*Xho*I fragment in plasmid pETSynB53Km with the fragment that was amplified by using primers *iota*/IaN and *iota*/IaC from *C. perfringens* DNA and was treated with *Bam*HI and *Xho*I.

pETTeNT-L12Km and pETTeNT-H4Km encoding the light and heavy chains

of tetanus toxin, respectively, were generated by direct cloning of fragments amplified from *C. tetani* DNA into expression vector pET28b(+). A fragment encoding the light chain of tetanus toxin after amplification was treated with *Nde*I and *Hind*III restriction endonucleases and was joined with a large *Nde*I-*Hind*III fragment of plasmid pET28b(+). A fragment encoding the heavy chain of tetanus toxin after amplification was treated with *Stu*I and *Hind*III restriction endonucleases and joined with the large *Hind*III-*Eco*ICR fragment of plasmid pET28b(+).

Expression and purification of recombinant proteins. When cell cultures were at an absorbency at 600 nm of 0.4 to 0.5, protein expression was induced by adding isopropyl- β -D-thiogalactopyranoside (IPTG). Cells were harvested 90

min later. Light chains of BoNT/B and BoNT/E were recovered after inclusion bodies were dissolved in 7 M guanidine hydrochloride and renatured in 10 mM Tris-HCl-1 mM EDTA-300 mM arginine (pH 7.0). Proteins were further purified by using ion-exchange chromatography.

Proteolytic assay. Two recombinant proteins, SynB-receptor-associated protein (RAP) and 25-kDa synaptosome-associated protein (SNAP25)-RAP (unpublished data), which contained RAP (27, 28) fused with SynB and SNAP25, respectively, were used to detect the enzymatic activities of light chains of clostridial neurotoxins. The light chains of BoNTs were incubated with the appropriate substrate proteins in buffer containing 10 mM Tris-HCl (pH 6.8) and 1 mM ZnSO₄ at 37°C for 1.5 h. After incubation, the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by 4 to 20% Tris-glycine gels from Novex and were visualized by staining with Coomassie blue.

RESULTS

Amplification of tRNAs recognizing codons ATA, AGA, and CTA rarely used in *E. coli*. To investigate the impact of ATA, AGA, and CTA codon usage on translation of clostridial genes in *E. coli*, we amplified the *ileX*, *argU*, and *leuW* (as part of the *subB-E* tRNA operon) genes in *E. coli*. This was done by amplifying known sequences of interest (7, 11, 14, 15) by PCR and subsequently cloning the sequences into a multicopy plasmid. Plasmid pACYC184 was chosen as an appropriate vector because it is compatible with the pBR-based vectors that we used for cloning and expression of clostridial proteins in our studies. To investigate the role of each of the three rarely used codons (ATA, AGA, and CTA) on expression of clostridial genes in *E. coli*, plasmids encoding the *ileX* (pACYC-Ile7), *argU* (pACYC-Arg34), and *leuW* (pACYC-L10) genes separately or in the combinations *ileX-argU* (pACYC-IleArg10), *argU-leuW* (pACYC-RL5), and *ileX-argU-leuW* (pACYC-IleArgLeu17 and pACYC-IRL10) were constructed (Fig. 1) and introduced into *E. coli* BL21(λDE3). We did not observe any decrease in the growth rate of cells containing the amplified *ileX* gene, which is different than the results reported previously (19, 32). Indeed, cells containing plasmid pACYC-Ile7 grew at a rate as similar to the rate of growth of cells containing plasmid pACYC184 (data not shown). Similar results were obtained with cells containing plasmids pACYC-Arg34, pACYC-L10, and pACYC-IRL10. We observed an almost 50% decrease in the growth rate of cells containing plasmid pACYC-IleArgLeu17. Because plasmid pACYC-IRL10 is a derivative of plasmid pACYC-IleArgLeu17 and because the growth rate of cells containing plasmid pACYC-IRL10 was normal, we concluded that the decrease in the growth rate in the case of plasmid pACYC-IleArgLeu17 was related to amplification of the part of *subB-E* operon, which is missing in plasmid pACYC-IRL10 (Fig. 1) and is different from the *leuW* gene.

To confirm that amplification of tRNA genes resulted in increased accumulation of the corresponding tRNAs, we performed an RT-PCR analysis of total RNA isolated from BL21(λDE3) cells carrying either pACYC184 or pACYC-IRL10. Our analysis revealed that in order to obtain equal concentrations of PCR-amplified fragments corresponding to products of the *ileX*, *argU*, and *leuW* genes, three or four additional PCR cycles were needed for RNA from cells carrying pACYC184 than for RNA from cells carrying pACYC-IRL10 (data not shown). Furthermore, to confirm that the amplified genes encode functional tRNAs, we constructed three plasmids that encode a fragment of *Pseudomonas* exotoxin A, pETI₁₀PA10, pETR₁₀PA25, and pETL₁₀PA32. The N-terminal regions of the proteins encoded by these plasmids contained stretches of 10 isoleucine (codon ATA), arginine (codon AGA), and leucine (codon CTA) residues, respectively. Figure 2 shows data for expression of *Pseudomonas* exotoxin A derivatives encoded by these plasmids in BL21(λDE3) cells containing either plas-

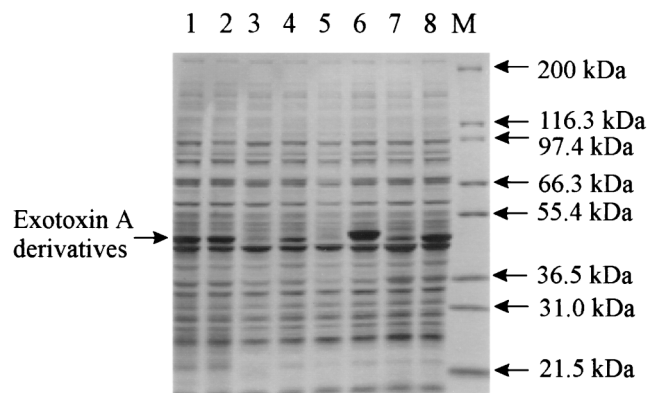


FIG. 2. Effect of amplification of the *ileX*, *argU*, and *leuW* genes in *E. coli* BL21(λDE3) on production of different derivatives of *Pseudomonas* exotoxin A. BL21(λDE3) cells were cotransformed with a plasmid encoding a derivative of *Pseudomonas* exotoxin A (lanes 1 and 2, pETPA5; lanes 3 and 4, pETI₁₀PA10; lanes 5 and 6, pETR₁₀PA25; lanes 7 and 8, pETL₁₀PA32) and with either pACYC184 (lanes 1, 3, 5, and 7) or pACYC-IRL10 (lanes 2, 4, 6, and 8). Cells were induced with IPTG and lysed, and cell proteins were separated by 4 to 20% gradient SDS-PAGE and visualized by Coomassie blue staining. Lane M contained the Mark12 wide-range protein standard from Novex.

mid pACYC184 or plasmid pACYC-IRL10. Production of exotoxin A derivatives encoded by plasmids pETI₁₀PA10, pETR₁₀PA25, and pETL₁₀PA32 was more efficient in cells containing plasmid pACYC-IRL10 than in cells containing control plasmid pACYC184. Protein encoded by parent plasmid pETPA5 was produced with the same efficiency in cells containing pACYC184 and in cells containing pACYC-IRL10. These data confirm that amplification of the *ileX*, *argU*, and *leuW* genes increased the functional levels of the corresponding tRNAs in *E. coli*.

Construction of plasmids encoding fragments of BoNTs and expression of these fragments in *E. coli*. As described above, we constructed a set of plasmids encoding the light chains of BoNT/A, BoNT/B, BoNT/C, and BoNT/E, as well as the heavy chains of BoNT/B, BoNT/C, and BoNT/E. Fragments encoding the light and heavy chains of BoNTs were amplified from clostridial genomic DNA by using primers listed in Table 1. Amplified fragments were cloned into expression vectors to create BoNT fragment-encoding genes whose transcription was under control of the efficient bacteriophage T7 promoter, and the region around the start codon was also optimized to ensure efficient initiation of translation. To analyze expression of our recombinant genes, we introduced these plasmids into BL21(λDE3) cells that simultaneously were transformed with either pACYC184 or derivatives of this plasmid containing the *ileX*, *argU*, and *leuW* genes, and the proteins produced were analyzed by SDS-PAGE. As shown in Fig. 3 and 4, cells cotransformed with both a BoNT fragment-encoding plasmid and plasmid pACYC184 produced proteins of interest in such low quantities that they were not detectable on Coomassie blue-stained gels. Also, we were not able to detect substantial amounts of BoNT fragments in the cells containing plasmid pACYC-Arg34, pACYC-L10, or pACYC-RL5 instead of pACYC184. In contrast, production of recombinant BoNT fragments was substantially greater in cells cotransformed with either pACYC-Ile7, pACYC-IleArg10, or pACYC-IRL10 and BoNT fragment-encoding plasmids. Thus, amplification of the *ileX* gene plays a major role in increasing the production of BoNT fragments. Also, cells containing plasmid pACYC-IleArg10 or pACYC-IRL10 produced proteins of interest at slightly (up to twofold) higher levels than cells containing plas-

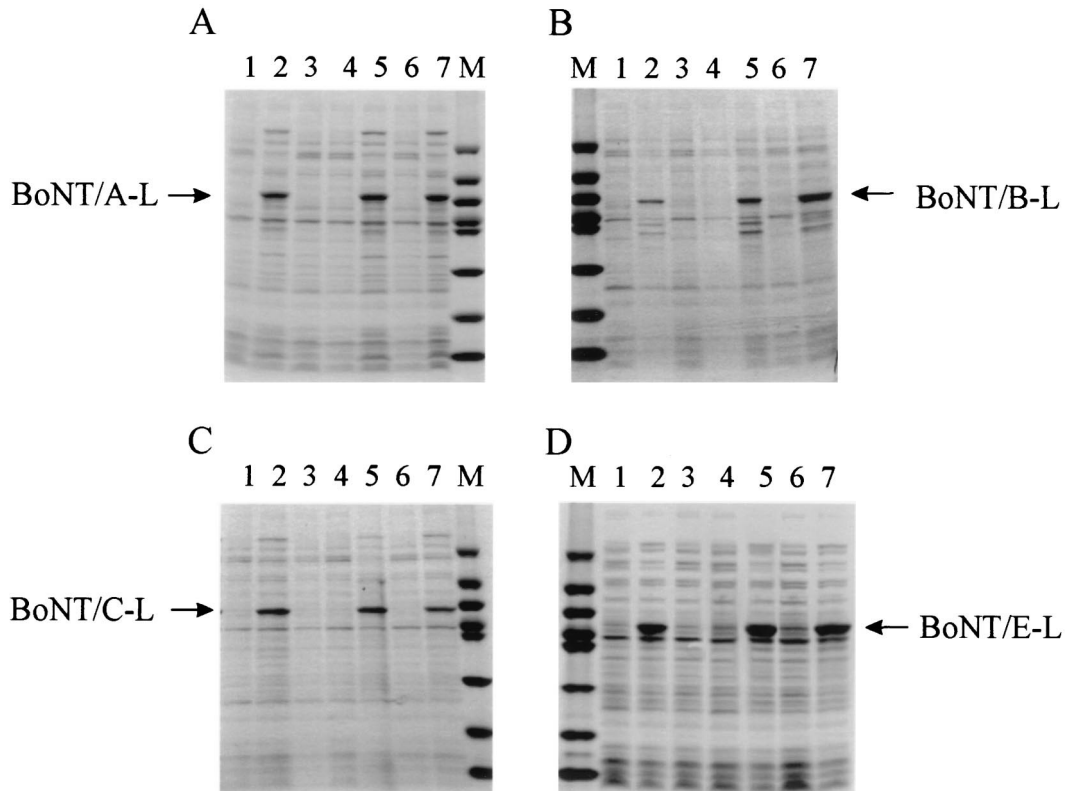


FIG. 3. Effect of amplification of the *ileX*, *argU*, and *leuW* genes in *E. coli* BL21(λ DE3) on production of light chains of BoNT/A (A), BoNT/B (B), BoNT/C (C), and BoNT/E (D). The expression of each protein was evaluated in the presence or absence of amplified *ileX*, *argU*, or *leuW* genes, as follows: lane 1, no amplification (pACYC184); lane 2, pACYC-Ile7; lane 3, pACYC-Arg34; lane 4, pACYC-L10; lane 5, pACYC-IleArg10; lane 6, pACYC-RL5; and lane 7, pACYC-IRL10. Cells were induced with IPTG and lysed, and the total cell proteins were separated by 4 to 20% gradient SDS-PAGE and visualized by Coomassie blue staining. The arrows indicate the locations of the various neurotoxin proteins. The molecular weight markers used (lane M) were phosphorylase *b* (molecular weight, 97,400), bovine serum albumin (66,200), glutamate dehydrogenase (55,000), ovalbumin (42,700), aldolase (40,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

mid pACYC-Ile7 produced these proteins. This improved production effect was observed with cells that were grown for 1.5 h after induction of expression with IPTG but not in cells grown for 16 h after induction of expression when no significant accumulation of proteins of interest was observed (data not shown).

The identities of the proteins were confirmed with specific antibodies. Furthermore, to ensure the functional integrity of the toxin products as proteases, we carried out enzymatic activity tests as described above. Recombinants BoNT/A-L, BoNT/B-L, and BoNT/E-L were recovered from inclusion bodies by

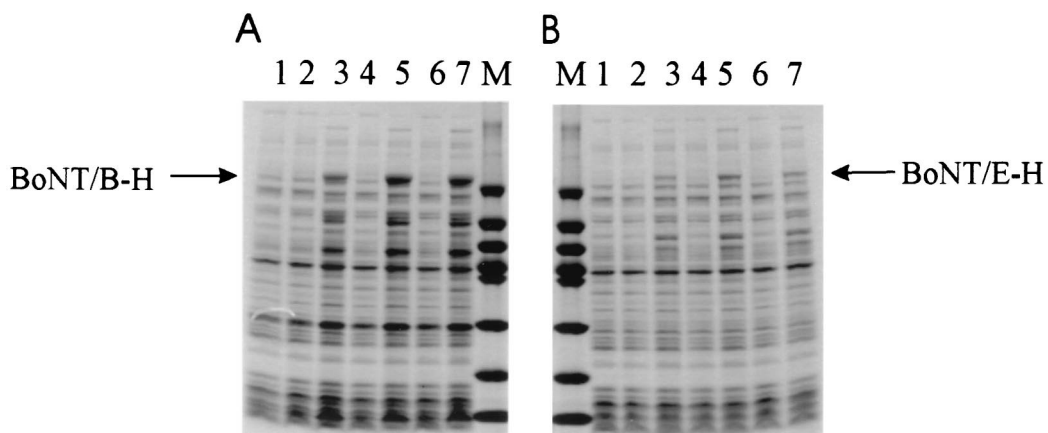


FIG. 4. Effect of amplification of the *ileX*, *argU*, and *leuW* genes in *E. coli* BL21(λ DE3) on production of heavy chains of BoNT/B (A) and BoNT/E (B). The expression of each protein was evaluated in the absence or presence of the amplified *ileX*, *argU*, or *leuW* genes, as follows: lane 1, no amplification (pACYC184); lane 2, pACYC-Ile7; lane 3, pACYC-Arg34; lane 4, pACYC-L10; lane 5, pACYC-RL5; lane 6, pACYC-IleArg10; and lane 7, pACYC-IRL10. Cells were induced with IPTG and processed for SDS-PAGE as described in the legend to Fig. 2. The arrows indicate the locations of the expressed proteins BoNT/B-H and BoNT/E-H. The molecular weight markers used (lane M) were the molecular weight markers described in the legend to Fig. 3.

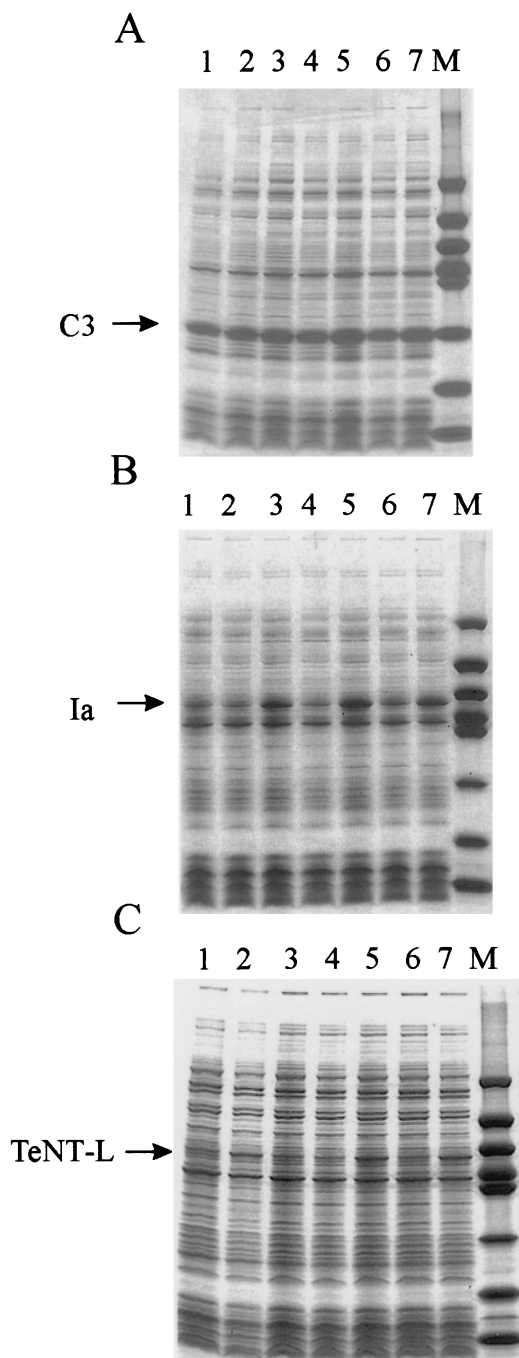


FIG. 5. Effect of amplification of the *ileX*, *argU*, and *leuW* genes on production of the botulinum C3 protein (A), the iota toxin Ia protein (B), and the light chain of tetanus toxin (C) in BL21(λ DE3) cells. The expression of each protein was evaluated in the absence or in the presence of amplified *ileX*, *argU*, or *leuW* genes, as follows: lane 1, no amplification (pACYC184); lane 2, pACYC-Arg34; lane 3, pACYC-Ile7; lane 4, pACYC-L10; lane 5, pACYC-IleArg10; lane 6, pACYC-RL5; and lane 7, pACYC-IRL10. Cells were induced with IPTG and processed for SDS-PAGE as described in the legend to Fig. 2. The arrows indicate the locations of proteins C3 and Ia and the light chain of tetanus toxin (TeNT-L). The molecular weight markers used (lane M) were the molecular weight markers described in the legend to Fig. 3.

using the denaturation-renaturation procedure described above, and their enzymatic activities were tested. Figure 5 shows that BoNT/B-L was active in cleavage of the SynB-RAP fusion protein but was not active when the SNAP25-RAP fusion pro-

tein was the substrate. In contrast, BoNT/A-L (Fig. 6) and BoNT/E-L (data not shown) cleaved SNAP25-RAP and but did not cleave the SynB-RAP fusion protein. The activities of recombinant light chains were completely inhibited by EDTA. The specificities of BoNT/B-L and BoNT/E-L were also confirmed *in vivo* by using previously described systems (10, 13).

Construction and expression in *E. coli* of plasmids encoding proteins from different representatives of the genus *Clostridium*. We hypothesized that amplification of tRNAs that recognize rarely used codons may be a useful strategy which has general applicability for improving the efficiency of heterologous expression in *E. coli*. To study this, we tested the applicability of this procedure for expression of other clostridial toxins. We used the C3 protein (16) from *C. botulinum*, the light and heavy chains of tetanus toxin from *C. tetani*, and the iota toxin Ia protein from *C. perfringens* (17) as prototypes. The corresponding sequences were amplified from the total DNAs of the corresponding microorganisms by using PCR and the specific primers listed in Table 1 and were placed under control of a bacteriophage T7 promoter with an efficient translation initiation site as described above. The resulting plasmids, pTSC3-7 (encoding the C3 protein), pETTeNT-L12Km and pETTeNT-H4Km (encoding the light and heavy chains of tetanus toxin, respectively), and pETiota11Km (encoding the iota toxin Ia protein), were introduced into BL21(λ DE3) cells containing either pACYC184 or tRNA-encoding derivatives of this plasmid, and production of the corresponding recombinant proteins was analyzed by SDS-PAGE. Our analysis revealed that unlike plasmids encoding fragments of BoNTs, plasmids pTSC3-7, pETiota11Km, pETTeNT-L12Km (Fig. 5), and pETTeNT-H4Km (data not shown) gave relatively efficient production of recombinant proteins in *E. coli* cells that contained normal quantities of tRNA genes. Amplification of either *ileX*, *argU*, or *leuW* separately did not result in increased production of the C3 protein. Simultaneous amplification of the *ileX* and *argU* genes did slightly improve production of this protein. In the cases of the pETiota11Km, pETTeNT-L12Km (Fig. 5), and pETTeNT-H4Km (data not shown) plasmids, amplification of the *ileX* gene had a positive effect on production of recombinant proteins. Amplification of the *argU* gene in addition to amplification of the *ileX* gene also allowed us to improve production of the heavy chain of tetanus toxin encoded by pETTeNT-H4Km.

DISCUSSION

Low levels of expression of clostridial neurotoxins in traditional organisms such as *E. coli* may explain why our understanding of the mechanism of action of such an important class of toxins is lagging behind our understanding of the mechanism of action of other toxins. The fact that efficient expression of a tetanus toxin fragment was achieved after complete de novo synthesis of the coding sequence and adjustment of codons in the sequence on the basis of *E. coli* codon usage indicated the importance of the codons used in the sequence for efficient production of this protein (12). Whether this was fully attributable to codon usage or to other factors, such as the mRNA secondary structure, was not clear. Furthermore, this approach is expensive and requires a substantial amount of preliminary work before each protein can be expressed. In this study, we examined whether rarely used codons play a major role in decreasing the efficiency of production of clostridial neurotoxins in *E. coli*. By amplifying tRNA genes whose products recognize rarely used codons, we were able to significantly improve production of clostridial neurotoxin fragments in *E. coli*. We did this without changing the coding sequences of

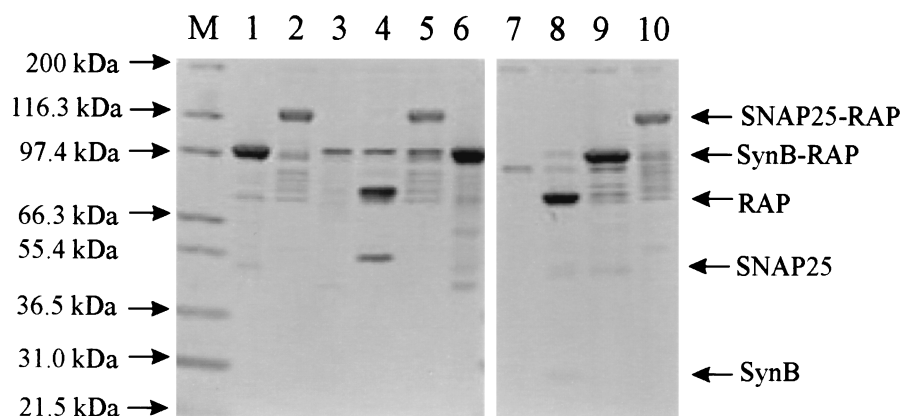


FIG. 6. Enzymatic activities of recombinant light chains of BoNT/A and BoNT/B. Substrate proteins SynB-RAP (lane 1) and SNAP25-RAP (lane 2) and light chains of BoNT/A (lane 3) and BoNT/B (lane 7) were included. Also included were a mixture of BoNT/A-L and SNAP25 in the absence (lane 4) and in the presence (lane 5) of EDTA (lane 5) and a mixture of BoNT/B-L and SynB-RAP in the absence (lane 6) and in the presence (lane 9) of EDTA. Lanes 6 and 10 contained a mixture of BoNT/A-L and SynB-RAP and a mixture of BoNT/B-L and SNAP25, respectively. Proteolytic activity was determined in the presence of 10 mM Tris-HCl (pH 6.8) and 1 mM ZnSO₄ for 1.5 h at 37°C. Proteolytic products were separated by 4 to 20% gradient SDS-PAGE, and proteins were visualized with Coomassie blue as described in the text. The arrows on the left indicate the positions of molecular weight markers (lane M) obtained from the Mark12 wide-range protein standard (Novex), and the arrows on the right indicate the locations of substrate proteins and their products.

neurotoxin fragments, which confirmed that rarely used codons play a major role in efficient expression of clostridial neurotoxin-encoding genes in *E. coli*. Table 2 shows the frequencies of rarely used codons in the sequences and the effect of amplification of tRNA-encoding genes on the level of expression. The data show that there is a definite correlation between the frequency of a particular codon in the reading frame and the effect of amplification of the corresponding tRNA-encoding gene on the production of the corresponding protein. Indeed, of the three codons examined (ATA, AGA, and CTA), the ATA codon encoding isoleucine is the most frequently used codon in neurotoxin-encoding sequences, and amplification of the *ileX* gene has the most dramatic effect on the level of expression of this codon. The AGA codon is less prevalent than the ATA codon, and thus the effect of *argU* gene amplification is more modest. Nevertheless, there is not a strong correlation between the frequencies of rarely used codons in each gene and the effects of the corresponding tRNA amplification on the efficiency of expression. Indeed, although ATA

occurs more frequently with the genes encoding the light chain of tetanus toxin (4.8%) and the iota toxin Ia protein (5.1%) than with the genes encoding the light chains of BoNT/A (3.8%) and BoNT/E (4.0%), amplification of the *ileX* gene had more dramatic effect on production of the last two proteins than on production of the first two. In addition, even though all of the clostridial genes examined in this study contain the CTA codon, expression of neither of them was improved by amplification of the *leuW* gene.

It is noteworthy that the disruptive effect of rarely used codons on translation efficiency depends not only on the prevalence of these codons but also on their relative locations in the gene. Indeed, in recombinant genes encoded by plasmids pETBoNT/E-L31 and pETR₁₀PA25, the frequencies of the AGA codon are practically the same (2.4 and 2.5%, respectively). Nevertheless, amplification of the *argU* gene had a more pronounced effect on expression of the recombinant gene from plasmid pETR₁₀PA25, in which all 10 AGA codons were clustered together, than on expression of the recombi-

TABLE 2. Codon usage in recombinant genes and effect of amplification of tRNA-encoding genes on expression in *E. coli*

Plasmid	Codon usage (%)			Effect of tRNA gene amplification on expression ^a		
	ATA	AGA	CTA	<i>ileX</i> (ATA)	<i>argU</i> (AGA)	<i>leuW</i> (CTA)
PETBoNT/A-L22Km	3.8 (19) ^b	2.0 (10)	1.0 (5)	++	-	-
PETBoNT/B-L10	6.2 (29)	2.3 (11)	0.2 (1)	++	+	-
pETBoNT/C-L20	5.9 (28)	3.1 (15)	0.8 (4)	++	-	-
pETBoNT/E-L31	4.0 (18)	2.4 (11)	1.5 (7)	++	+	-
pETBoNT/B-H18	6.4 (57)	1.9 (17)	1.0 (9)	++	+	-
pETBoNT/C-H14	5.0 (48)	2.5 (24)	0.9 (9)	++	+	-
pETBoNT/E-H10	4.4 (40)	2.2 (20)	0.3 (3)	++	+	-
pTSC3-7	2.4 (6)	3.2 (8)	0.8 (2)	+/-	+/-	-
pETiota11Km	5.1 (23)	1.7 (8)	0.8 (4)	+	-	-
pETTeNT-L12Km	4.8 (24)	1.8 (9)	1.4 (7)	+	-	-
pETTeNT-H4Km	5.7 (53)	1.8 (17)	1.1 (11)	++	+	-
pETPA5	0	0	0	-	-	-
pETI ₁₀ PA10	2.5 (10)	0	0	++	-	-
pETR ₁₀ PA25	0	2.5 (10)	0	-	++	-
pETL ₁₀ PA32	0	0	2.5 (10)	-	-	++

^a ++, strong effect; +, moderate effect; -, no effect; +/-, weak effect.

^b The values in parentheses are numbers of codons in the coding sequence.

nant gene from plasmid pETBoNT/E-L31, in which 11 AGA codons were randomly spreaded throughout the coding sequence.

The lack of an effect of *leuW* gene amplification on expression of clostridial proteins suggests that the disruptive effect of rarely used codons becomes noticeable only when the total number and relative frequency of these codons in the gene are higher than certain minimum values. Our results for expression of exotoxin A derivatives encoded by plasmids pET₁₀PA10, pETR₁₀PA25, and pETL₁₀PA32 also suggest that the effective number of codons varies with each type of codon. Indeed, of the three types of codons tested, the ATA codon had the most dramatic effect on production of proteins in *E. coli* cells. A stretch of 10 of these codons was sufficient to completely inhibit production of an exotoxin A derivative in *E. coli* cells that contained normal level of tRNAs. When the same protein was encoded by a gene that contained 10 CTA codons instead of ATA codons, production of the protein was significantly enhanced in the same *E. coli* cells. Our results also demonstrate that amplification of tRNA-encoding genes for rare codons can be used for optimization of protein production and may be applicable to many other genes for production of proteins that have great commercial value.

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