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Effects of Minor Arginyl tRNA and Isoleucyl tRNA on the Expression of *Clostridium botulinum* Neurotoxin Light Chain in *Escherichia coli*

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Abstract Botulinum neurotoxin type A (BoNT/A) is an extremely potent toxin, which is produced by *Clostridium botulinum*. The light chain of this protein (BoNT/A LC), which is known as a zinc endopeptidase, cleaves SNAP-25 involved in the exocytosis process. In this work, the expression of recombinant BoNT/A LC in *E. coli* is described. The BoNT/A LC gene of *C. botulinum* contains a high frequency of the arginine AGA and isoleucine ATA codons that are rarely used in genes of *E. coli*, hampering the translation of recombinant protein. The *argD* and *ileX* tRNA genes were cloned into pACYC184 vector, resulting in pACD13IX plasmid. The translational stress of the toxin gene related to codon bias was reversed by supplementation of the AGA arginyl tRNA of T4 phage and AUA isoleucyl tRNA of *E. coli*. This system may be applicable for the expression of a variety of AT-rich heterologous genes in *E. coli*.

Key words: Neurotoxin, codon-bias, tRNA, *Clostridium botulinum*

Botulinum neurotoxins (BoNTs), which are produced by various strains of *Clostridium botulinum*, are extremely potent substances that cause the neuroparalytic illness in humans and animals known as botulinum [18]. *C. botulinum* produces seven neurotoxin serotypes, designated by the letters A, B, C, D, E, and G [6], which are serologically distinct, but possess similar molecular weights [19]. In type A, BoNT is synthesized as a relatively inactive single-chain polypeptide with a molecular weight of 150 kDa. To become active, the toxin undergoes post-translational processing, in which the molecule is cleaved by a protease to yield a heavy chain (100 kDa) and a light chain (50 kDa) linked by a disulfide bond [2]. In its mode of action, the heavy chain functions in binding of the toxin to specific receptors on peripheral cholinergic nerve cells and forms channels in phospholipid membranes, facilitating internalization of the light chain [3]. The light chain (BoNT/A LC), identified as a zinc protease, cleaves SNARE proteins such as SNAP-25 that are essential for synaptic vesicle fusion, thus blocking neurotransmitter release, which leads to paralysis [16].

The expression of the BoNT/A LC gene of *C. botulinum* in *Escherichia coli* has been tried, but it showed poor results. It has been postulated that the poor expression might be related to the high number of AGA/AGG and AUA codons present in the BoNT gene. *Clostridium botulinum* has a relatively AT-rich genome of about 80% [7] and certain codons that are preferentially used by *C. botulinum* are rarely used by *E. coli* in highly expressed genes [2]. Rare codons have been shown to greatly diminish expression levels of foreign proteins in *E. coli*, because of a poor supply of the corresponding tRNAs in translational machinery [8, 10]. The rare AGA $tRNA^{Arg}$ of *E. coli* constitutes about 3% of the total arginine tRNA, and its cognate codon, AGA, also accounts for about 4% of the arginine codons [4]. The isoleucine AUA codon is the next rarest codon in *E. coli* and is used at a frequency of less than 0.4% [21].

To address the poor expression problem of the BoNT gene in *E. coli*, the plasmid carrying the *argD* of T4 phage [12], encoding rare arginine AGA tRNA and *ileX* gene encoding rare isoleucine AUA tRNA, was introduced into the *E. coli* host and the effect was analyzed. The supplementation of the rare arginyl tRNA and isoleucyl tRNA was shown to relieve the translational stress present during BoNT/A LC gene expression in *E. coli*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Escherichia coli DH5α [9] was used for the gene cloning experiments, and *E. coli* ER2566 (F-λ-*fhuA2[lon] ompT lacZ::T7 gal sulA11 Δ(mcrC-mrr)114::IS10 endA1*) as a host for the expression of BoNT. *E. coli* ATCC 11303 was

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infected with T4 phage (ATCC 11303-B4) to isolate phage DNA. pACYC184 was from NEB (Bervery, U.S.A.), and pET15b was from Novagen (Madison, U.S.A.). pHNT [15] was used for general PCR DNA cloning. The pGXS25 containing the human SNAP-25 cDNA and pBTHL plasmid containing the BoNT/A gene of *C. botulinum* were a generous gift from K. W. Lee (Neurotics, Korea).

Cloning and Expression of a Gene Encoding a BoNT Light Chain

The light chain DNA of the *botulinum* neurotoxin A, nucleotide position 19-1,255 corresponding to Gln7-Gly418, was amplified by PCR with BoNT/A DNA of pBTHL plasmid. The forward primer was 5'-ACGACTCGAGCA-ATTTAATTATAAAGATCCTGT-3' which adds a *Xho*I restriction site to the 5' end and the reverse PCR primer was 5'-ACTAGGATCCTATCCTTCATCTAATGATTTAG-3' which adds a *Bam*HI site to the 3' end. The 1,236-bp PCR product was inserted into the pET15b expression vector using the *Xho*I and *Bam*HI restriction sites, generating a pBTL15XB recombinant. pBTL15XB was transformed into *E. coli* ER2566 and the cells were grown at 37°C in LB broth with ampicillin (50 µg/ml) until OD_{600} reached to 0.6-0.8, followed by induction with 0.5 mM IPTG. Samples (1 ml) were harvested after 3 h and subjected to SDS-PAGE [14]. For the purification, *E. coli* cells were lysed and the lysate was purified with Ni-NTA agarose resin (Qiagen, U.S.A.) according to the manufacturer's instructions.

Nucleotide Sequence Analysis

The nucleotide sequences of the light chain gene of BoNT was determined by Solgent Co. (Daejeon, Korea) and deposited in the GenBank database under accession No. AY166872.

Cloning of *argD* tRNA and *ileX* tRNA Genes

A DNA fragment containing the *argD* $tRNA^{Arg}$ gene including its own promoter sequences was isolated from T4 phage DNA by PCR. The forward primer (*argD*1-5) 5'-GTAGAAGCTTCCCAATTCGGTATGATGT-3' and reverse primer (*argD*1-3) 5'-ACGCGGATCCTAAATGTAGCCT-AGAGAGGT-3' were used to amplify the *argD* gene from T4 phage DNA. A PCR fragment of expected size of 580 bp was digested with *Hin*dIII and *Bam*HI, and the digest was ligated into the pACYC184, generating a pAAD13 plasmid. The *ileX* $tRNA^{Ile}$ gene, which encodes the tRNA recognizing the isoleucine codon AUA, was amplified from *E. coli* genomic DNA by PCR with forward primer (*ileX*-5) 5'-ACTTCGGCCGGATTGCGACACGGAGTT-3' and reverse primer (*ileX*-3) 5'-AGACGTCGACGAAAAA-GGCTGACGATTTCT-3'. A 160-bp amplified DNA fragment was digested with *Eag*I and *Sal*I, and the digest was ligated into the *Eag*I, *Sal*I-double digested pAAD13, producing a pAAD13IX plasmid.

RT-PCR Analysis

Expression of *argD* and *ileX* was analyzed by an RT-PCR with arginyl or isoleucyl-internal 5' and 3' primers. Total RNA was isolated from 400 µl of cell culture with MICROBExpress bacterial mRNA purification kit (Ambion, U.S.A.) according to the manufacturer's instructions. A DNase incubation step (1 unit/sample, 1 h) was added to eliminate DNA contamination. cDNA synthesis was performed with 5 µg of purified total RNA and Superscript RT II (Invitrogen, U.S.A.), followed by PCR amplification with their internal primers. RT-PCR samples were visualized on 2% agarose gel.

Western Blot Analysis

Polyclonal antibody against *C. botulinum* neurotoxin Type A was purchased from Metabiologics (Madison, U.S.A.). The protein samples in a 10% polyacrylamide gel were transblotted on to PVDF membrane, and BoNT LC was detected with a chemiluminescence system (KPL, U.S.A.). A primary rabbit polyclonal anti-BoNT/A, followed by a secondary goat anti-rabbit antibody coupled to horse peroxidase was used. Chemiluminescence was detected by exposure of the blot coated with a luminol-based solution to Kodak X-OMAT X-ray film.

Endopeptidase Activities

E. coli ER2566 containing pGXS25 was induced by addition of 0.5 mM IPTG at 37°C for 3 h. Recombinant His_6-tagged SNAP-25 was isolated using a Ni-NTA agarose column according to the vendor's manual. Endopeptidase activities were carried out in a 50-µl reaction mixture containing 4 µg of SNAP-25, 0.5 µg of purified recombinant BoNT LC, or 0.2 units of native BoNT/A (Allergan, Ireland). After incubation in a buffer (0.03 HEPES, 3 mM DTT, 0.2 mM zinc chloride, pH 7.3) at 37°C, reactions were stopped at various times by adding SDS-PAGE loading buffer. The reactants were resolved on a 12% SDS-polyacrylamide gel.

Results and Discussion

Cloning of BoNT Light Chain Gene and Sequence Analysis

From the gene for BoNT type A of *C. botulinum* containing the light and heavy chains, two oligonucleotides were designed to amplify the BoNT light chain gene by PCR. The length of the cloned BoNT LC gene is 1,344 bp, and it encodes a 448-amino-acid protein with a calculated molecular mass of 51,325 Da. The deduced amino acids sequence was compared to the sequences in protein databases, and showed 99% identity to the neurotoxin type A of *C. botulinum*, indicating that this protein is a variant of BoNT type A. DNA sequence analysis indicates that the A+T content in this gene is 71.1%. Table 1 compares the codon

Table 1. Usage of Arg and Ile codons in genes of *E. coli* and the BoNT LC gene of *C. botulinum*.

Amino acid	Codons	Fractions (%) in *E. coli*[1]	Fractions (%) in *C. botulinum*[2]
Arg	AGG	3	14
	AGA	4	72
	CGG	8	0
	CGU	42	7
	CGA	5	7
	CGC	37	0
Ile	AUA	7	61
	AUU	47	39
	AUC	46	0

[1]Taken from Glick and Pasternak (1998).
[2]BoNT/A gene of *C. botulinum*.

usage of Arg and Ile for the BoNT gene of *C. botulinum* with protein-coding genes of *E. coli*, indicating that the rare arginine AGA/AGG and isoleucine AUA codons represent 86% and 61% of the total Arg and Ile codons of *C. botulinum*, and occur at 18 and 9 times the average level of *E. coli* genes, respectively.

Expression of Arginyl and Isoleucyl tRNA

In order to test the effects of the addition of tRNAs for the rare codons AGA and AUA on the expression of light chain of BoNT in *E. coli*, the pAAD13IX plasmid carrying the *argD* tRNA gene of T4 phage was constructed under the control of its natural promoter and cognate *ileX* tRNA gene of *E. coli*. A simplified map of this construction is shown in Fig. 1. RT-PCR was used to confirm the expression of *argD* and *ileX* tRNA genes in *E. coli* ER2566. The 75-bp and 76-bp fragments corresponding to AGA $tRNA^{Arg}$ and AUA $tRNA^{Ile}$, respectively, were found in RNA samples isolated from cells containing pAAD13IX. No such fragment was seen in cultures of cells lacking the cloned tRNA genes (Fig. 2). Since the amplified DNA fragment did not originate from pACYC184 plasmid DNA, it was concluded that there was an accumulation of the rare arginyl AGA tRNA and isoleucyl AUA tRNA in cells bearing this plasmid. The absence of an amplified *ileX* tRNA from the control cells (Fig. 2, lane 4) might be the result of the presence of only single copy of *ileX* on the host chromosome. The *ileX* promoter on the plasmid may be more proliferative, resulting in high expression of *ileX* tRNA. While overexpression of tRNA genes has been reported to be detrimental to the cell [20], expression of the tRNAs from the indigenous *argD* and *ileX* promoter in pACYC plasmid appeared to have a minor effect on the growth of ER2566 (data not shown).

Although the *ileX* gene encoding $tRNA^{Ile}$ contained a CAU anticodon, which is characteristic of the methionine tRNA gene, the C residue in the first position of the anticodon is post-transcriptionally modified to lyosine, base pairing with adenosine instead of guanosine in the third position

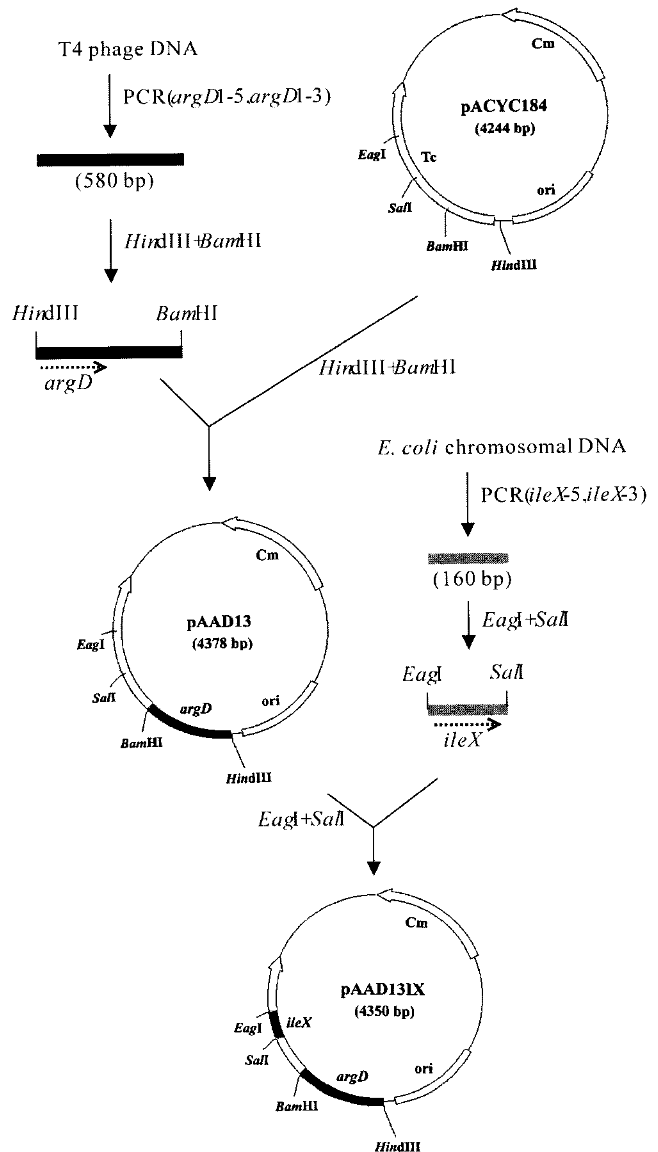

Fig. 1. Construction of the recombinant plasmid, pAAD13IX containing *argD* and *ileX* tRNA genes.

of codon [13]. The isoleucyl tRNA was not isolated to determine whether C residues were lysinylated or not, but the expression result (Fig. 3) suggests indirectly that supplementary cognate tRNA for AUA was also lysinylated and then recognized the AUA codons of the BoNT LC gene.

Expression of Light Chain of BoNT/A

Li and Singh [11] showed that the expression of BoNT/A light chain was obtained by extended postinduction of 15 h at 30°C. But under the same condition as they described, the preliminary experiments designed to express the light chain of BoNT gene of *C. botulinum* in *E. coli* ER2566 expression system were unsuccessful (data not shown). It was suspected that failure to express was due to the

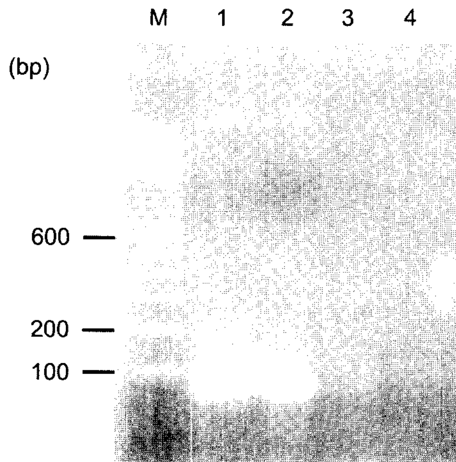

Fig. 2. RT-PCR analysis of *E. coli* ER2566 transformants.
Total RNA samples were isolated and incubated with internal primers specific to the tRNA sequences within the *argD* (lanes 1 and 3) or *ileX* (lanes 2 and 4). Lanes: M, DNA 100-bp ladder marker; 1 and 2, RNA from ER2566 cells containing pAAD13IX; 3 and 4, RNA from ER2566 cells containing pACYC184 control plasmid.

presence of a high fraction of rare codons found in this gene, as shown in Table 1. To relieve the codon-biased translational stress in *E. coli* during BoNT LC gene expression, a pAAD13IX was introduced into the *E. coli* host strain ER2566 harboring a BoNT/A LC gene in an expression plasmid pBTL15XB. The pAAD13IX plasmid is a pACYC184 derivative that carries the p15A origin of replication and is thus compatible with the pBTL15XB vector, which is of ColE1 origin of replication. The ER2566 strain co-transformed with the pAAD13IX and pBTL15XB were compared with cells co-transformed with the pACYC184 and pBTL15XB. Figure 3A shows that BoNT LC began to express in the presence of the pAAD13IX plasmid. With the pAAD13 plasmid harboring only an *argD*, the protein was barely detected. In the presence of control pACYC184 plasmid alone, no induced band could be seen. BoNT/A LC, on the other hand, accumulated to approximately 10% of the total cell protein, when coexpressed with the *argD* and *ileX* genes. This result indicated that the input of only AUA arginyl tRNA was not enough to express the codon-biased BoNT/A LC in *E. coli*, that is, both AUA Arg tRNA and AUA Ile tRNA should be supplemented to overcome the translational stalling.

Western blot analysis (Fig. 3B) was carried out using anti-native BoNT/A rabbit serum to assay the immunological reactivity. BoNT LC expressed in *E. coli* was specifically recognized by the sera and had the predicted molecular size (51 kDa), confirming the expression of full-length light chain of BoNT.

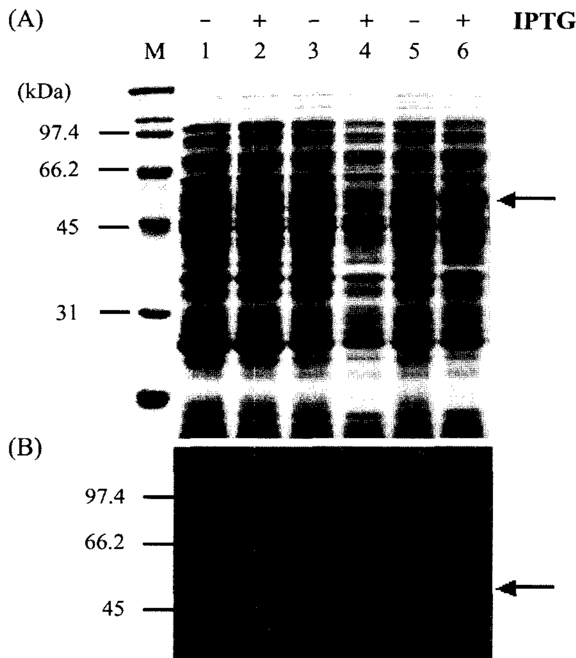

Fig. 3. SDS-PAGE gel (A) and Western blot analysis (B) of ER2566 cells containing the BoNT LC gene with and without pAAD13IX plasmid.
Whole cells induced without (lanes 1, 3, 5) or with IPTG (lanes 2, 4, 6) were resolved on 10% polyacrylamide gel. Lanes: M, protein molecular mass markers; 1 and 2, cells with pACYC184 control plasmid; 3 and 4, cells with pAAD13 plasmid; 5 and 6, cells with pAAD13IX. The arrows indicate the BoNT LC protein bands.

Digestion of SNAP-25 with Recombinant BoNT LC

The BoNT/A protein is known to cleave a single site near the C-terminal of SNAP-25 [1]: the glutaminyl-arginine peptide bond corresponding to residues 197 and 198 of

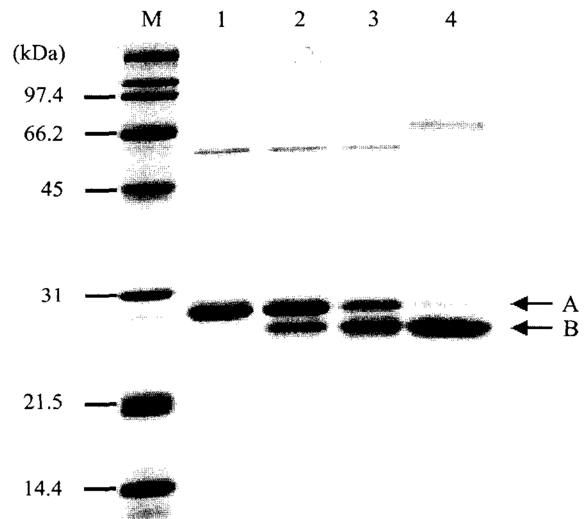

Fig. 4. Cleavage of recombinant SNAP-25 by recombinant BoNT/A LC protein. SNAP-25 protein was incubated with recombinant LC (lanes 1, 2, and 3) or native BoNT/A (lane 4).
The reaction was terminated by adding the sample buffer at 0 (lane 1), 30 (lane 2), and 60 (lanes 3 and 4) min. The arrows A and B indicate uncleaved and cleaved SNAP-25, respectively.

SNAP-25. The SNAP-25 gene cloned in pET15b vector was expressed in *E. coli* and purified for use as a substrate for the BoNT/A LC. Thus, the recombinant *E. coli* harboring pET15b-SNAP25 was lysed and partially purified as a His-tagged recombinant protein by chromatography on Ni-NTA-agarose. The size of SNAP-25 protein fused with His-Tag peptide of pET15b is 25,600 Da. When BoNT/A cleaves the peptide bond between Gln^{217} and Arg^{218} of recombinant SNAP-25, this protein would be reduced to a molecular mass of 24,600 Da. The endoproteolytic activity of the recombinant BoNT LC and native BoNT/A were tested by assaying the cleavage of SNAP-25 protein. Figure 4 shows that the recombinant light chain partially cleaved SNAP-25, but in a way similar to that of native BoNT/A, suggesting it retained a functional property. The incomplete digestion of SNAP-25 by recombinant BoNT LC was suspected to be due to some loss of activity during the purification and renaturation process.

In conclusion, this study demonstrates that the pAAD13IX containing *argD* and *ileX* tRNA genes appears to overcome difficulties in the translation of heterologous genes, having a high proportion of arginyl AGA and isoleucyl AUA codon that are rarely used by *E. coli*. Compared with re-engineering AT-rich genes into the optimal codon bias of *E. coli*, the pAAD13IX system has advantages in terms of time and expense. Therefore, this system would be applicable for the expression of a variety of AT-rich heterologous genes in *E. coli*.

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