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Cloning, Overexpression and Purification of *Bacillus subtilis* Elongation Factor Tu in *Escherichia coli*

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To establish the overexpression and one-step purification system of *Bacillus subtilis* elongation factor-Tu (EF-Tu), the EF-Tu gene was amplified with or without own ribosome binding site (rbs) by PCR and the only PCR product without rbs was subcloned successfully. For the expression of the EF-Tu gene cloned after PCR amplification, a constitutive expression system and inducible expression system with His_6 tag at N-terminus or C-terminus, or glutathione-S-transferase (GST) fusion system were examined in *E. coli* and *B. subtilis*. Except GST fusion system in *E. coli*, however, all other trials were unsuccessful at the step of plasmid construction for the EF-Tu expression. The GST/EF-Tu fusion proteins were highly expressed by IPTG induction and obtained as both soluble and insoluble form. From the soluble GST/EF-Tu fusion protein, EF-Tu was obtained to near homogeneity by one-step purification with glutathione-sepharose affinity column chromatography followed by factor Xa treatment. The purified EF-Tu showed high GDP binding activity. These results indicate that the GST/EF-Tu fusion system is favorable to overexpression and purification of *B. subtilis* EF-Tu.

Keywords: *B. subtilis*; EF-Tu Gene; Overexpression and Purification.

Introduction

In prokaryotic cells, elongation factor Tu (EF-Tu) plays a central role in the elongation cycle of protein synthesis by promoting the binding of aminoacyl-tRNA to the A site of ribosome. In *E. coli*, it seems that almost all aminoacyl-tRNAs, including charged suppressor tRNAs, can bind to EF-Tu readily, and only two exceptions, common in prokaryotic cells, are known. These exceptions are formylmethionylated initiator tRNA ($tRNA_i^{fMet}$) and charged selenocysteine tRNAs (Ser-$tRNA^{Sec}$ and Sec-$tRNA^{Sec}$), and these charged tRNAs do not bind to EF-Tu during protein synthesis in the cells. The former one entered the P site of ribosome directly after binding with initiation factor 2 (IF2) and the later is transferred to the A site of ribosome by interaction with Sel B protein (Forchhammer *et al*., 1990). Although both EF-Tus of *E. coli*, a typical gram negative bacteria and *B. subtilis*, a typical gram positive bacteria show the same function in the translation, high amino acid homology and strong immunological cross-reaction (Wenzig and Schleifer, 1989), they also show some different characteristics. One of different features in the two EF-Tus is the interaction with misacylated tRNA. *B. subtilis* produces misacylated glutamyl-$tRNA^{Gln}$ (Glu-$tRNA^{Gln}$) as an intermediate for the production of correctly charged glutaminyl-$tRNA^{Gln}$ (Gln-$tRNA^{Gln}$) (Wilcox, 1969). The conversion of glutamic acid bound to $tRNA^{Gln}$ to glutamine is catalyzed by Glu-$tRNA^{Gln}$ specific amidotransferase (Glu-AdTase) (Curnow *et al*., 1998), and total reaction is summarized as misacylation/transamidation. It was suggested that the misacylated Glu-$tRNA^{Gln}$ in this system should not bind to EF-Tu before the conversion of glutamic acid to glutamine on the $tRNA^{Gln}$ under physiological conditions. The suggestion was strongly supported by the report of Stanzel *et al*. (1994), since eukaryotic organelles use the same misacylation/transamidation reaction to produce the Gln-$tRNA^{Gln}$ (Schön *et al*., 1988). The chloroplast EF-Tu from *Pisum sativum* did not bind with misacylated Glu-$tRNA^{Gln}$

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Abbreviations: EF-Tu, elongation factor-Tu; GST, glutathione-S-transferase.

of the chloroplast whereas *E. coli* EF-Tu interacted with the mischarged $tRNA^{Gln}$ well. These data indicated that the chloroplast EF-Tu has different features from *E. coli* EF-Tu, and EF-Tus of gram positive bacteria and eukaryotic organelles discriminate selectively against misacylated $tRNA^{Gln}$. However, it remains to be determined whether the correctly charged Gln-$tRNA^{Gln}$ can bind to EF-Tu after conversion of the glutamic acid, and if so, the mechanism by which the EF-Tu can distinguish the same $tRNA^{Gln}$ charged by different amino acids, glutamine and glutamic acid for the selective discrimination should be investigated.

Another different property between *E. coli* and *B. subtilis* EF-Tu is the susceptibility to antibiotics such as efrotomycin, kirromycin and pulvomycin. In general, EF-Tu of gram positive bacteria is less sensitive to these antibiotics (Landini *et al*., 1993). Although some amino acids in EF-Tu of gram positive bacteria interfering the binding of those antibiotics has been suggested (Krásnný *et al*., 1998), it is still unclear how those amino acids affect the interaction since a three-dimensional structure of ternary complex of the EF-Tu is not available up to now.

To investigate the precise mechanism leading to the differences, cloning and expression of the EF-Tu gene and an easy purification system for the EF-Tu expressed from the gene mutated are largely required. Nucleotide sequence of *B. subtilis* EF-Tu gene has been known (Ludwig *et al*., 1990) and purification of *B. subtilis* EF-Tu with conventional liquid chromatography from the bacterial cells was performed successfully in other research groups (Wenzig and Schleifer, 1989). However, there has been no reports on the cloning of the complete EF-Tu gene as a single DNA fragment, the overexpression, nor the easy purification system for the EF-Tu expressed from the EF-Tu gene cloned.

In this study, therefore, we tried to amplify the EF-Tu gene by PCR, and to construct various expression systems, including a constitutive and inducible expression system. His_6 tagging at N-terminus or C-terminus, and glutathione-S-transferase (GST) fusion systems were used for the inducible expression system. However, only a GST/EF-Tu fusion overexpression system was constructed. The GST/EF-Tu was expressed successfully and purified to near homogeneity using glutathione-sepharose affinity column chromatography. The purified EF-Tu showed strong GDP binding activity. These results indicate that the GST/EF-Tu fusion system is favorable for overproduction and purification of *B. subtilis* EF-Tu from *E. coli*, and the expression system allows further functional analysis of the EF-Tu engineered genetically.

Materials and Methods

Bacterial strains and plasmids *B. subtilis* M168 (Bacillus Genetic Stock Center, Ohio State Univ.) was used as the gene source for the PCR of the EF-Tu gene. *E. coli* DH5α was used for maintenance and amplification of the plasmids used. *B. subtilis* LKS 87 (Kim *et al.*, 1997) was used as the host strain for the cloning of the EF-Tu gene in pUSH-1, pUSH-2 (Schön and Schumann, 1994), and pRB374 (Brückner, 1992). The pUSH-1 and pUSH-2 are IPTG inducible expression vectors containing the His_6 tag at N-terminus and C-terminus, respectively, and pRB374 is a constitutive expression vector that can be replicated in both *E. coli* and *B. subtilis*. *E. coli* BL21 was used for the overexpression of GST/EF-Tu fusion protein from pGEX derivatives. pGEX-5X-1 (Amersham Pharmacia Biotech Ltd. UK) was used for the overexpression of *B. subtilis* EF-Tu with GST fusion protein and pGEXFXtufA (Knudsen *et al.*, 1992) is an overexpressing plasmid of *E. coli* EF-Tu (kindly provided by Dr. Knudsen).

Chemicals and enzymes Radioactive materials including ^{3}H-GDP (10.1 Ci/mmol) and α-^{35}S-dATP (1000 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Amersham Pharmacia Biotech Ltd., UK). ATP, dNTPs and DTT were obtained from Boehringer Mannheim (Boehringer Mannheim Gmbh, Germany). Synthetic oligonucleotides used for PCR and for DNA sequencing were prepared by Bioneer (Taejon, Korea). Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase were obtained from NEB (New England Biolabs, Inc., USA). *Taq* DNA polymerase, and rRNasin were purchased from Promega (Promega, USA). Glutathione sepharose 4B was obtained from Amersham Phamacia Biothech (Amersham Pharmacia Biotech Ltd. UK). Other chemicals were obtained from Sigma (Sigma, USA) as molecular biological grade.

Expression and purification of *B. subtilis* and *E. coli* EF-Tu The respective BL21 transformant harboring pGEXFXtufA for *E. coli* EF-Tu expression or pGEXBSEF for *B. subtilis* EF-Tu expression was cultured overnight in 2YT media supplemented with 100 μg/ml of ampicillin at 30°C. Ten ml of the culture was transferred to 1 L of the same media and cultured at 30°C with vigorous shaking. When cell growth in the culture reached 0.8 optical density at 600 nm, the gene expression was induced by the addition of IPTG to 1 mM. After 3 h of induction, cells were harvested and washed with 50 mM Tris-HCl (pH 7.6). The cell pellet was re-suspended in 30 ml of buffer A (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 100 mM NaCl, 15 μM GDP, 1% triton X 100) and the cells were disrupted by sonication. The crude extract was obtained by centrifugation with 20,000 rpm, at 4°C for 30 min. and then applied to affinity chromatography with glutathione sepharose 4B (1.5 cm × 10 cm). The column was washed with buffer B (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 100 mM NaCl, 15 mM GDP) and the bound GST/EF-Tu was eluted with washing buffer A containing 5 mM reduced glutathione. Fractions containing the fusion protein were pooled and dialyzed against washing buffer B to remove glutathione. The GST portion of the fusion protein purified was cleaved by treatment with factor Xa (fusion protein : factor Xa = 200 : 1) at 25°C for 2 h and the proteins was applied again to the glutathione affinity column to remove the uncleaved fusion protein and the GST cleaved from EF-Tu.

GDP binding assay of purified EF-Tu The GDP binding activity of EF-Tu was measured by the method of Miller and Weissbach (1974) with a slight modification. Since EF-Tus

interact with different guanosine nucleotides such as GDP, GTP and ppGpp or aminoacyl-tRNAs in the cell, the standardization of purified EF-Tus is required in order to measure their activity. For this reason, EF-Tu·GDP had been formed by saturation of EF-Tu with 10-fold excess of GDP followed by dialysis against binding buffer. To measure ^{3}H-GDP exchanging activity, 200 μl of buffer containing 1 μM EF-Tu·GDP and 2 μM ^{3}H-GDP was incubated at 30°C. An aliquot of 50 μl was withdrawn after 5 min, 10 min and 15 min and rapidly transferred to a nitrocellulose membrane (Millipore Corp. type HA with 25 mm diameter and 0.45 μm pore). The membrane was immediately washed three times with 10 parts washing buffer (10 mM Tris-HCl, pH 7.4, 10 mM NH_4Cl, 10 mM $MgCl_2$). The membrane was dried at room temperature and the amount of retained ^{3}H-GDP on the membrane was measured by scintillation counting (Packard, TriCrab 2100).

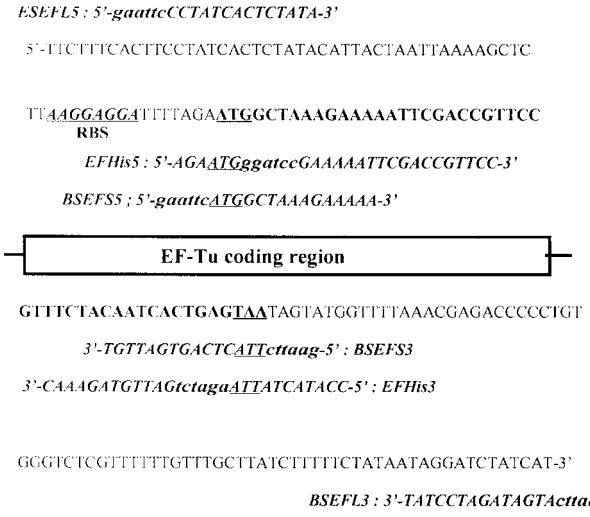

Fig. 1. DNA sequences of synthetic oligonucleotides for PCR corresponding to the flanking region of *B. subtilis* EF-Tu gene. The DNA sequence of *B. subtilis* EF-Tu is known (GenBank accession No. D64127). Underlined letters indicate the start and stop codons of the gene, and ribosome binding site is represented as underlined, italic letters. The synthetic PCR primers are shown as italic letters, and the small letters in the respective oligonucleotides are additional restriction enzyme sites.

Results

PCR cloning and construction of overexpression system of the EF-Tu Chromosomal DNA of *B. subtilis* M168 was used as a template DNA for PCR amplification of the gene encoding EF-Tu. The coding and flanking sequences of 5′ and 3′ of the EF-Tu gene as provided by Ludwig *et al*. (1990; personal communication) were used as the basis of the design of three different sets of PCR primer (Fig. 1). The first set of primers, BSEFL-5 and BSEFL-3 were targeted at upstream ribosome binding site (rbs) and downstream transcription termination signal, respectively. The second set of primers (BSEFS-5 and BSEFS-3) were only targeted at the ORF region of the EF-Tu from start codon to stop codon. Six bases for *Eco*RI site were added and represented with small letters in the primer sequence as shown in Fig. 1. The last set of primers EFHis5 and EFHis3, also targeted the ORF region, and *Bam*HI and *Bgl*II sites were created, respectively. These restriction sites were located in the coding sequence as illustrated in Fig. 1. After PCR with the respective primer set, about 1.2 kb DNA fragment was obtained. The DNA fragment was phosphorylated and ligated with pUC18, and digested with *Sma*I. The PCR product amplified with BSEFS or EFHis primer set was cloned successfully and the resulting plasmids were named pBSTUS and pBSTUHis, respectively. However, no clone was obtained from over 30,000 colonies in the case of the PCR product amplified with the primer set of BSEFL. Nucleotide sequences of the clones were determined and these were exactly the same as that of the *B. subtilis* EF-Tu gene as published by Ludwig *et al*., 1990. To construct the GST/EF-Tu expression system, the EF-Tu insert was obtained from digestion of pBSTUS with *Eco*RI and recloned at the same restriction site of pGEX-5X-1. The recombinant plasmids containing the EF-Tu gene were isolated and the direction of the insert was determined. The correct clones were confirmed by DNA sequencing, and pGEXBSEF was obtained. In the case of pBSTUHis, the recombinant plasmid obtained was digested with *Bam*HI and *Bgl*II, and 1.2 kb DNA fragment was eluted from agarose gel. The DNA fragment was ligated with pUSH1, and pUSH2 had been digested with *Bam*HI, and introduced into *B. subtilis* LKS87 or *E. coli* DH5α. In all the clones obtained, however, the insert showed the reverse direction from the promoter (data not shown).

Overexpression and purification of *E. coli* and *B. subtilis* EF-Tu *E. coli* and *B. subtilis* EF-Tu were overexpressed from the respective BL21 transformant harboring pGEXFXtufA or pGEXBSEF by the addition of IPTG to 1 mM. Disrupted cells were separated to soluble and insoluble fraction by centrifugation, and the expression level of the EF-Tu in each fraction was analyzed by SDS-PAGE. The amount of the soluble GST/EF-Tu reached 10–15% of the insoluble GST/EF-Tu as shown in Fig. 2A. The soluble fraction was applied to glutathione sepharose 4B column and the fractions containing GST/EF-Tu were identified by SDS-PAGE. The selected fractions purified to near homogeneity (Fig. 2B, lane 5) were pooled, dialyzed and treated with factor Xa (GST/EF-Tu fusion protein : Factor Xa = 200 : 1) to cleave the GST portion (Fig. 2B, lane 6). The proteins treated with factor Xa were applied again to glutathione sepharose 4B column to remove the uncleaved fusion proteins and cleaved GST. The fractions containing EF-Tu still showed two more protein bands whose apparent molecular weights were about 34 and 38

kDa, respectively (Fig. 2B, lane 7). To remove these contaminants, the EF-Tu solution was applied to FPLC Mono Q ion exchange chromatography. Factor Xa and the contaminants were removed completely from the EF-Tu solutions (Fig. 2C). Although Fig. 2B only shows the purification of *B. subtilis* EF-Tu, the purification of *E. coli* EF-Tu was almost the same as that of *B. subtilis* EF-Tu (Fig. 2C). The apparent molecular weight of the purified *B. subtilis* EF-Tu was slightly larger than that of *E. coli* EF-Tu.

GDP binding activity of purified EF-Tu For measuring the ^{3}H-GDP exchanging activity of the purified EF-Tu, 200 µl of buffer containing 1 µM EF-Tu·GDP and 2 µM ^{3}H-GDP was incubated at 30°C. After 5 min, the radioactivity of the reaction containing the respective EF-Tu was about 30-fold higher than that of the control without EF-Tu, and there was no significant increase in radioactivity after 5 min (Table 1). This result indicated that the GDP exchanging reaction was almost completed in 5 min due to the high GDP binding activity of the purified EF-Tu.

Discussion

Cloning of *B. subtilis* genes encoding EF-Tu EF-Tu genes from various origins such as *E. coli* (Bloor and Barber, 1993; Knudsen *et al.*, 1992), *Thermus thermophilus* (Blank *et al.*, 1995), *Mycobacteria* (Luneberg *et al.*, 1991), mitochondria (Kuhlman and Palmer, 1995; Woriax *et al.*, 1995), chloroplasts (Ursin *et al.*, 1993) were cloned and expressed successfully as authentic form or tagged with His_6 or GST fusion protein. In the case of typical gram positive eubacteria, *B. subtilis* or *B. stearothermophilus*, there were no reports on the cloning of the EF-Tu gene as a full length DNA or on a successful expression system with the cloned gene, although their nucleotide sequences are known. Moreover, it was reported that the cloning of the genes encoding *B. subtilis* (Ludwig *et al.*, 1990, personal communication) or *B. stearothermophilus* EF-Tu (Krásnný *et al.*, 1998) as a single DNA fragment in *E. coli* had been unsuccessful. In our results, the clones were not obtained in the amplified PCR fragment containing even ribosome binding sites

Table 1. GDP binding activity of the purified EF-Tus.

Reactions	Radioactivity (cpm) after indicated reaction time		
	5 min	10 min	15 min
Control	1,305 ± 76	1,055 ± 123	1,234 ± 46
EF-Tu (bs)	31,946 ± 137	32,374 ± 142	32,226 ± 156
EF-Tu (ec)	31,976 ± 143	32,417 ± 157	32,444 ± 161

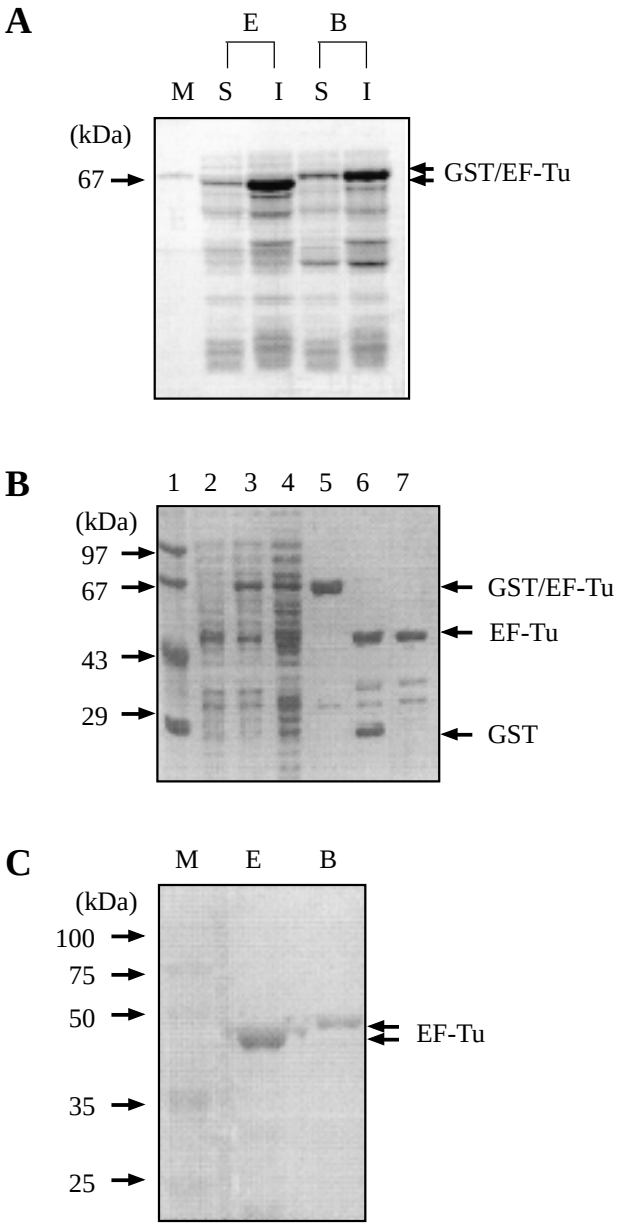

Fig. 2. Overexpression and purification of *B. subtilis* EF-Tu in *E. coli*. **A.** The overexpression of *E. coli* (E) and *B. subtilis* EF-Tu (B) as fusion proteins with GST. Soluble (S) and insoluble (I) fractions were obtained as mentioned in Materials and Methods, and M is a bovine serum albumine as a molecular weight marker protein. **B.** The purification of *B. subtilis* EF-Tu. The GST/EF-Tu of *B. subtilis* was overexpressed by induction with 1 mM IPTG (lane 3) whereas no expression of the fusion protein was found without IPTG induction (lane 2). Almost all of the expressed GST/EF-Tu shown in the lane 2 were bound to the glutathione sepharose 4B affinity column (lane 4) and eluted from the column by 5 mM glutathione (lane 5). The GST/EF-Tu was cleaved by the treatment of factor Xa to EF-Tu and GST (lane 6), and EF-Tu was separated from GST using the glutathione sepharose 4B affinity chromatography (lane 7). **C**. The purified the *E. coli* (E) and *B. subtilis* EF-Tu (B) after removal of the contaminants as shown in the lane 7 of **B** using FPLC Mono Q column.

(data not shown). In addition, all the EF-Tu clones in pUC18 showed the reverse direction from the β-galactosidase promoter. The same situation was found when pUSH1 or pUSH2 was used for the cloning of the PCR product in *E. coli*. Therefore, it was suggested that even small amounts of EF-Tu of *B. subtilis* or *B. stearothermophilus* might be highly toxic to *E. coli* cells. However, the reason is still not clear. When *B. subtilis* was used as host cell for the cloning of EF-Tu gene into pUSH1 or pUSH2, it was also unsuccessful in obtaining the correct clones. In this case, it was assumed that the imbalance of EF-Tu and Glu-AdTase in *B. subtilis*, caused by the expression of additional EF-Tu encoded on the recombinant plasmid, might have induce the incorporation of mischarged Glu-tRNA^{Gln} to the A site of the ribosome through the formation of Glu-tRNA^{Gln}·EF-Tu·GTP ternary complex. Similarly, it has been reported that moderate expression of cloned EF-Tu gene is enough to lead to a misappropriation of methionyl-tRNA$_i^{fMet}$ in the elongation process (Guillon *et al.*, 1996). In this case, however, binding Met-tRNA$_i^{fMet}$ to EF-Tu is not highly toxic to cell because the methionine is still being used for correct decoding of the methionine codon of mRNA on the ribosome.

Expression and purification of the EF-Tu *B. subtilis* EF-Tu was expressed successfully as a GST/EF-Tu fusion protein in *E. coli* as well as *E. coli* EF-Tu, and about 15% of overexpressed GST/EF-Tu was obtained as a soluble form (Fig. 2A). The apparent molecular weight of *B. subtilis* EF-Tu fused with GST was about 67 kDa, slightly larger than that of *E. coli*. After removal of the GST portion, the EF-Tu of purified *B. subtilis* might contain five additional amino acids, Gly-Ile-Pro-Glu-Phe (633 Da), at the N-terminus, with apparent molecular mass of 47 kDa, while that of *E. coli* EF-Tu was 45 kDa (Fig. 2C). The calculated molecular mass of the amino acid of *B. subtilis* and *E. coli* EF-Tu was 43,593 Da and 43,313 Da, respectively.

Features of the GST/EF-Tu expression system This is the first report on the successful expression of plasmid encoded *B. subtilis* EF-Tu in *E. coli* with a simple purification system. It was suggested that the expression of *Bacilli* EF-Tu in *E. coli* might be highly toxic to host cells even in small amounts. However the EF-Tu of *B. subtilis* fused with GST was highly expressed in *E. coli*. Therefore, it was assumed that the soluble GST/EF-Tu has no EF-Tu function. Knudsen *et al.* (1992) reported that *E. coli* EF-Tu fused with GST did not bind with kirromycin, and this implies that the GST/EF-Tu may not bind with aminoacyl-tRNAs. After removal of GST from the soluble GST/EF-Tu, however, the EF-Tu showed high GDP binding activity. This is the most important advantage of this system because purified EF-Tu is active but EF-Tu expressed as GST fusion protein is prevented from taking part in protein synthesis. Therefore, this expression and purification system of EF-Tu might be very useful for obtaining various mutant EF-Tus encoded by recombinant plasmid for genetic analysis, and for functional studies of EF-Tu originating from various gram positive bacteria as well as *B. subtilis*. For this reason, we are trying to express various mutant EF-Tus of *B. subtilis* and *B. stearothermophilus* to understand not only the precise mechanism of the selective discrimination of the EF-Tu on the mischarged Glu-tRNA^{Gln} but also the mechanism of the kirromycin resistance of the EF-Tu of gram positive bacteria.

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